Elucidating the Molecular Mechanisms for Lipoprotein Processing and Localisation of Factor H binding protein in *Neisseria meningitidis*

A thesis submitted in partial fulfilment for the degree of Doctor of Philosophy

By

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Declaration

This thesis entitled ‘Elucidating the Molecular Mechanisms for Lipoprotein Processing and Localisation in *Neisseria meningitidis*’ is based upon the work conducted in the Faculty of Science, Engineering and Computing at Kingston University London and in the Centre for Biomolecular Sciences at The University of Nottingham. All of the work described here is the candidate’s own original work unless otherwise acknowledged in the text or by references. None of the work presented here has been submitted for another degree at this or any other university.

To my family
and my chosen family, friends.
You were light when everything else at times seemed so dark.

'Everything that has a beginning has an ending. Make your peace with that and all will be well'
- Jack Kornfield, *Buddha's Little Instruction Book*

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Publications

Variant Signal Peptides of Vaccine Antigen, FHbp, Impair Processing Affecting Surface Localization and Antibody-Mediated Killing in Most Meningococcal Isolates

da Silva, R.A.G., Karlyshev, A.V., Oldfield, N.J., Wooldridge, K.G., Bayliss, C.D., Ryan, A., Griffin, R. (2019). *Front. Microbiol.* 10: 2847. doi: 10.3389/fmicb.2019.02847

The role of apolipoprotein N-acyl transferase, Lnt, in the lipidation of factor H binding protein of *Neisseria meningitidis* strain MC58 and its potential as a drug target

da Silva, R. A. G., Churchward, C. P., Karlyshev, A. V., Eleftheriadou, O., Snabaitis, A. K., Longman, M. R., Ryan, A., and Griffin, R. (2017). *British Journal of Pharmacology*, 174: 2247–2260. doi: 10.1111/bph.13660.

Presentations

Factor H binding protein, is typically a non-lipidated precursor that localises to the meningococcal surface by Slam

Poster presentation. Microbial Adhesion and Signal Transduction Gordon Research Conference. Salve Regina University. Newport, RI, US. July 2019.

Identification of a novel, unprocessed species of Factor H binding protein on the surface of meningococcal strains Vaccine antigen,

Poster presentation. 5th Young Microbiologist Symposium. Queen's University. Belfast. Northern Ireland, UK. August 2018.

The role of Lnt in lipidation of FHbp in *Neisseria meningitidis* and its potential as a drug target

Oral presentation. Invited speaker. Middlesex University, London, UK. January 2017.

The role of Lnt in lipidation of FHbp in *Neisseria meningitidis* and its potential as a drug target

Oral presentation. Early Career Scientist Conference of the Society of Applied Microbiology. Royal Society of Medicine, London, UK. October 2016.

List of Abbreviations

ACP: Adhesin Complex Protein
AP: Alternative pathway
App: Adhesion and Penetration Protein
ATP: adenosine triphosphate
BACTH: Bacterial two-hybrid
BBB: Blood brain barrier
BCSFB: Blood-CSF barrier
bp: Base pairs
BSA: Bovine serum albumin
C4bp: Complement regulatory C4b-binding protein
cDNA: Complementary DNA
CEACAM: Human carcinoembryonic antigen
CFU: Colony forming units
CP: Classical pathway
CSF: Cerebrospinal fluid
DE: Differentially expressed
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic Acid
ECM: Extracellular matrix
EDTA: Ethylenediaminetetraacetic acid
FACS: Fluorescence-activated cell sorting
FBA: Fructose-1,6-bisphosphate aldolase
FH: Factor H
FHbp: Factor H binding protein
Fwd: Forward
GAPA-1: Glyceraldehyde 3-phosphate dehydrogenase
GC: Giolitti-Cantoni
HCEC: Human corneal epithelial cells
HEPES: N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid
HrpA-HrpB: Haemagglutinin/Haemolysin-related proteins A and B
hSBA: Human SBA
HSPG: Heparan sulphate proteoglycan
HUVEC: Human umbilical vein endothelial cells
IL: Interleukin
IM: Inner membrane
IPTG: Isopropyl-β-D-thiogalactopyranoside
kDa: Kilodalton
KLD: Kinase-, ligase- and DpnI-treated
KO: Knock Out
LB: Lysogeny broth

Lgt: Phosphatidylglycerol-prolipoprotein diacylglyceryl transferase
Lnt: Apolipoprotein N-acyltransferase
lnt: Linear non-threshold
LOS: Lipo-oligosaccharide
LP: Lectin pathway
LspA: Lipoprotein signal peptidase
MAC: membrane attack complex
MCS: Multiple cloning siteMH: Mueller Hinton
MIC: Minimum inhibitory concentration
MOI: Multiplicity of infection
MspA: Meningococcal Serine Protease A
NadA: Neisserial adhesin A
Ng*: Neisseria gonorrhoeae*
NhhA: Neisseria Hia Homologue A
Nm: *Neisseria meningitidis*
NS: Not significant
OD: Optical density
OM: Outer membrane
Opa: Opacity protein A
Opc: Opacity protein B
PAMPs: pathogen-associated molecular patterns
PBS: Phosphate-buffered saline
PCR: Polymerase chain reaction
PMA: Phorbol 12-myristate 13-acetate
PorA: Porin A
PorB: Porin B
PRRs: pattern recognition receptors
qRT-PCR: Quantitative reverse transcription polymerase chain reaction
Rev: Reverse
RIN: RNA integrity number
rMFI: Relative Mean of Fluorescence Intensity
RNA: Ribonucleic acid
RNAseq: RNA sequencing
RPM: Rotations per minute
RPMI: Roswell Park Memorial Institute Medium
RT-PCR: Reverse transcription polymerase chain reaction
SAS: Subarachnoid space
SBA: Serum bactericidal assay
SDM: Site-directed mutagenesis
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sec: Secretory
SEM: Standard error of the mean

Slam: Surface Lipoprotein Assembly Modulator
SNP: Single nucleotide polymorphism
SOE: Splicing by Overlap Extension
SP: Signal peptide
TAE: Tris-acetate-EDTA
TBE: Tris borate EDTA
TEM: Transmission electron microscopy
TEMED: Tetramethylethylenediamine
THP-1: Human monocytic cell line
TLR: Toll-like receptors
Tn: Transposon
TPS: Two-partner secretion
TspA: T-cell stimulating protein A
WB: Western immunoblotting
WC: Whole cell
WCL: Whole cell lysate
WGS: Whole genome sequencing
WHO: World Health Organisation
WT: Wild type

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Abstract

Neisseria meningitidis (Nm) is accountable for thousands of meningitis cases that lead to high mortality in children and young adults every year. Currently, as part of the efforts to combat this infectious disease caused by serogroup B strains, two vaccines have been licensed in recent years containing meningococcal lipoprotein Factor H binding protein (FHbp). Trumemba vaccine (Pfizer) has FHbp as its sole antigen and the Bexsero vaccine (GSK) has FHbp as one of four antigens in its vaccine formulation. The success of these vaccine formulations, in particular Trumemba, depends on sufficient surface display of FHbp in order to elicit protective serum antibody response. The expression level of FHbp varies between and within strains and the molecular mechanisms regulating this have been established. The aims of the work presented in this thesis were to elucidate the molecular mechanisms involved in the processing and surface localisation of FHbp, which is key for target recognition following immunisation with FHbp-based vaccines.

Firstly, the generation of a transposon (Tn) library in strain MC58 enabled the discovery of the gene responsible for triacylation of FHbp in Nm. The impact of disruption of Lnt, which likely triacylates all other Nm lipoproteins was investigated and the data suggest the potential of Lnt as a novel drug target. Secondly, the identification of single nucleotide polymorphisms (SNPs) in the signal peptide (SP) of FHbp in strain L91543 led to the discovery of unprocessed FHbp on the cell surface and this finding was applicable to others Nm isolates. This has implications for current FHbp-based vaccines.

In further detail in Chapter 3, the generation of a Tn library in strain MC58 is described. One mutant that lacked binding to the anti-FHbp monoclonal antibody (mAb) JAR4 was found to have been disrupted in the *lnt* gene. Experimental evidence was provided to support the predicted role of Lnt in acylating FHbp in Nm. In addition, data are shown that Lnt disruption affects FHbp expression at RNA and protein level suggesting a wider impact on the Nm cell than loss of ability to add the third fatty acid to FHbp. This chapter also presents data concerning the surface exposure of diacylated FHbp in the Lnt mutant which indicates a more flexible Lol machinery in the meningococcus than in *E. coli*, for which Lnt is essential.

Due to the importance of lipoproteins for maintaining membrane integrity and likelihood of Lnt disruption affecting all Nm lipoproteins, it is speculated that the mutant would be significantly affected in membrane homeostasis. This is investigated in Chapter 4. RNAseq analysis of MC58Lnt reveals 183 genes to be DE, including genes encoding lipoproteins and genes related to RNA biology, and involved in adhesion and survival of the meningococcus. The biological impact of Lnt disruption is investigated in adhesion and invasion assays using of HCECs and HUVECs. While the mutant is shown to invade HCECs less, it invaded HUVECs more than when compared to the WT. HCECs and HUVECs also presented different pattern of expression of IL-6 and IL-8. HUVECs expressed more of these inflammatory proteins and this was suggested to be linked with higher invasion of MC58Lnt in this type of cells. Whereas survival and replication in THP-1 cells by the mutant was not affected, it displayed reduced ability to form biofilms on an abiotic surfaces and reduced virulence in the tested

Galleria mellonella model. Importantly the disruption of Lnt leads to increased susceptibility of Nm to several antibiotics tested, supporting the potential of Lnt as a novel drug target.

To further investigate molecular mechanisms that govern FHbp processing and surface localisation, the rest of the thesis is focused on the SP of FHbp. In Chapter 5, strain L91543 containing SP SNPs is taken for analysis. Site-directed mutagenesis (SDM) shows that these SNPs in the SP of FHbp are indeed responsible for processing and surface localisation. Specifically, a single SNP in the hydrophobic region of the SP, common to 88% of the UK isolates analysed (1742/1895), abolishes FHbp processing. This has important implications for Trumemba which consists of acylated FHbp. We show by FACS reduced display of FHbp in strains with SP SNPs and corresponding reduced susceptibility to killing by FHbp-specific antibodies in SBA assays.

Chapter 1
Introduction

1.1- The meningococcus

The genus *Neisseria* is part of the family of microorganisms Neisseriaceae and includes, at least, 17 species including *Neisseria lactamica* and, the medically relevant organisms, *N. gonorrhoeae* (Ng) and *N. meningitidis* (Nm) (Tønjum and van Putten, 2017). Nm, otherwise known as the meningococcus, was identified for the first time in 1887 by Weichselbaum in a cerebrospinal fluid of a patient. It is a fastidious, gram-negative β-proteobacterium, oxidase-positive microorganism. The meningococcus shares about 90% homology at the nucleotide level with Ng and has high plasticity which contributes to the diversity between strains of this organism (Rhouphael and Stephens, 2012).

Structurally, like all other gram-negative bacteria, Nm has a subcapsular cell envelope that consists of an outer membrane (OM), a peptidoglycan layer, and a cytoplasmic or inner membrane (IM) (Fig 1.1). In order to infect mucosal surfaces and invade the human host, Nm uses several virulence factors present in its envelope. It has developed genetic mechanisms that provide molecular mimicry of the host, antigenic variation, and high frequency phase variation[8] of genes which switch on and off the expression of a number of virulence factors (Stephens, 2009).

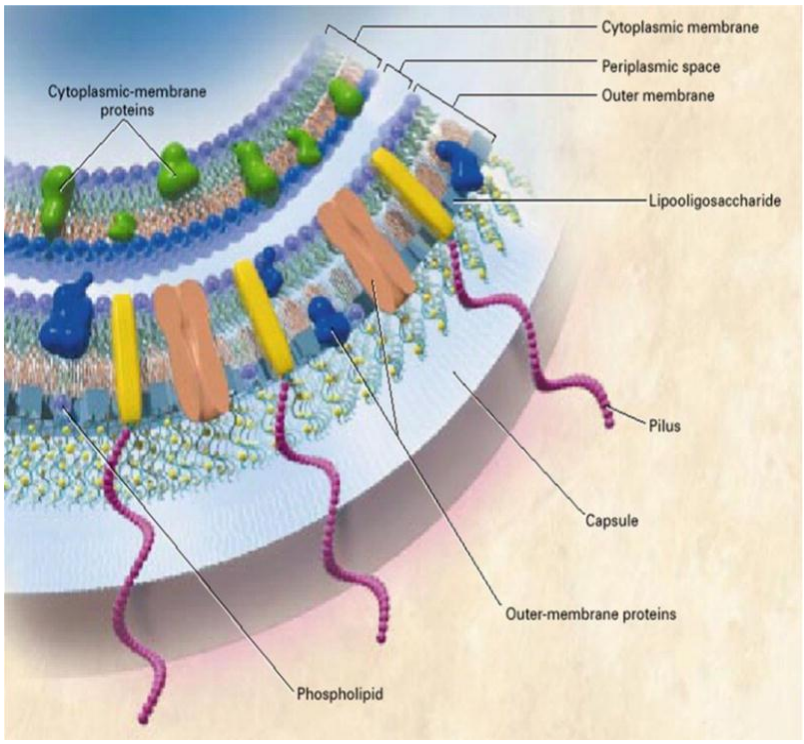


Figure 1. 1- Cross-sectional view of the meningococcal envelope

Structures such as OM, IM, outer membrane proteins (OMPs), capsule, pilus and lipooligosaccharide are shown (image from Rouphael and Stephens, 2012).

Nm is an obligate human pathogen and commensally colonizes the nasopharyngeal mucosa. It can be found asymptomatically in approximately 10% of the population at any given time (WHO, 2018). The meningococcus is one the leading causes of bacterial meningitis and sepsis, accounting for 9.1–36.2% of the worldwide cases in all age groups (Oordt-Speets *et al.*, 2018). Despite all the current immunization programs available against Nm, over 30,000 cases are still reported in sub-Saharan Africa each year (WHO, 2018). Invasive meningococcal disease has high mortality (between 10 and 15% when treated or up to over 60% when untreated) and high frequency (more than 10%) of morbidity that includes severe sequelae such as

cognitive dysfunction, hearing loss, and limb amputation. (Rouphael and Stephens, 2012; Pizza and Rappuoli, 2015; WHO, 2018).

This organism is classified into 12 different serogroups depending on its capsular polysaccharide (Harrison *et al*., 2013). Invasive meningococci typically express polysaccharides, A, B, C, W or Y (Jolley *et al*., 2007). Globally, the serogroup B is one of the most widespread serogroups (Fig 1.2) and one of the main groups responsible for outbreaks in developed countries (Pelton, 2016).

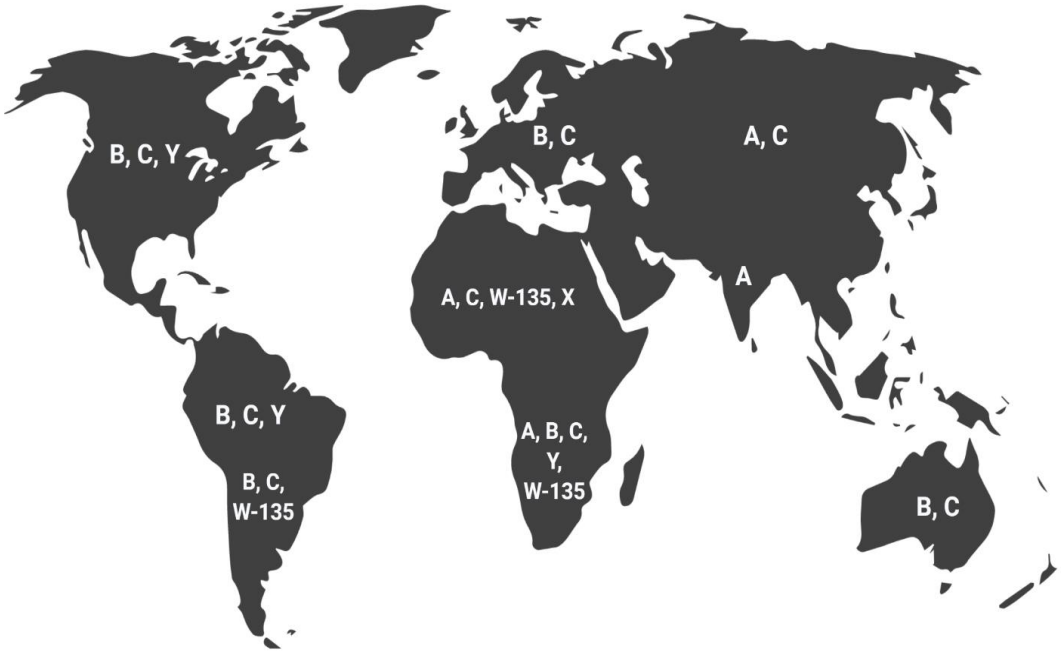


Figure 1. 2- Worldwide serogroup distribution of invasive meningococcal disease (adapted from Jafri et al., 2013)

1.2- Pathogenesis: routes of interaction between the meningococcus and host

In order to colonise the host, Nm has evolved several structures that favour its establishment in the upper-respiratory tract of the host. These include virulence factors that not only interact with epithelial cells but also adhere to and invade endothelial cells once the mucosal barrier is crossed. Nm deploys cunning mechanisms to avoid the immune system in order to survive and multiply in the blood stream causing septicaemia (Hill *et al.*, 2010). When Nm crosses the blood brain barrier (BBB), it can survive and multiply as well in the meninges causing what is known is meningitidis: a serious inflammatory disease that can rapidly lead to death (Kim, 2008). Schematic diagram of the pathogenic route used by Nm is shown (Fig 1.3) and the contribution of the important virulence factors at each step.

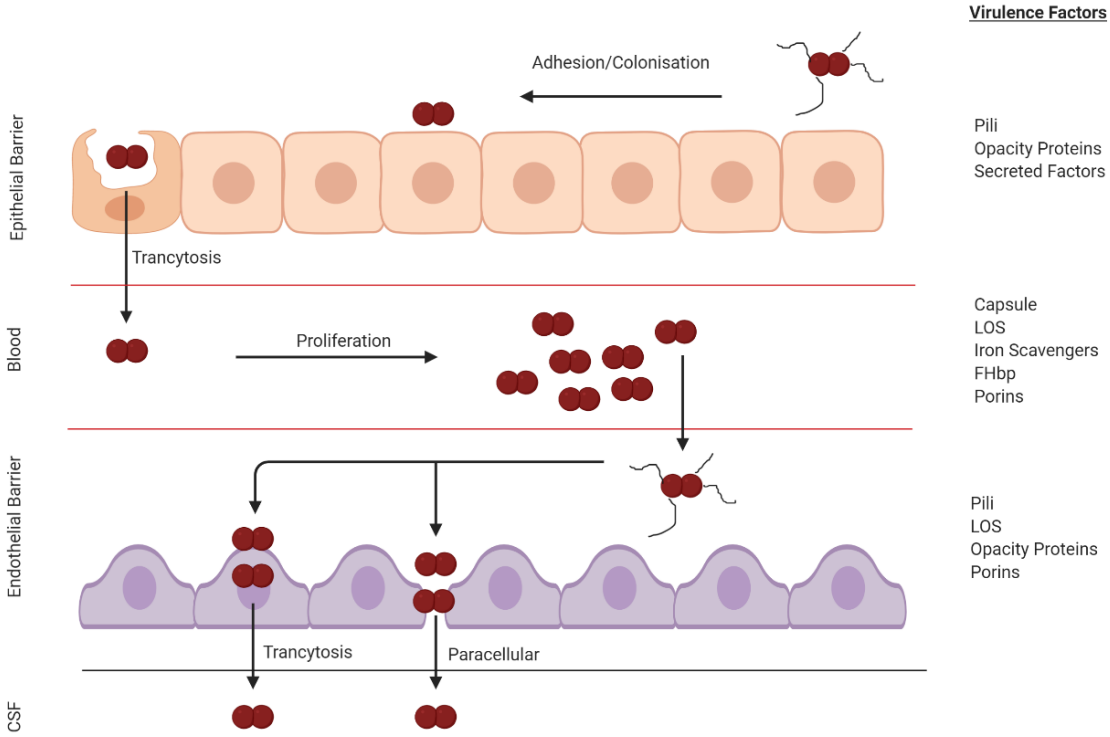


Figure 1. 3- Schematic diagram of meningococcal pathogenesis (adapted from Vassey, 2014)

1.2.1- Adhesion and invasion of epithelial cells

Nm typically asymptomatically colonises nasopharyngeal epithelial cells (Rouphael and Stephens, 2012). The mucosal membrane of the upper-respiratory tract is its natural habitat which is opportunistic for transmission to other humans upon intimate contact or by respiratory droplets (Stephens and Farley, 1990). Successful Nm colonisation of epithelial cells follows after intimate adhesion following initial adhesion (Deghmane *et al.*, 2002; Nassif *et al.*, 1994). The way Nm adheres and invades epithelial cells is similar to how it interacts with other types of cells.

The main structures involved in adhesion of Nm to epithelial cells are pili. They support initial adhesion of capsulated and non-capsulated Nm by providing strong and efficient binding to host receptors (Virji *et al.*, 1993). By retraction, after initial adhesion, pili are also responsible for bringing the organism closer to its target giving opportunity for a more intimidate adhesion by other adhesins such as Opacity proteins Opa and Opc. Piliation is then lost after close adhesion is established (Nassif *et al.*, 1999).

As reviewed by Hung and Christodoulides (2013), initial adhesion is also mediated by several other adhesins, incuding NadA (neisserial adhesin A), NhhA (Neisseria Hia Homologue A), App (Adhesion and Penetration Protein), MspA (Meningococcal Serine Protease A), HrpA-HrpB (Haemagglutinin/Haemolysin-related proteins A and B), FBA (fructose-1,6-bisphosphate aldolase), GAPA-1 (Glyceraldehyde 3-phosphate dehydrogenase), TspA (T-cell stimulating protein A) and ACP (Adhesin Complex Protein). These adhesins have all been shown experimentally to contribute to initial adhesion to a plethora of different receptors in the cell.

Interestingly, cytosolic proteins such as enolase, DnaK and peroxiredoxin have been shown to be used by the meningococcus for plasminogen recruitment (Knaust *et al.*, 2007). Plasminogen is a key proenzyme component of the extracellular matrix (ECM) of the mucous barrier but also is present in the basal laminae of epithelial and endothelial cells. Nm is able to use this proenzyme to localise enolase, DnaK and peroxiredoxin in the OM despite typically being cytoplasmic proteins. Acting as plasminogen receptors, they convert plasminogen to plasmin (its active protease form) on the surface of Nm allowing exploitation for enhanced adherence and colonisation of the host (Knaust *et al.*, 2007).

After initial adhesion, capsule (introduced in section 1.4.1) expression is typically downregulated since its presence can affect intimate adhesion by OM proteins such as Opa and Opc (Virji *et al.*, 1993). Both proteins interact independently with different host receptors. Other adhesins may help with this process but their roles remain to be fully characterised (Hung and Christodoulides, 2013).

Aggregation follows adhesion of the target cell with microcolony formation in the host. Surface HrpA has been shown to be involved in this process (Neil and Apicella, 2009). Pili components such as PilX and PilQ contribute towards biofilm formation after successful establishment of Nm (Hélaine *et al.*, 2005; Lappann *et al.*, 2006; Yi *et al.*, 2004). The notion of biofilm is defined as the intimate contact of associated bacterial cells within an exopolymeric matrix that contains a complex mixture of biomolecules such as lipids, polysaccharides and proteins produced by the organism (Hall-Stoodley *et al.*, 2004). Biofilm formation by Nm is supported by the fact that

successful colonisation persists to up 10 months in the host (Greenfield *et al.*, 1971). Such persistence can only be explained by proper aggregation and colonisation of the meningococcus in order to resist biophysical clearance mechanisms employed by the host.

Invasion of Nm may follow after intimate adhesion is established. As reviewed by Merz and So (2002), non-piliated bacteria dissociate from the microcolonies and tightly associated with the host plasma membrane. Upon this host-bacteria contact, there is an expansion of the host membrane followed by engulfment of the organism by the epithelial cells. Internalised Nm can survive and multiply successfully in epithelial cells (Lin *et al*., 1997a). Epithelial traversal by the meningococcal occurs mainly by invasion and transcytosis, whereby bacteria cross through the cellular layer (Sutherland *et al.*, 2010). Paracellular route (traverse between cells) may also occur, however evidence of this route being used with epithelial cells by the meningococcus is conflicting. If the paracellular route is used, it is without clear disruption of lateral junction as seen for endothelial cells (Merz *et al.*, 1996; Pujol *et al.*, 1997; Sutherland *et al.*, 2010).

1.2.2- Survival and multiplication of the meningococcus in the bloodstream

After invading and crossing the epithelial layer, Nm reaches the bloodstream. Survival of Nm in the bloodstream depends on expression of virulence factors and host innate and adaptive immune[1,4] **(Glossary; p.266)** mechanisms. Several virulence factors help the meningococcus to evade the immune system (Fig 1.4).

Capsule and lipo-oligosaccharide (LOS), for instance, are the most crucial virulence factors to prevent complement-mediated killing and to promote survival in the bloodstream (Kugelberg *et al.*, 2008). Expression of capsule is again switched on and its presence induces lower deposition of complement component C4b, limiting the ability of antibodies to recognise Nm (Agarwal *et al.*, 2014). Sialylated LOS was shown to bind factor H (FH) and prevent complement-mediated killing by the alternative pathway (Lewis *et al.*, 2012). Several other virulence factors have evolved that bind complement regulatory proteins in order to down regulate the complement cascade and subvert the immune system. Porin A (PorA) is one of these factors. PorA can bind the main inhibitor of the classical pathway C4bp (complement regulatory C4b-binding protein) (Jarva *et al.*, 2005). Factor H binding protein (FHbp), Porin B (PorB), Neisserial Surface Protein A (NspA), and Neisseria Heparing-binding Antigen (NHBA) all bind to FH and down-regulate the alternative pathway (Pizza and Rappuoli, 2015). Moreover, Nm employs an additional mechanism to evade complement-mediate killing by using the serine protease autotransporter NalP to cleave the complement

element C3 and lowering its deposition on the surface of the cell (Del Tordello *et al.*, 2014).

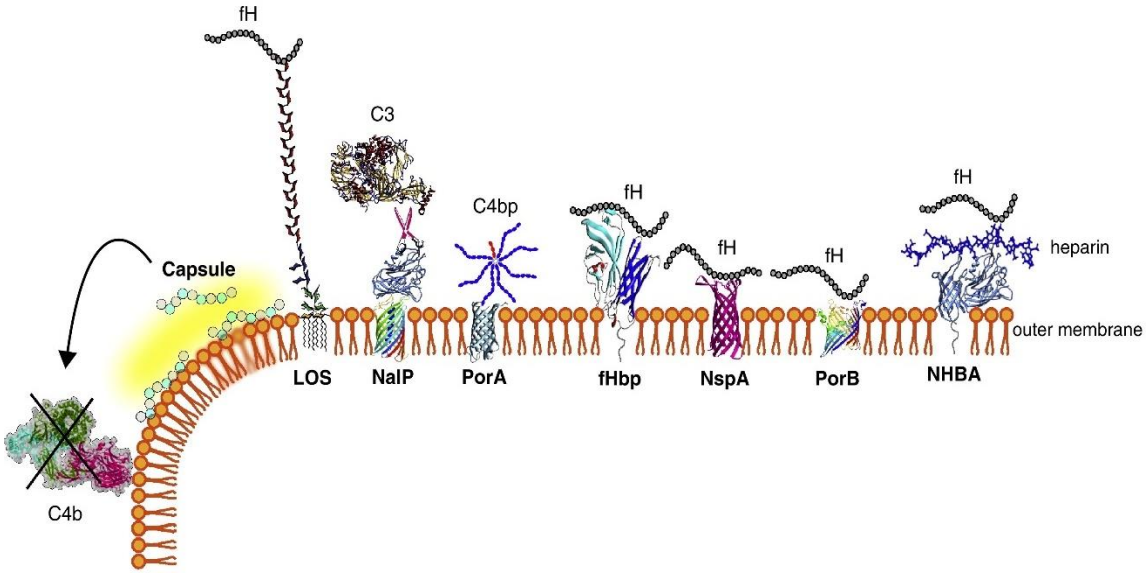


Figure 1. 4- Schematic illustration of Nm virulence factors interacting with complement components

The image shows some of the neisserial virulence factors introduced in this thesis that interact with complement molecules in order to defend the microorganism from targeting by the host immune system. LOS, FHbp, NspA, PorB, NHBA which interact with the complement component Factor H are shown. The virulence factors capsule and PorA, which prevents C4b deposition and interact with C4bp are also shown. (image from Pizza and Rappuoli, 2015).

The number of virulence factors used by Nm to evade the immune system illustrates how complex the survival mechanisms are for successful meningococcal replication in the host.

1.2.3- Adhesion and invasion of endothelial cells

Transcytosis through the epithelial cell layer allows Nm to reach the bloodstream and consequently interact with endothelial cells. Vascular colonisation follows after rapid growth of the meningococci in the blood. Similarly, to how it happens with epithelial cells, colonisation involves initial adhesion, proliferation, aggregation and invasion. Again pili, Opa and Opc are the key adhesins that promote adhesion of Nm to endothelial cells (Melican *et al.*, 2013; Virji *et al.*, 1993b). Interestingly, it was reported that while Opc is more efficient in promoting adhesion of Nm to endothelial cells. Opa has higher binding affinity for epithelial cells (Virji *et al.* 1993b). However, both Opa and Opc are implicated in adhesion of endothelial cells (Virji *et al.*, 1993b).

After colonisation of endothelium, Nm has the propensity to cross this cell layer. Different routes have been shown that could allow traversal of the meningococcus through endothelial cells. Meningococci can traverse the vascular endothelium by trafficking between endothelial cells (paracellular route) (Dunn *et al.*, 1995), or moving through cells (transcytosis) *(Eugène et al.*, 2002). Carriage across cellular barriers leukocytes (Trojan horse mechanism) can also happen (Coureuil *et al.*, 2012) . Transcytosis through endothelial cells likely follows after microbial uptake by protrusions of residual epithelial microvilli (Eugène *et al.*, 2002). Paracellular trafficking, on the other hand, occurs as a result of LOS damage that disrupts the intercellular tight junctions (Dunn *et al.*, 1995).

1.2.4- Invasion and crossing of the brain vascular endothelial layer

The last barrier crossed by Nm before accessing the cerebrospinal fluid (CSF) is the Blood-CSF barrier (BCSFB) also known as Blood Brain Barrier (BBB). Disruption of the brain endothelial vascular layer is characteristic of meningococcal invasive disease (Hung and Christodoulides, 2013). Nm has been shown to maintain tight binding to cerebral endothelial cells experimentally under conditions of high shear force[9] of a flow (Mairey *et al*., 2006). PilQ and Porin A are two of the virulence factors shown to bind to the laminin receptor of brain microvascular endothelial cells (BMEC) (Orihuela *et al.*, 2009).

Although a specific mechanism of neisserial invasion of the subarachnoid space (SAS) has not been fully demonstrated yet, it is known that bacteremia is a pre-requisite for invasion of the BBB (Christodoulides *et al.*, 2002). It is thought that similar to how leukocytes cross from the blood to enter the CSF, bacteria may enter the SAS. This could happen by traversing the BBB or by disrupting intercellular junctions (Carbonnelle *et al.*, 2009). Schematic diagram adapted from Hill *et al.*, 2010 showing the crossing of the meningococcus from the brain vascular endothelial layer to the SAS (Fig 1.5).

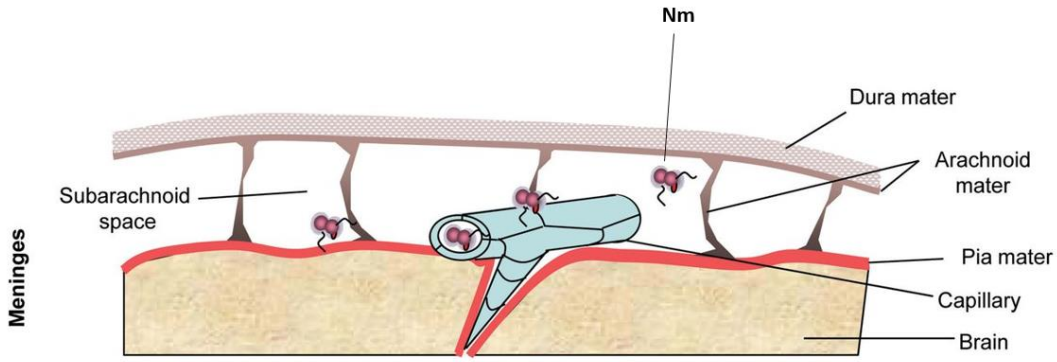


Figure 1. 5- Meningococcal penetration of the BBB and interaction with the meninges (adapted image from Hill *et al.*, 2010)

Once in the SAS space, pili have been shown to have high ligand affinity for the leptomeninges[5] and meningioma cells (Hardy *et al.*, 2000). Conversely, Opa and Opc are not as relevant in this context and do not seem to play a role in adhesion to the meningioma (Hardy *et al.*, 2000; Oldfield et *al.*, 2007). The only other adhesin known to be relevant to binding of meningioma cells *in vitro* is ACP (Hung *et al.*, 2013). More remains to be elucidated about Nm survival and interaction with cells in the SAS. Several of the virulence factors discussed in the above sections will be described more fully later in this chapter (section 1.4).

1.3- Host response to *N. meningitidis*

Initial clearance of Nm in the host is mediated by innate immune recognition of the organism which triggers complement-mediated killing (Schneider *et al.*, 2007). Individuals that have deficiencies in different components of the complement system are more susceptible to invasive meningococcal disease, suggesting this mechanism of defense by the host is crucial for combating Nm (Ram *et al.*, 2010). Indeed, complement proteins are essential for targeting and eliminating invasive bacteria. They represent the first line of defence against many pathogenic bacteria and, in relation to the meningococcus, have been demonstrated as the single most important factor that can dictate disease outcome (Lewis and Ram, 2014).

Initially, the complement system is activated by pattern recognition receptors (PRRs) such as the complement-related protein C1 (Johswich, 2017). PRRs can recognise pathogen-associated molecular patterns (PAMPs) such as LOS and peptidoglycan. Once complement PRRs identify microbial PAMPs, they can initiate complement-mediated response (Lewis and Ram, 2014). After activation of the system, the complement proteins protect the host by providing the signal to phagocytise bacteria when C3 convertases opsonise[6] (label) the bacteria's surface, or by direct lysis of the invaders when C5 convertases generate the membrane attack complex (MAC) (Ermert *et al.,* 2019). Complement can be activated via the classical pathway (CP), the lectin pathway (LP) or the alternative pathway (AP). There are more than 30 proteins involved in this innate response (Lewis and Ram, 2014). Below an adapted image from Koelman *et al.* (2019) that

illustrates some of the components of the complement-mediated killing (Fig 1.6).

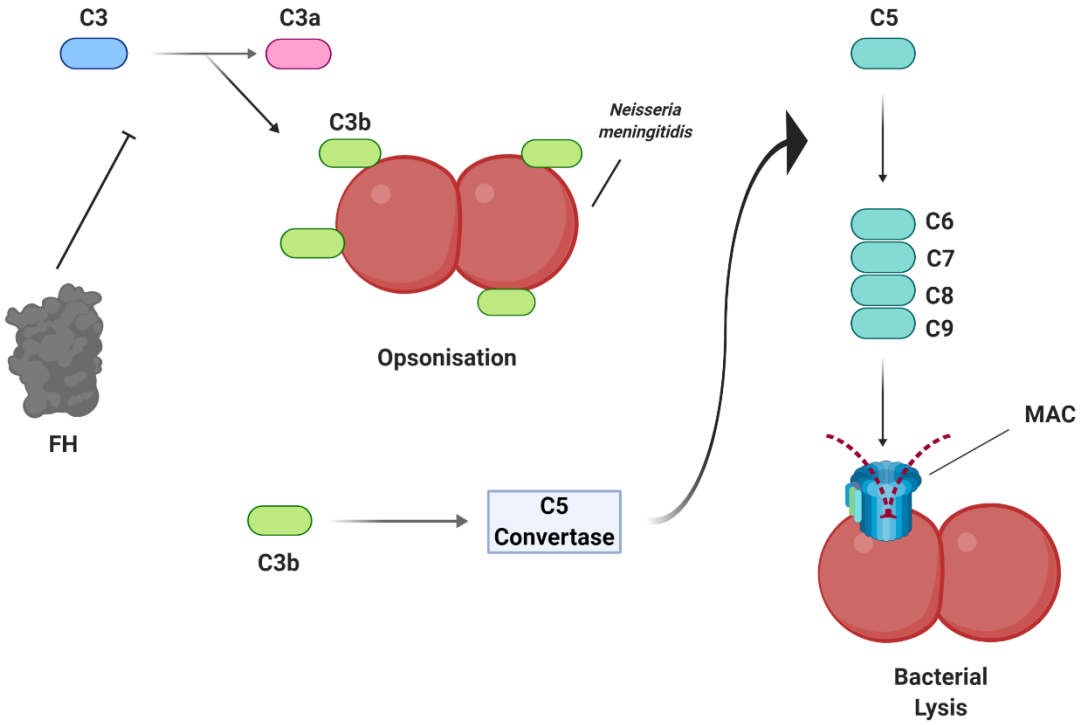


Figure 1. 6- Schematic diagram of activated complement cascade

All three complement cascade pathways converge to C3 convertases which are responsible for the opsonisation (labelling) of the target. This leads to identification of the microorganism for phagocytosis. By the end of the pathway, C5 convertases generate the MAC that culminates with death of the microorganism. Complement inhibition can be achieved by host molecules such as FH (image adapted from Koelman *et al.*, 2019).

One element of the complement cascade of important relevance to the meningococcus is Factor H (FH). The protein FH is central for regulating the alternative pathway inhibiting its action. FH is a 150 kDa single-chain

glycoprotein that contains 20 complement control protein (CCP) domains responsible for interacting with C3b (Ripoche *et al.*, 1988). Pathogens can bind to all 20 domains, however, normally, they have higher affinity for domains 5–7 and 19–20 (Ermert *et al.*, 2019). Not surprisingly, Nm has evolved several virulence factors like FHbp to bind this molecule and evade killing by complement (Granoff *et al.*, 2009).

1.3.1- Inflammation response to *N. meningitidis*

Nm dissemination causes exacerbated inflammatory responses in the host (Johswich, 2017). The main PRRs that take part in identifying Nm PAMPs are Toll-like receptors (TLR), including TLR2, TLR4 and TLR9 (Mogensen *et al.*, 2006). Upon binding the microorganism, they trigger an inflammatory response by inducing the expression of many genes such as those encoding cytokines and chemokines.

Chemokines and cytokines are small cell-signaling proteins that are responsible for recruiting immune cells to the site of invasion and activating them to differentiate (Turner *et al.*, 2014). Whilst chemokines are classified into different families based on the position of the N-terminal cysteine residue, cytokines are classified according to the type of immune response they induce. Cytokines can be adaptive immunity interleukins, or pro-inflammatory and anti-inflammatory molecules (Turner *et al.*, 2014). In invasive meningococcal disease high levels of virtually all chemokines and cytokines are observed, particularly cytokine IL-6 and chemokine IL-8 when the meningococcus causes bacteremia/sepsis (Møller *et al.*, 2005; Schultz *et al.*, 2008; Waage, 1989). Interestingly, expression of these proteins has been linked to cytokine-promoted damage that occurs upon meningococcal transit through cell layers (Hill *et al.,* 2010a).

Some of the virulence factors which are described in section 1.4 have been shown to induce inflammation. Neisserial LOS is recognised by TLR4 in macrophages and peripheral blood mononuclear cells and evokes expression of cytokines such as TNF, IL-6 and IL-1α (Ingalls *et al.*, 2000; Janeway and Medzhitov, 2002; Zughaier *et al.*, 2004). TLR9 has been

shown to be able to recognise genomic neisserial DNA in PBMCs and HEK cells provoking expression of IL-8, IL-6, TNF and IP-10 (Mogensen et al., 2006). PorB and NhhA have been shown to signal via TLR2 in human airway epithelial cells (Liu *et al.*, 2010) and macrophages (Wang *et al.*, 2016), respectively.

1.4- Virulence factors

Over the past few decades, many antigens have been identified as the molecular basis for the virulence of the meningococcus, including the capsular polysaccharide, major surface adhesion proteins including pili, Opa and Opc, and several minor adhesion molecules such as NadA, NhhA, App, and MspA. Other virulence factors include endotoxin (LOS), iron-binding proteins, Porins A and PorB and the lipoprotein FHbp (Fig 1.7; Stephens, 2009).

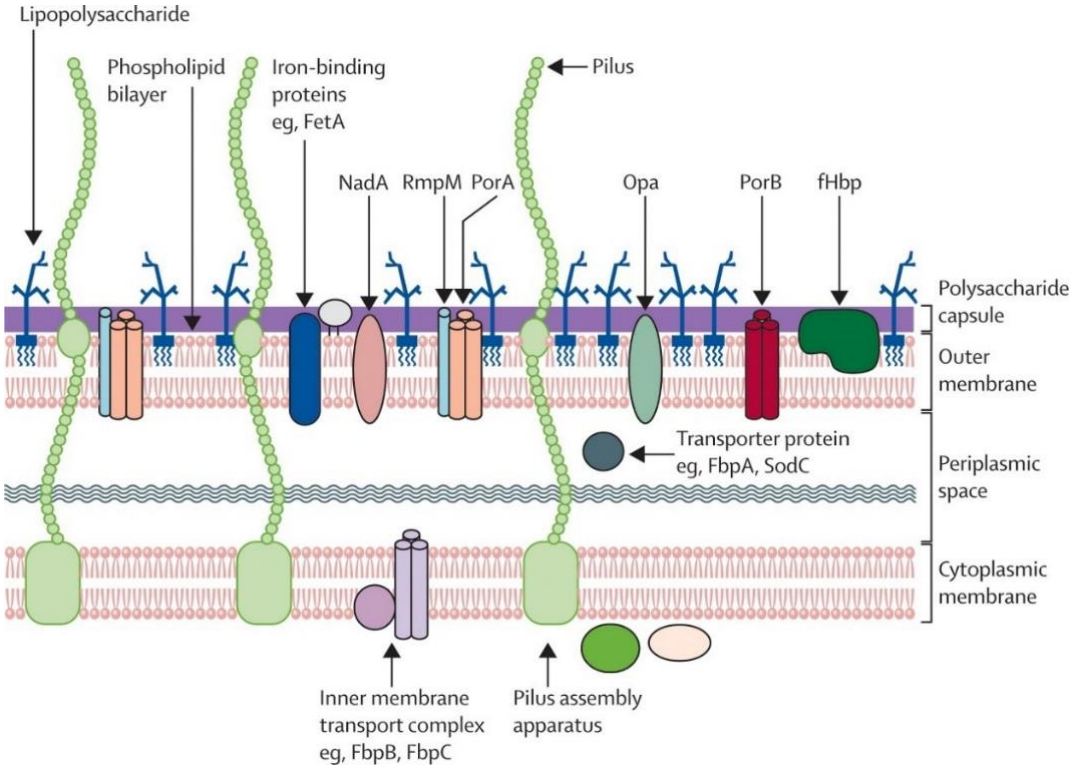


Figure 1. 7- Transverse section of the meningococcal cell envelope

Several virulence factors are shown such as pilus, Opa and FHbp (image from Sadarangan *et al.*, 2010).

1.4.1- Capsule

Invasive strains of Nm are always encapsulated. The presence of capsule allows survival of the meningococcus in the bloodstream protecting it against complement-mediating killing and phagocytosis (Uria *et al*., 2008). The main meningococcal capsular polysaccharides are composed of sialic acid derivatives such as N-acetylneuraminic acid (Neu5Ac), except capsule A, which is formed of N-acetyl-mannosamine-1-phosphate instead (Tzeng *et al.*, 2016). Neu5Ac is one of the main types of sialic acid found in humans, and the meningococcus uses it to mimic the host and avoid being

recognised as foreign (Blacklow and Warren, 1962; Estabrook *et al.*, 1997; Kahler *et al*., 1998).

Depending on the serological reaction to the capsule, Nm can be classified into 13 different serogroups; A - E, I, K, L, H, X, W, Y, Z. Their expression and surface localisation are regulated by genes clustered at a locus named *cps*. Each region of this locus has been reported to be responsible for an aspect of the capsule biogenesis (Fig 1.8). For instance, genes in region A encode enzymes related to capsule biosynthesis while regions B and C have been identified as being related to translocation of the capsule to the OM and to the expression of the isomerase required for 3-deoxy-D-manno-octulosonic acid (Kdo) biosynthesis, respectively (Harrison *et al*., 2013; Tzeng *et al.*, 2016). Expression of the genes linked to Kdo biosynthesis is important since their inactivation affects both sialic acid and non-sialic acid capsules expression (Tzeng *et al.*, 2002). The meningococcus can switch capsule expression on and off by phase variation and it uses this mechanism to modulate capsule expression to help evade host immunity (Frosh and Vogel, 2006).

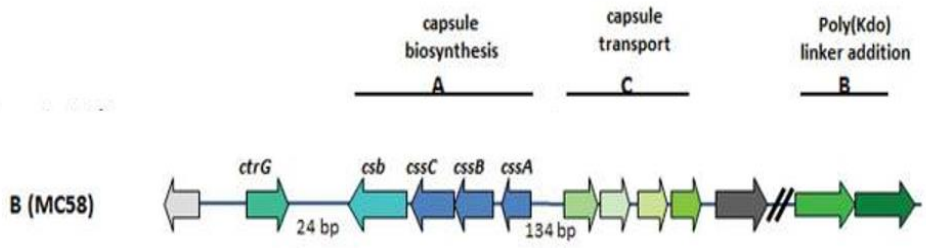


Figure 1. 8- Genetic organization of the capsule loci of Nm serogroup B

Neisserial capsule genes locus are organised in regions A, B and C on the *cps* locus. Gene cluster organisation of capsule B is shown as well as some of the known shared genes implicated in capsule biosynthesis of region A (image adapted from Tzeng *et al.*, 2016).

1.4.1- Lipo-oligosaccharide

Similar to the capsule, LOS helps in the survival of Nm in the host. Specifically, LOS is involved in the defense against cationic antimicrobial peptides (CAMPs) expressed by macrophages and neutrophils (Rouphael and Stephens, 2012) and its sialylation helps the organism to evade host defenses by mimicry as with the capsule (Mandrell *et al.*, 1988). LOS is composed of three parts, lipid A, a core oligosaccharide and heptose residues (Fig 1.8; Kahler and Stephens, 1998) that can bind to different human receptors such as CD14 and Toll-like receptor 4 (TLR4) and induce expression of important inflammatory proteins in the host, including IL-6 and TNF-α (Braun *et al*., 2002; Zughaier *et al*., 2004). Phase variation is also employed to regulate the expression of genes responsible for LOS biogenesis such as the *lgt* genes which are involved in the extension of saccharide chains (Fig 1.9; Jennings *et al.*, 1999).

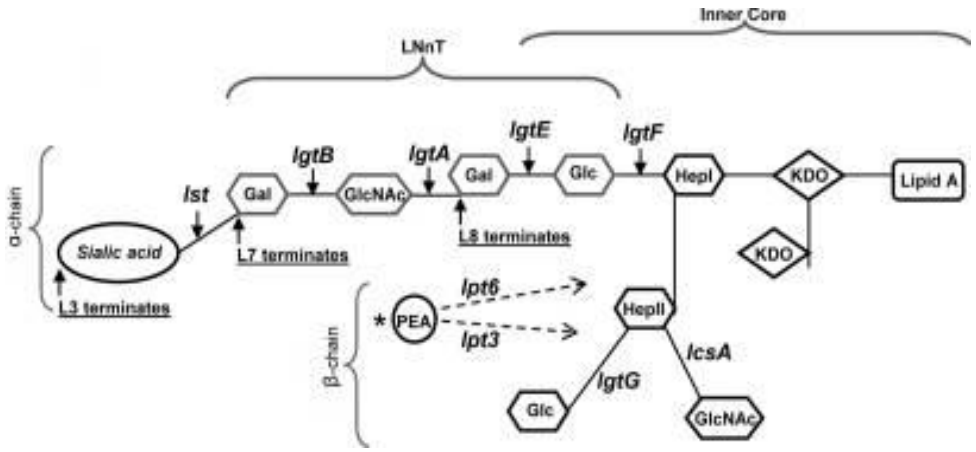


Figure 1. 9- Nm LOS structure

LOS is composed of three parts, lipid A, a core oligosaccharide and heptose residues. Several enzymes involved in the biosynthesis of LOS are shown (image from Hill *et al.*, 2010).

1.4.2- Major adhesins: Pilus and Opacity Proteins

Several surface structures, besides capsule and LOS, are involved in host cells interactions and pathogenicity. Nm exploits these structures as a mechanism to subvert the immune system, evade killing and promote colonisation. Among these structures, adhesins such as Opa, Opc and pili are the main ones used by the meningococcus to adhere to and invade host cells (Pizza and Rappuoli, 2015). While Pili and Opa are related to tissue specificity helping the binding to mucosal non-ciliated epithelial and endothelial cells, Opa and Opc help with cell invasion of these tissues. Antigenic and phase variations of these structures play an important role in regulating their expression so they can work in an orchestrated way to colonise the host (Carbonnelle et al., 2009).

Pili mediate adhesion but are also responsible for DNA uptake, twitching motility and bacterial migration (Pizza and Rappuoli, 2015). Pili can be projected several nanometres beyond the capsule and can be found post-transcriptionally modified with sugars. Piliated cells adhere to and invade host cells more effectively than non-piliated cells (Marceau *et al.*, 1998; Stephens and McGee, 1981). More than 20 genes are involved in pilus biogenesis (Brown *et al.*, 2010). PilE is the major structural subunit (Kolappan *et al.*, 2016). PilQ forms the pore for pilus extrusion, and pilus tip-located PilC is thought to play an essential role in pilus-mediated adhesion (Rudel *et al.*, 1995; Scheuerpflug *et al.*, 1999) Other proteins such as PilF and PilT promote extension, retraction, and loss of pili (Fig 1.10; Carbonnelle *et al.*, 2009; Freitag *et al.*, 1995).

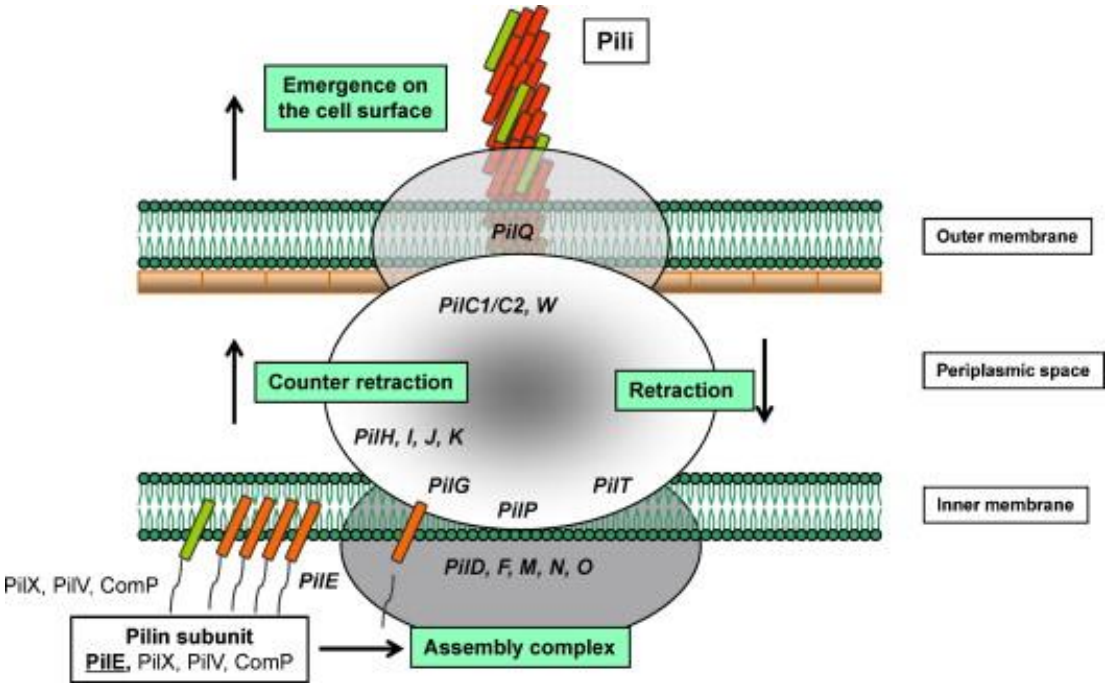


Figure 1. 10- Pilus Biogenesis

An illustration of the complex molecular mechanism of pilus biogenesis. Several proteins are involved in pilus assembly and function. PilE is the major subunit but requires a platform of other genes (eg. PilD, PilF, PilM, PilN, PilO, PilP) for its correct scaffolding and localisation (image from Carbonnelle *et al.*, 2009).

Whilst pili promote initial adhesion to host cells, Opa and Opc are involved in intimate adhesion which are easy to detect on acapsulate strains (Virji *et al.*, 1992). Opa and Opc are the most abundant adhesins in the OM. Both genes are regulated by phase variation. Three to four copies of Opa genes can be found in the neisserial genome, but, by contrast, Opc is expressed by one single gene (Hung and Christodoulides, 2013). They act independently to promote microbial adhesion and possess affinities for different human receptors. Most Opa proteins bind primarily to members of the human carcinoembryonic antigen (CEACAM) receptor family (Hill *et al*.,

2010a). Variations in the quantity and in the amino acid sequence of CEACAM molecules influence how effectively Opa will bind to a cell (Popp *et al.*, 1999; Rowe *et al.*, 2007). Opc can bind to a variety of different human cell receptors including heparan sulphate proteoglycan (HSPG), integrins, ECM proteins such as vitronectin and fibronectin (de Vries *et al.*, 1998; Virji *et al.*, 1994). Low expression of Opc permits more Opa-dependent adhesion, however it is not completely clear how both adhesins coordinate their function for successful adhesion and invasion (de Vries *et al.*, 1996).

1.4.3- Minor adhesins

Several other proteins have been shown to assist in meningococcal adhesion by capsulated neisserial cells including NadA, NhhA, App and MspA. NadA and NhhA aid neisserial binding to epithelial cells. Specifically, NadA binds to β1 integrins (Nägele et al., 2011) and NhhA is able to bind to laminin and heparin sulphate (Scarselli et al., 2006). MspA helps with adhesion to epithelial and endothelial cells (Turner *et al.*, 2006). App, which is homologous of MspA, is known to facilitate colonisation and spread (Serruto *et al*., 2003). Host receptors for these adhesins have not yet been identified (Hung and Christodoulides, 2013).

1.4.4- Other virulence factors

Several other virulence factors have been shown to be crucial for Nm survival *in vivo*. The ability to survive in the hostile environment of the host depends on the efficient acquisition of nutrients like iron and subverting the immune system. Here other virulence factors are also introduced.

1.4.4.1- Porins

The OM proteins, PorA and PorB, are important virulence factors involved in cell signaling and transport of small molecules through the meningococcal membrane (Jarva *et al.,* 2005; Massari *et al.*, 2000). Whereas PorA has been shown to inhibit complement-mediated killing via interaction with the complement regulator C4b-binding protein (C4bp) (Jarva *et al*., 2005), PorB has been demonstrated to be important for protecting host cells from

apoptosis (Massari *et al.*, 2000). The involvement of PorA and PorB in invasion is thought to be through actin manipulation of host cells during infection (Nassif *et al*., 1999). PorA expression is phase variable (van der Ende *et al*., 1995) and both porins have been reported to be absent in some strains (Peak *et al*., 2016).

1.4.4.2- Iron transport systems

There is little freely available iron in the host. Due to the importance of iron in several meningococcal metabolic processes such as DNA replication and electron transfer in the respiratory chain, Nm has evolved several mechanisms to acquire iron from its host (Schoen *et al.*, 2014). There are at least five known meningococcal receptors capable of sequestering iron from the host, including TonB-dependent receptors, transferrin-binding protein A and B (TbpA/TbpB), lactoferrin-binding protein A and B (LbpA/LbpB), hemoglobin-binding outer membrane protein (HmbR), and haptoglobin-binding protein A and B (HpuA/HpuB) (Perkins-Balding *et al.*, 2004). Some of these iron acquisition systems comprise two proteins, a surface-exposed lipoprotein and an integral OMP (Tommassen and Arenas, 2017).

Iron acquisition is vital for meningococcal survival *in vivo.* A study published by Echenique-Rivera *et al.* (2011), showed that a mutant of TbpB was unable to grow in blood. Iron is mainly found bound to proteins in the host, for instance, to transferrin in serum, lactoferrin in mucosal surfaces and hemoglobin and ferritin within cells (Perkins-Balding *et al*., 2004). The iron is bound with high affinity to these proteins (Perkins-Balding *et al*., 2004).

The iron receptors expressed by Nm directly hijack iron from the host proteins (Perkins-Balding *et al.*, 2004; Schoen *et al.*, 2014).

1.4.4.3- Secretion Systems

Nm possesses at least three secretion systems that are important for its virulence. The autotransporter (AT) pathway responsible for secreting IgA protease and Neisseria autotransporter NalP; the two-partner secretion (TPS) system known as hemagglutinin/hemolysis-related protein A and B (HrpA/HrpB); and the type 1 secretion system (T1SS). These are important systems involved in pathogenesis, for instance, the TPS system have been implicated in biofilm formation (Neil and Apicella, 2009), and to adhesion to epithelial cells (Schmitt *et al*., 2007) and are found in invasive clonal complexes of Nm (van Ulsen *et al.*, 2008). Moreover, whilst NalP has been demonstrated to subvert the immune system by ultimately, taking part in the cleavage of the complement element C3 (Roussel-Jazédé *et al.*, 2010), IgA protease has been shown to not only be responsible for promoting bacterial survival (Lin *et al.*, 1997b) but also for facilitating adhesion and biofilm formation (Tommassen and Arenas, 2017).

1.4.5- Lipoproteins

Lipoproteins are a diverse class of multifunctional, membrane-associated molecules which constitute a significant fraction of the OM of Gram-negative bacteria (Nakayama *et al*., 2012). They are covalently modified at the periplasmic side of the IM by the addition of a lipid moiety and most are thought to then be translocated to the OM. The lipid moiety allows anchorage to IM or OM. (Konovalova and Silhavy, 2015; Macek *et al.*, 2019). Lipoproteins have diverse functions ranging from maintaining envelope architecture and stability to mediating host-pathogen interactions. (Okuda and Tokuda, 2011; Nakayama *et al*., 2012; Zückert, 2014). In Nm some important examples of lipoproteins implicated in pathogenesis include TbpB, LbpB, and HpuA which are involved in iron acquisition, and the autotransporter NalP (Tommassen and Arenas, 2017). Other examples include the adhesin NHBA, and the surface-exposed FHbp (Tommassen and Arenas, 2017), which are both involved in subverting the immune system recruiting FH.

1.5- Meningococcal Vaccines

Many of the virulence factors that elicit immunogenic response have been investigated as vaccine candidates. Several capsular polysaccharide-based vaccines are licensed for use against serogroups A, C, Y and W. They have proved to be effective against invasive meningococcal diseases caused by these serogroups (Snape and Pollard, 2005; Cooper *et al.*, 2011). Nowadays, however, a protein-polysaccharide conjugate version of these vaccines is preferred for offering improved longer protection by enhanced antibody response (Dretler *et al.*, 2018). Two of the major companies that manufacture meningococcal vaccines are Pfizer and GlaxoSmithKline (GSK). Their quadrivalent meningococcal polysaccharide vaccines that cover serogroups A, C, Y, and W consist of the polysaccharide conjugated to tetanus toxin (Pfizer) or *Corynebacterium diptheriae* CRM197 toxin (GSK), respectively.

Due to the similarity of the serogroup B polysaccharide to structures found on neural cells, this capsule is not suitable as vaccine against serogroup B strains (Finne *et al.*, 1987; Yongye *et al*., 2008). Non-capsular, conserved, surface antigens such as NadA, PorA, NHBA, and FHbp were tested as vaccine candidates against organisms expressing group B capsule (McNeil *et al*., 2013). FHbp is one of four antigens of the Bexsero (GSK) vaccine that also includes NadA, NHBA, and outer membrane vesicle (OMV) expressing PorA from the New Zealand strain NZ PorA P1.4 (Feavers and Maiden, 2017). FHbp is also the sole antigen of Trumenba vaccine (Pfizer) which is composed of two common variants of FHbp both of which are lipidated, unlike FHbp in Bexsero (Feavers and Maiden, 2017).

FHbp is one of the most well studied vaccine antigens to date. FHbp is a surface-exposed lipoprotein and is the focus of this thesis. The investigation of how bacteria process and localise lipoproteins antigens to the cell surface could provide useful knowledge to those developing lipoprotein-based vaccines and valuable insight for improving FHbp-based vaccines such as Trumemba. In the following sections, how bacterial cells manage to export and surface localise lipoproteins is introduced.

1.6- Lipoprotein Biogenesis

The mechanism of lipidation of lipoproteins has been well studied in the gram-negative organism *E. coli*. The lipidation process involves three main steps following translocation of the preprolipoprotein (lipoprotein precursor) from the cytoplasm to the IM by the general secretion pathway (Sec-pathway) or twin arginine translocation (Tat-pathway) pathway (Natale *et al.*, 2008). Sec typically exports unfolded proteins while Tat is described as the typical export pathway for folded proteins (Nakayama *et al.*, 2012). Export of preprolipoproteins by Sec is driven by the SP which incorporates the lipobox, a conserved motif with the typical consensus sequence $[LVI]^{(-3)}[ASTVI]^{(-2)}[GAS]^{(-1)}C^{(+1)}$ (Qi *et al*, 1995; Babu *et al.*, 2006).

1.6.1- Translocation across the IM via Sec translocon

Post-translational modification and correct localisation of lipoproteins require a number of different mechanisms in the cell that include several proteins in the cytoplasm, IM and OM. The unfolded preprolipoproteins contain a cleavable SP that is recognised as soon as it exits the ribosome by proteins of the Sec translocon (Fig 1.11; Tsirigotaki *et al.*, 2017). SecA binds the signal peptide and SecB binds to the remaining nascent preprolipoprotein which prevents it from folding (Chatzi *et al.*, 2014; Chatzi *et al.*, 2013; Huber *et al.*, 2011). SecA is the ATPase-motor protein responsible for proof-reading the nascent proteins and for delivering them to the SecYEG translocase machinery (Economou and Wickner, 1994; Schiebel *et al.*, 1991). Translocation of preprolipoprotein through SecYEG of the plasma membrane occurs via repeated cycles of ATP binding and hydrolysis by SecA and proton motive force (Chatzi *et al.*, 2013; Economou and Wickner, 1994; Schiebel *et al.*, 1991). Once in the IM, preprolipoproteins undergo processing including diacylation, signal peptide cleavage and further acylation. Most mature lipoproteins are then sorted to the OM (Auclair *et al.*, 2012; Tsirigotaki *et al.*, 2017).

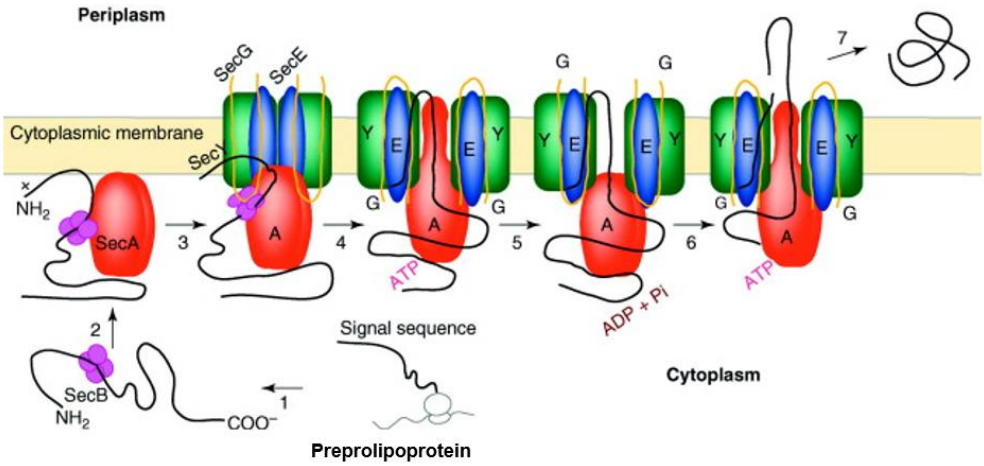


Figure 1. 11- Translocation of preprolipoproteins across the cytoplasmic membrane (IM) by the Sec translocon

The key steps of targeting and sorting by cytoplasmic proteins, SecA and SecB (1-3); and translocation by SecYEG (4-7) (image from Mori and Ito, 2001).

Several other proteins have been reported to be involved in translocation across the IM. Targeting, for instance, can be achieved by the trigger factor (Oh *et al*., 2011) or by the signal recognition particle (SRP) and its membrane receptor FtsY (Akopian *et al.*, 2013). Translocation is enhanced by the presence of the auxiliary proteins in the cytoplasm such as SecDF–YajC and YidC (Akopian *et al.*, 2013; Schulze *et al.*, 2014). Besides the main translocation machinery SecYEG, only the ATPase-motor SecA has been shown to be essential (Tsirigotaki *et al*., 2017).

1.6.2- Pathway for di- and tri-acylation of lipoproteins in *E. coli* and other bacteria

Upon translocation to the IM, lipoproteins undergo maturation and transportation to their final destination. The mechanism of lipid modification (Fig 1.12) involves the transfer of a diacylglyceryl group to the cysteine of the lipobox by the enzyme lipoprotein diacylglyceryl transferase (Lgt) (Mao *et al.*, 2016). This generates the prolipoprotein (Wu and Tokunaga, 1986).

Following this, the signal peptide at the *S*-diacylglyceryl cysteine is cleaved by the lipoprotein signal peptidase (LspA) which generates the substrate for the next enzyme in this pathway, an apolipoprotein with a new N-terminal cysteine at +1. The third step involves the addition via amide linkage of another acyl group by the lipoprotein n-acyl transferase (Lnt) to the available amino group of the *S*-diacylglyceryl cysteine of the apolipoprotein. The fully mature triacylated lipoprotein may then go on to be sorted to the OM where the lipid moiety anchors the mature lipoprotein into the OM (Zückert, 2014). Here the majority of lipoproteins face the periplasm, however three lipoproteins in *E. coli* are flipped to be partially exposed at the cell surface; Rcs-signaling pathway protein F (RscF), Braun lipoprotein (Lpp) and outer membrane protein assembly complex C (BamC) as reviewed by Hooda and Moraes (2018).

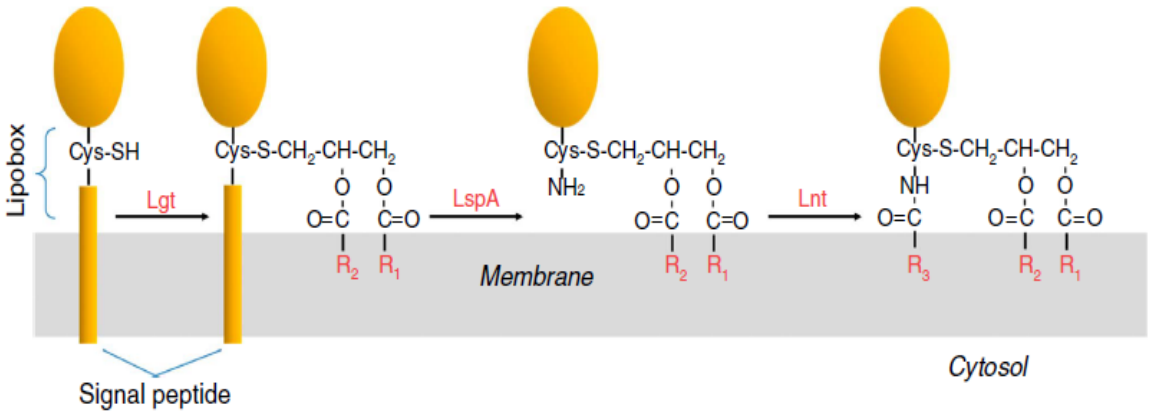


Figure 1. 12- Pathway for di- and tri-acylation of lipoproteins in *E. coli* and other bacteria

The figure shows the pathway for lipidation of a mature lipoprotein. Lgt adds a diacyl group to the preprolipoprotein, next LspA cleaves the signal peptide of the prolipoprotein and Lnt adds the third acyl group to the apolipoprotein generating the now mature triacylated lipoprotein. (image from Mao *et al.*, 2016).

All three enzymes are considered essential in *E. coli*, however recent studies have demonstrated that certain Gram-negative organisms, such as Ng and *Francisella tularensis*, and low-GC-content Gram-positive bacteria (Firmicutes[2]), can survive without the expression of Lnt (LoVullo *et al.*, 2015; Chahales and Thanassi, 2015). By contrast, in Gram-positive bacteria, Lgt and Lsp seem to be essential in some of the tested Actinobacteria but not in low-GC-content species (Nakayama *et al*., 2012). Due to their importance in this thesis, each enzyme is reviewed in the following sections.

1.6.2.1- Diacylglyceryl transferase (Lgt)

Lgt is an IM enzyme (Pailler *et al.*, 2012) that utilizes negatively charged phospholipids as lipid substrates (Sankaran and Wu, 1994). After translocation to the IM of the preprolipoprotein, Lgt is the enzyme responsible for transferring the diacylglyceryl group from membrane phospholipids to the thiol group of the invariable cysteine residue of preprolipoproteins, producing prolipoproteins (Mao *et al*., 2016).

A study of *E. coli* Lgt's structure (Fig 1.13) by Mao and co-workers (2016) has revealed the periplasmic location of key protein structures of Lgt that are essential for transacylation. This study also revealed how specific lateral entry of Lgt substrates is required for successful catalytic activity by Lgt.

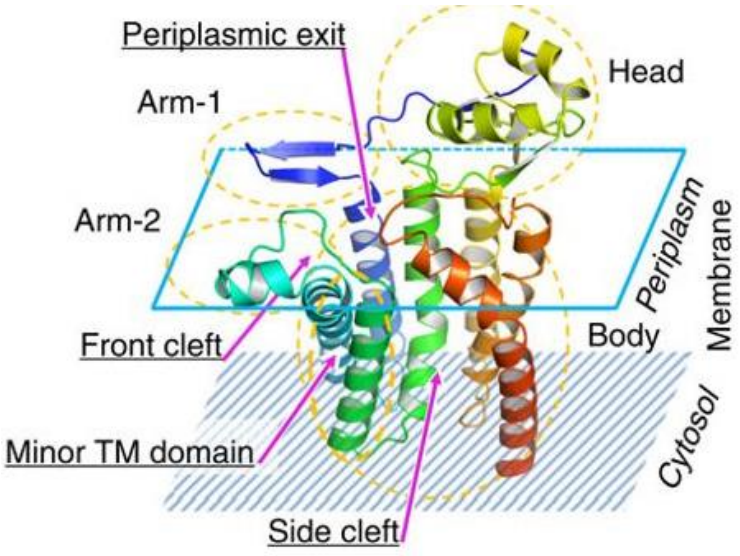


Figure 1. 13- Resolved structure of Lgt

Cytosolic and periplasmic portions of Lgt can be observed (image from Mao *et al.,* 2016).

Lgt is indispensable in many gram-negative bacteria such as *E. coli*, and has been proposed to be a possible target of new drugs (El Arnaout and Soulimane, 2019). It is thought to not be essential for Firmicutes and high-GC-content Actinobacteria, except *M. tuberculosis* and *S. coelicolor* for which Lgt absence causes major global growth defect (Nakayama *et al.*, 2012; Tschumi *et al.*, 2012).

1.6.2.2- Signal Peptidase (LspA)

After initial diacylation of preprolipoproteins by Lgt, LspA (a type II signal peptidase) is responsible for cleaving the signal peptide from the amino acid immediately upstream of the now lipidated cysteine residue, generating apolipoproteins. This enzyme is known to not be essential for some gram-positive bacteria but it is thought to be essential for gram-negative bacteria (Nakayama *et al*., 2012).

LspA is a small protein (169 amino acids in *P. aeruginosa*) that is an integral IM protein with two main domains (cytoplasmic and periplasmic domains) (Fig 1.14) and a transmembrane domain (Vogeley *et al.*, 2016). Structural experiments by Vogeley and colleagues (2016) identifying important amino acid residues in this protein suggested the catalytic site of this enzyme has two crucial aspartate residues Asp124 and Asp143. Importantly, it has been shown that LspA requires diacylated substrate to function indicating how crucial lipidated substrates are for this enzyme (Vogeley *et al.*, 2016).

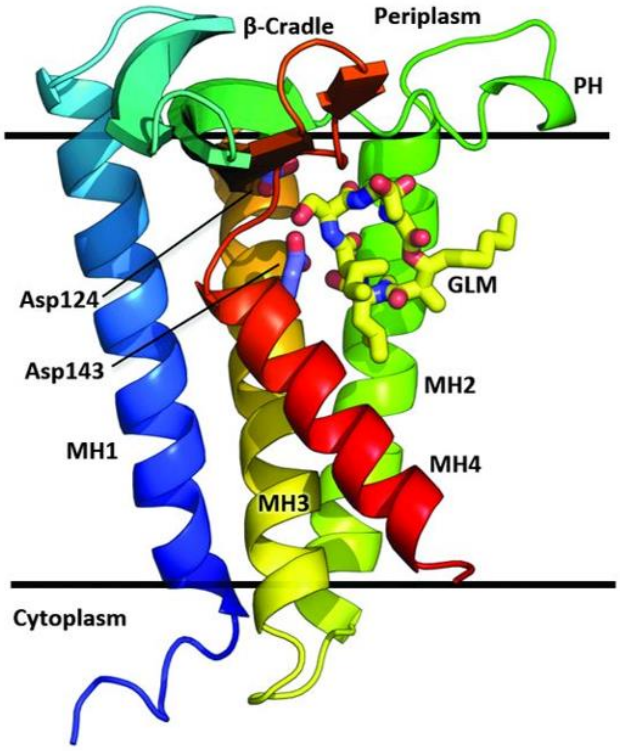


Figure 1. 14- Resolved structure of LspA

LspA structure with globomycin (GLM) binding to catalytic site. Key residues Asp124 and Asp143 are shown. Transmembrane helices are labelled MH1 to MH4 and periplasmic helix is labelled PH (image from Vogeley *et al.*, 2016).

Due to its essentiality in gram-negative bacteria, LspA is being investigated as a drug target. Studies with globomycin, known to inhibit LspA, show that the blocking the function of this protein causes accumulation of prolipoprotein within the periplasm and cell death in *E. coli* (Hussain *et al.*, 1980; Lai *et al.*, 1981; Yakushi *et al.*, 1997).

1.6.2.3- Apolipoprotein N-acyltransferase (Lnt)

After diacylation and SP cleavage, apolipoproteins are modified with the addition of a third acyl group from a phospholipid at α-amino group of the diacylated cysteine by Lnt (Nakayama *et al*., 2012).

Like Lgt and LspA, Lnt is an integral IM protein. Crystal structures of *E. coli* Lnt showed that Lnt has six trans-membrane spanning domains with a lateral opening for substrate entrance and a substrate exit to the periplasm (Fig 1.15; Lu *et al.,* 2017). The largest domain of Lnt was identified as a nitrilase-like domain bearing the catalytic site. Lnt catalyses the hydrolysis of nitriles to carboxylic acids and ammonia (Brenner *et al.*, 2016).

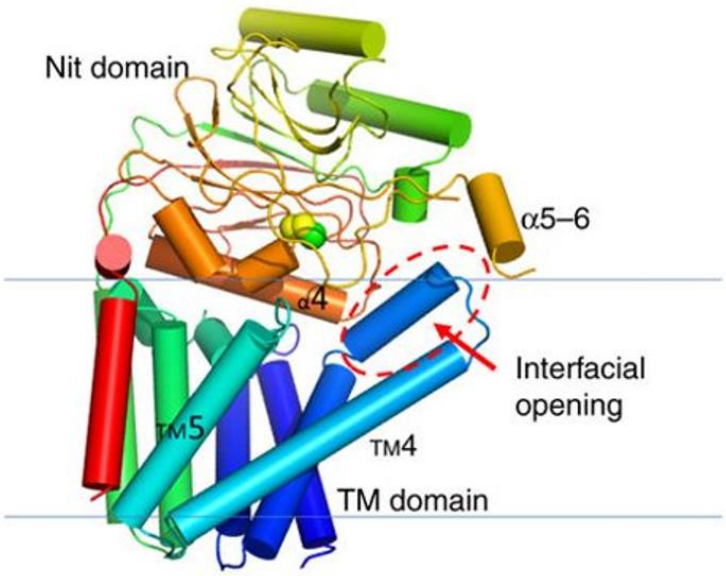


Figure 1. 15- Resolved structure of Lnt

Nitrilase (Nit) domain, interfacial opening, transmembrane domains (TM) and alpha (α) helixes of Lnt are shown (image from Lu *et al.,* 2017).

Studies in *E. coli* show that N-acylation by Lnt is crucial to ensure correct and efficient sorting of lipoproteins to the OM (Narita and Tokuda, 2011).

1.6.3- Sorting of lipoproteins to the OM

Most triacylated (mature) lipoproteins are next translocated (sorted) to the OM by the lipoprotein outer membrane localization (Lol) apparatus. The final destination of lipoproteins to either the IM or OM is determined by the amino acid sequences that follow the Cys residue of the lipobox. In *E. coli* the presence of an Aspartic Acid (D) residue at position +2 causes IM retention of the mature lipoprotein (Yamaguchi *et al.*, 1988). Most lipoproteins are destined to the OM, unless the above so-called or other 'Lol avoidance' signal is present (Zückert, 2014).

In *E. coli,* five proteins are involved in the Lol pathway, Lol A-E. After preprolipoproteins are transported via Sec to the IM (Fig. 1.16) and lipidated, the mature triacylated lipoprotein is extracted from the IM by the LolCDE complex. This an ABC transporter-like complex that releases the lipoprotein into the periplasm and delivers this to the chaperone LolA which, in turn, delivers it to the acceptor protein LolB which finally inserts the lipoprotein into the OM (reviewed by Konovalova and Silhavy, 2015).

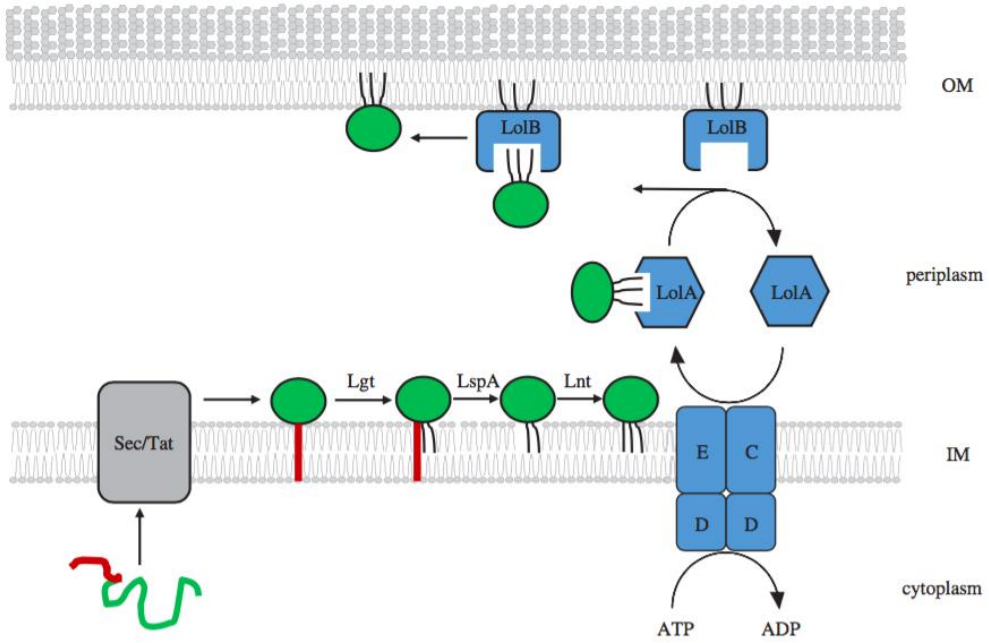


Figure 1. 16- Lipoproteins lipidation pathway and sorting to the OM

Following lipidation and cleavage of the signal peptide by enzymes Lgt, LspA and Lnt, the now mature lipoprotein is recognised by the Lol machinery, responsible for sorting it to the OM. LolCDE removes the new lipoprotein from the IM and delivers it to LolA which is responsible for translocation of the new lipoprotein across the periplasm. LolA then delivers the new lipoprotein to LolB which will then facilitate the new lipoprotein insertion in the inner part of the OM (image from Konovalova and Silhavy, 2015).

1.6.4- Surface Lipoprotein Assembly Modulator (Slam)

Whilst in *E. coli* only three lipoproteins are partially surface-displayed (Hooda and Moraes, 2018), in Nm eight lipoproteins are flipped to face the exterior (Hooda *et al*., 2017a). For decades, the mechanisms used by bacteria to perform this mechanism remained elusive. Recently, Hooda and co-workers (2016) showed that the protein named Slam is responsible for performing the flipping of two neisserial lipoproteins, TbpB and FHbp (Hooda et al., 2016). Hooda *et al.* (2016) showed that Slam alone is able to localise both proteins to the surface of *E. coli* that otherwise would not surface-display them. They have suggested that the signal for recognition by Slam is at the C-terminus of these proteins. Moreover, in a different study, they demonstrated Slam-like proteins to be widely present in several different gram-negative microorganisms (Hooda *et al.*, 2017).

1.7- Factor H binding protein (FHbp) as a vaccine antigen

In the quest for suitable vaccine candidates to target serogroup B strains, whole genome sequencing (WGS) of Nm enabled the selection of new antigens by reverse vaccinology (Pizza *et al*., 2000). Following the identification of surface-exposed FHbp in laboratory reference strain MC58, its prevalence and surface localisation were investigated in diverse strains by flow cytometry and its ability to induce protective antibody response (Masignani *et al*., 2003; Borrow and Miller, 2006).

FHbp is classified as two main families (A and B) and as three main subvariants (var1, var2 and var3), which are further divided into sub-groups due to high sequence variation between strains (Brehoni *et al.*, 2009). FHbp is lipid-modified with three palmitoyl fatty acids (Mascioni *et al*., 2010). Currently, there are two approved vaccines consisting of recombinant FHbp. Novartis bought by GSK developed the 4CMenB vaccine, Bexsero, containing non-lipidated FHbp and three other meningococcal antigens, and Pfizer vaccine Trumenba comprises two lipidated subvariants of FHbp (reviewed by McNeil *et al*., 2013; and Seib *et al.*, 2015).

A concern was raised with the Bexsero vaccine after a study was discontinued in infants when cases of unexpected reactogenicity arose even under low dosage administration of the vaccine were observed (McNeil *et al*., 2013; Martino-Torres *et al.*, 2014; Seib *et al.*, 2015). Overall, Bexsero confers strong serum bactericidal assay (SBA) responses and has generated encouraging results as an immunization tool in infants and adolescents after at least two doses of the vaccine and data indicate that its implementation diminishes the transmission in the tested groups (Read *et*

al., 2014). However, a recent study carried by Marshall *et al.* (2018) showed that no herd immunity was reached when vaccinating teens with the 4CmenB vaccine. This study highlights the importance to continue to improve the current methods of immunization against meningococcus group B.

The development of Trumenba was based on observations that the lipidated form of FHbp elicited greater immunogenicity and breadth of protection compared with the non-lipidated version in mice (Fletcher *et al*., 2004). This vaccine was licensed in the USA in October 2014 and approved for use in individuals aged 10 to 25 by the Food and Drug Administration agency (FDA). The potency of this vaccine and its ability to protect against diverse strains of Nm is still under investigation.

Concerningly, in addition to the variation in sequence of FHbp between strains which could lead to failure of Trumemba to provide sufficiently broad protection, different strains express different levels of FHbp (Fletcher *et al.*, 2004; Biagini *et al*., 2016). For some isolates, the level of expression is below that required for recognition by FHbp-specific antibodies and this could lead to emergence of vaccine escape strains (Newcombe *et al.*, 2014)

1.7.1- Regulation of FHbp

FHbp regulatory pathways in Nm are not fully understood. Newcombe *et al.* (2014), for instance, showed poor surface display of FHbp in the OM in strain L91543. Griffin and co-workers identified SNPs in the SP of FHbp of this strain which could potentially affect its ability to be transported across the IM and subsequently surface (Karlyshev *et al.*, 2015).

Until now, environmental factors such as oxygen, temperature and iron availability are known to influence FHbp expression levels. Oriente *et al*. (2010) showed that *fHbp* has two different transcripts, one of them having its promoter regulated by oxygen limitation in an FNR-dependent manner. Low oxygen induces the expression of this transcript and this suggests that FHbp expression may be important in the low oxygen environments found in the host during infection, specifically after crossing mucosal barrier (Oriente *et al.*, 2010).

Temperature was showed to be important in regulating the translation of *fHbp* with expression induced between 37°C and 42°C. Higher temperatures are triggered during inflammation caused by the bacterium which could mean FHbp is playing an important role to evade the immune system at the time of infection (Loh *et al*., 2013). Sanders *et al*. (2012) demonstrated that iron availability is also a key factor that impacts on *fHbp* expression. The mechanism by which it occurs varies among strains. The availability of iron can induce an over-expression of FHbp in strains belonging to clonal complex 32 (cc32) like MC58 (Sanders *et al.*, 2012).

Although the key molecular mechanisms determining FHbp expression have now been elucidated, the molecular pathways of transport across the IM followed by lipidation, sorting to the OM and ultimately surface display, remain to be elucidated. Inevitably this pathway requires a plethora of genes These genes themselves may be subject to regulation subsequently affecting FHbp expression levels.

1.8- Aims of this study

The main aim of this thesis is to elucidate the molecular mechanisms involved in the processing and localisation of FHbp. In addition, the impact of disrupting one of the key genes required for processing is investigated at the molecular, cellular and biological level.

Chapter 2
Materials and Methods

A list of commonly used solutions and reagents is provided in Appendix I. Vector maps and a list of plasmids generated throughout this thesis can be found in Appendix II.

2.1- Bacterial strains

E. coli strain JM109 single use competent cells were purchased from Promega and used for transformations. *E. coli* BTH101 (Euromedex) was used as a reporter strain for BACTH assays. The strains of *N. meningitidis* used were MC58 (B:15:P1.7,16, ST-74; ET-5) purchased from LGC Standards, L91543 (C:2aP1.2, ST-11; ET-37) kindly provided by Professor McFadden (University of Surrey) and H44/76 (B:P1.7,16:F3-3: ST-32) gifted by Professor Rob Read (University of Sheffield). All other group B isolates were provided by Dr Chris Bayliss (University of Leicester) with the approval from Ray Borrow (Public Health England) (Table 2.1) and are listed on the Meningococcus Genome Library database (MRF collection). These latter isolates were obtained from patients in England, Wales, Northern Ireland and Scotland between 2009-2017.

Table 2. 1- Meningococcal serogroup B invasive isolates used in this study and the expressed variant of FHbp in these strains

Strain Number	Strain	Strain designation and clonal complex	Subfamily/variant of FHbp
1	H44/76	ST-15/ cc32	B/1
2	M10_240684	B: P1.5-1,10-8: F5-13: ST-11 / cc11	B/1
3	M10_240701	B: P1.7-2,14: F5-5: ST-213 / cc213	B/1
4	M02_241729	B: ST-11 / cc11	B/1
5	M10_240579	B: P1.7-2,4: F5-1: ST-11238 / cc269	B/1
6	M13_240525	B: P1.7,16: F3-3: ST-2931 / cc32	B/1
7	M04_241215	B: ST-41 / cc41|44	B/1
8	M11_241066	B: P1.7-2,4: F1-5: ST-41 / cc41|44	B/1
9	M13_240614	B: P1.7-2,4: F3-9: ST-10868 / cc41|44	B/1
10	M10_240750	B: P1.7-2,4: F1-5: ST-8203 / cc41|44	B/1
11	M13_240675	B: P1.7-2,4: F5-8: ST-1475 / cc41|44	B/1
12	M11_240236	B: P1.22,9: F5-12: ST-1161 / cc269	B/1
13	M12_240006	B: P1.22,9: F5-12: ST-1161 / cc269	B/1
14	M11_241033	B: P1.7-1,4-1: F5-1: ST-269 / cc269	B/1
15	M14_240367	B: P1.19-1,15-11: F5-36: ST-269 / cc269	B/1
16	M02_240210	ST-1195 / cc269	B/1
17	M11_240077	B: P1.19-1,15-11: F1-7: ST-269 / cc269	B/1
18	M13_240486	B: P1.7-2,13-1: F5-1: ST-269 / cc269	B/1

2.2- Culture Conditions

E. coli strains were grown in Lysogeny broth (LB) broth or agar (Merck), unless where stated, at 37°C with shaking at 200 rpm. All meningococcal strains were grown on GC agar plates or in GC broth (Difco) containing Kellogg's glucose and iron supplements (Kellogg *et al*., 1963) in a moist

atmosphere containing 5% CO_2 at 37°C or at 30°C for transformation experiments and with shaking at 220 rpm for broth cultures.

2.2.1- Determination of Colony Forming Units (CFU)/mL of *N. meningitidis*

Overnight cultures of Nm were standardised in LB to an OD_{600} of 0.1. Serial dilutions were prepared in phosphate buffered saline (Oxoid) covering dilution factors of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}. 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9}. In triplicate, 5µL of each dilution was dropped and spread onto LB agar and incubated overnight at 37°C. Colonies were counted, and the mean CFU/mL was determined using the following formula:

$$number\ of\ colonies \times dilution\ factor \times \frac{1000}{volume\ added\ to\ LB} = cfu/mL$$

2.3- Antibiotics

Antibiotics were purchased from Sigma and were added at the following concentrations: Kanamycin, 30 µg/ml and 60 µg/ml, Erythromycin, 300 µg/ml and 0.3 µg/ml for *E coli* and *N. meningitidis,* respectively; 100 µg/ml Ampicillin for *E. coli;* and 30 µg/ml Nalidixic acid for *E. coli* BTH101.

2.4- Cell suspensions

For each experiment, to adjust to the required optical density at A_{600}, meningococcal cell suspensions were made by resuspending a loop of cells from a freshly grown overnight plate in PBS. Serial dilutions of inocula were then plated to verify consistence in colony forming unit (CFU) counts between strains in triplicates.

2.5- Molecular Methods for DNA manipulation

2.5.1- *E. coli* transformation

Chemically competent *E. coli* were transformed by heat shock (Froger and Hall, 2007). Following incubation on ice for 30min with at least 25ng of DNA, cells were heat shocked at 42°C for 42s. 950µL of LB broth was added and the cells allowed to recover for 1h at 37°C. After the incubation time, they were then plated on agar with appropriate antibiotics.

2.5.2- *N. meningitidis* plate transformation

Nm was transformed following a similar approach to the one used by Zhang *et al.* (2010). Nm was grown overnight on GC agar plates at 30°C to favour piliation. A loop of cells was then re-streaked on a plain GC agar plate, and GC broth with supplemented with 10mM $MgSO_4$ containing at least 250ng DNA was spotted onto the designated area of the plate. A negative control of just GC broth with 10mM $MgSO_4$ was spotted onto a different designated area of the plate. After overnight incubation at 37°C, cells at the designated areas were collected and resuspended in PBS and plated on GC agar plates with the antibiotics of interest.

2.5.3- Genomic DNA extraction

Genomic DNA was extracted from Nm using the GenElute™ Bacterial Genomic DNA Kit Protocol (Sigma). Briefly, a loop of Neisserial cells from overnight plate culture was thoroughly resuspended in 180µL of Lysis Solution T. 20µL of RNase A Solution was added, mixed, and incubated for 2min at room temperature. Next, 20µL of the Proteinase K solution was added to the sample. It was well mixed and incubated for 30min at 55°C. 200µL of Lysis Solution C was then added, vortexed thoroughly (about 15s), and incubated at 55°C for 10min. While lysis was being performed, 500 µL of the Column Preparation Solution was added to a pre-assembled GenElute Miniprep Binding Column and centrifuged at 12,000 × *g* for 1min. The eluate was discarded. After lysis, 200µL of ethanol (95–100%) was added to the lysate and mixed thoroughly by vortexing for 5–10s. Following, the entire contents of the tube was transferred into the binding column, and

centrifuged at ≥ 6500 × *g* for 1 min. The collection tube containing the eluate was discarded and the column was placed in a new 2mL collection tube. 500µL of Wash Solution 1 was added to the column and centrifuged for 1min at ≥ 6500 × *g*. The collection tube containing the eluate was discarded and the column placed again in a new 2mL collection tube. 500µL of Wash Solution was added to the column and centrifuged for 3min at maximum speed (12,000–16,000 × *g*) to dry the column. The column was centrifuged for an additional 1 min at maximum speed if residual ethanol was seen. Finally, the collection tube was discarded containing the eluate and the column was placed in a new 2mL collection tube. 200µL of Nuclease-free water was added directly onto the centre of the column and centrifuged for 1min at ≥ 6500 × *g* to elute the DNA. DNA was quantified using NanoDrop™ Lite Spectrophotometer.

2.5.4- Plasmid DNA extraction

Plasmid DNA was extracted from *E. coli* using the Monarch® Plasmid Miniprep Kit (NEB). Briefly, bacterial culture of 1–5ml was pelleted by centrifugation for 30s at maximum speed. Supernatant was discarded. Pellet was resuspended in 200µl Plasmid Resuspension Buffer (B1). 200 µl Plasmid Lysis Buffer (B2) was added, and gently inverted 5–6 times, and the tube incubated at room temperature for 1 minute. 400µl of Plasmid Neutralization Buffer was added to the tube and the sample gently inverted until solution was neutralized. The tube then incubated at room temperature for 1min. The lysate was centrifuged for 2–5min at maximum speed. Supernatant was carefully transferred to the spin column and

centrifuged for 1min at maximum speed. Flow-through was discarded. Column was re-inserted in the collection tube and 200µl of Plasmid Wash Buffer was added and centrifuged for 1 minute at maximum speed. 400µl of Plasmid Wash Buffer 2 was added and centrifuge for 1min also at maximum speed. Column was transferred to a clean 1.5ml microfuge tube, and finally 30µl Nuclease-free water was used to elute the DNA. DNA was quantified using NanoDrop™ Lite Spectrophotometer.

2.5.5- Polymerase Chain Reaction (PCR)

PCRs were performed using Q5 High-Fidelity 2X Master Mix (NEB), unless otherwise stated, as per manufacturer's instructions in a Perkin-MJ Research PTC-200 Peltier Thermal Cycler or C1000 Touch™ Thermal Cycler (BioRad). Primers were purchased from Sigma and their sequences listed in Table 2.5 at the end of this chapter. When performing PCR, all components were mixed prior to use. They were mixed as shown below:

Table 2. 2- PCR reaction set-up

Component	25µl Reaction	50µl Reaction	Final Concentration
Q5 High-Fidelity 2X Master Mix	12.5µl	25µl	1X
10 µM Forward Primer	1.25µl	2.5µl	0.5µM
10 µM Reverse Primer	1.25µl	2.5µl	0.5µM
Template DNA	variable	variable	< 1,000 ng

Nuclease-Free Water	to 25µl	to 50µl	-

The reaction was gently mixed and the PCR tubes transferred to a PCR machine for thermocycling. Thermo-cycling conditions for a routine PCR were as shown below (Table 2.3):

Table 2. 3- Thermo-cycling conditions

STEP	TEMPERATURE	TIME
Initial Denaturation	98°C	30 seconds
Denaturation	98°C	5–10 seconds
Annealing	50–72°C	10–30 seconds
Extension	72°C	20–30 seconds/kb (back to denaturation for 25-35 cycles)
Final Extension	72°C	2 minutes
Hold	4–10°C	-

After thermo-cycling, PCR products were then run on an agarose gel and/or purified using the PCR Mini Elute kit (Qiagen) as per manufacturer's instructions and briefly described below.

2.5.5.1- DNA purification

PCR products or restricted digested DNA were purified using PCR MinElute (Qiagen). Initially, 5 volumes of Buffer PB was added to 1 volume of the PCR reaction and mixed. MinElute column was placed in a provided 2ml

collection tube in a suitable rack. To bind DNA, the sample was applied to the MinElute column and centrifuged for 1min. Flow-through was discarded and the MinElute column was placed back into the same tube. To wash, 750 µl Buffer PE was added to the MinElute column and centrifuged for 1 min. The flow-through was discarded and the MinElute column was placed back in the same tube. The column was centrifuged for an additional 1min at maximum speed. Finally, the MinElute column was placed in a clean 1.5ml microcentrifuge tube and, to elute the DNA, 10µl of Buffer EB (10mM Tris·Cl, pH 8.5) or water was added to the centre of the membrane was added, let it stand for 1min, and then centrifuged for 1min.

2.5.6- Agarose gel electrophoresis

Briefly, agarose gel (1% w/v) containing SYBR Safe (diluted 10,000x) was poured into a casting tray. After immersing the gel in 1% Tris-acetate-EDTA (TAE) buffer, samples containing 1× DNA loading dye were loaded alongside 1kb plus DNA Ladder (Invitrogen), unless otherwise mentioned. The gel was run at 100V for 60min. Finally, DNA bands were visualized using Gel Doc™ XR+ Gel Documentation System (BioRad). Bands were extracted from gel using Monarch DNA Gel Extraction Kit (NEB) when needed, following manufacturer's instructions as described below.

2.5.6.1- Gel extraction of DNA bands

DNA bands were excised and purified from agarose gels using Monarch DNA Gel Extraction Kit (NEB). Initially, the DNA fragment was excised from

the agarose gel, taking care to trim excess agarose. Band was transferred to a 1.5ml microfuge tube, and the gel slice was weighed. Four volumes of Gel Dissolving Buffer were added to the gel slice. The sample was incubated between 37–55°C (typically 50°C), vortexed periodically until the gel slice was completely dissolved (generally 5–10min). For DNA fragments > 8 kb, an additional 1.5 volumes of water were added after the slice was dissolved. Next, the column was inserted into the collection tube and the sample was loaded onto the column. It was centrifuged for 1min, then the flow-through was discarded. The column was re-inserted into the collection tube, and 200µl of DNA Wash Buffer was added. It was centrifuged for 1min. This cleaning step was then repeated. The column was transferred to a clean 1.5ml microfuge tube, and, at least, 6µl of DNA Elution Buffer or water were added to the centre of the matrix. One minute was waited and the column was centrifuged for 1min to elute DNA.

2.5.7- DNA digestion and dephosphorylation

Restriction enzymes from NEB were used unless otherwise stated. At least 10U of enzyme were added to the mix containing appropriate buffer and DNA. The mix was incubated for at least 1h at 37°C. After digestion, 1µL of Alkaline Phosphatase, Calf Intestinal (NEB) was added and incubated with the previous mix for 30min. Restriction digested DNA were purified using the PCR Mini Elute kit (Qiagen) as per manufacturer's instructions and as briefly described in section 2.5.5.1.

2.5.8- DNA ligation

T4 DNA ligase (Promega) was used according to the manufacturer's recommendations. Overnight incubation at 4°C of a 1:3 vector : insert ratio was performed. 5µL out of a total 10µL reaction were used to transform *E. coli.*

2.5.9- Site-Directed Mutagenesis

Site-directed mutagenesis was performed using the Q5® Site-Directed Mutagenesis Kit (NEB) according to the manufacturer's recommendations. Typically, 7ng of plasmid template and 0.5µM of each set of primers (Sigma) were used for amplification and incorporation of the desired mutation. Primers used for SDM are shown in Table 2.5. PCR was performed in a C1000 Touch™ Thermal Cycler (BioRad) with the following thermo-cycling conditions; 98°C for 30s followed by 25 cycles of 98°C for 10s (denaturation), 68°C for 15s (annealing), 72°C for 3 min and 25s (extension) and 72°C for 2min (final extension). 1µl of the PCR product was kinase-, ligase- and DpnI- treated (KLD treatment) and incubated for 5 min at room temperature. 5µl of the KLD mix was then used to transform competent cells. After plating on LB agar with kanamycin and incubating overnight, several colonies were isolated, grown individually in LB broth with kanamycin and plasmid DNA extracted and sequenced for verification.

2.5.10- Gene Splicing by Overlap Extension (SOE)

Gene Splicing by Overlap Extension (gene SOEing) was used to create a fusion PCR product to replace genes of interest in selected isolates with the kanamycin resistance gene (*kan*) from EZ::Tn5<KAN-2> insertion kit (Epicentre) following the approach described by Horton (1995). Generally, in the first round of PCRs, homology arms (HA1 and HA2) of approximately 600bp flanking genes of interest were amplified from genomic DNA of Nm L91543 or MC58 (Table 2.5) and the *kan* gene was amplified using primers Kan_fwd and Kan_Rev (Figure 2.1). In bold are the regions that will bind to kan gene, underlined the regions that will bind to HA of interest, and in italics the regions that over-lap as shown in the table for the respective primers used. The HA1 and *kan* products obtained were gene-cleaned then used as template for the second round of PCR with primers HA1_Fwd and Kan_Rev and the annealed product generated cleaned and used as template along with the HA2 PCR product for a third round of PCR with primers HA1_Fwd and HA2_Rev. The final PCR product generated containing HA1-kan-HA2 was gene cleaned and sequenced for confirmation. The verified construct was used to transform selected isolates with selection on Kanamycin. Deletion mutants were confirmed by PCR and DNA sequencing.

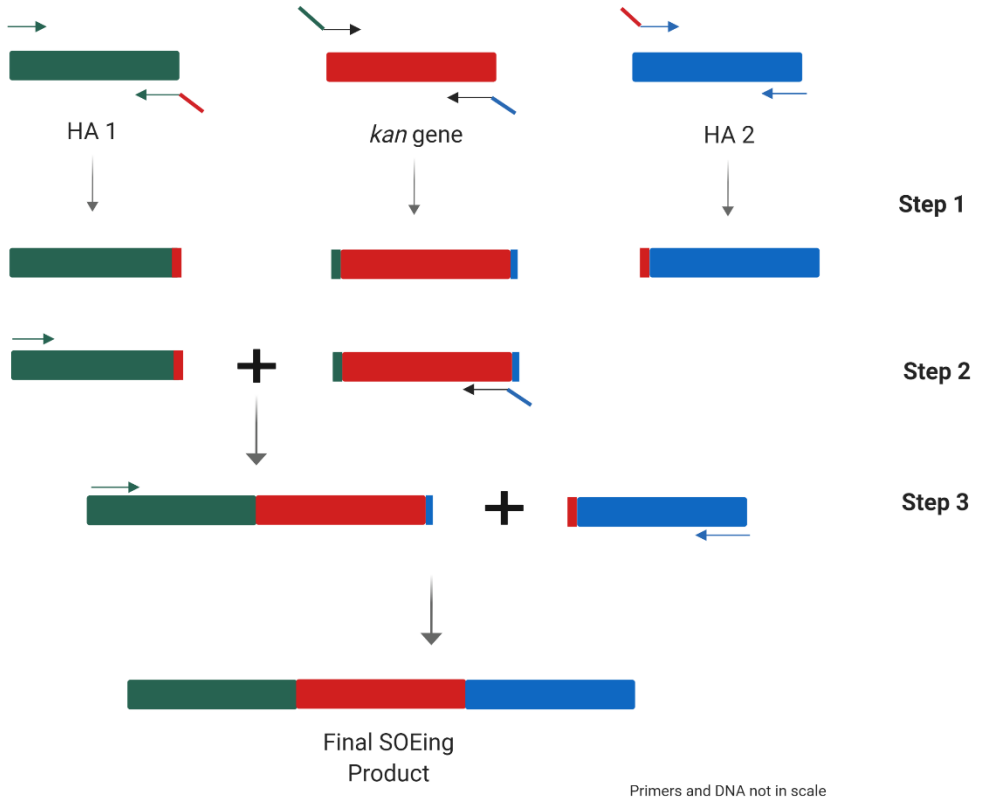


Figure 2. 1- Schematic figure of gene SOEing

After the initial amplification of HA1 (green), *kan* gene (red), and HA2 (blue) to incorporate areas of homology that will over-lap when performing the other steps (step 1), HA1 and *kan* gene were used as template and SOE'ed together (Step 2). The product containing HA1 and *kan* gene were then used as template to incorporate HA2 (Step 3). The final product was comprised of HA1-*kan*-HA2.

2.5.11- Quantitative RT-PCR (qRT-PCR)

2.5.11.1- RNA extraction and RNA quality assessment

RNA was extracted from 1ml cell suspensions of each strain standardised to A_{600} 0.65 (containing approximately 2 x 10^8 cells) using the RNeasy Mini kit (Qiagen) with enzymatic lysis and Proteinase K digestion. On column DNA digestion was performed using the RNase Free DNase set (Qiagen). The quality of the extracted RNA was assessed through agarose gel electrophoresis and recording the absorbance on Nanovue spectrometer values at 260/280 and 260/230 to check RNA purity.

2.5.11.2- Reverse transcription

One µg of cDNA was synthesised using the QuantiTect reverse transcription kit (Qiagen) in a two-step procedure.

The initial step is the genomic wipe-out and is performed as described below. All volumes must be adjusted accordingly to the initial concentration of the RNA extract as follows.

gDNA Wipeout buffer (7X)	2 µl
RNA	? µl equivalent to1 µg total
RNase-free water	? µl to make the total volume 14 µl

After adding all reagents, the tubes were incubated at 42°C for 2min and placed immediately on ice.

Then, the reverse transcription step was set up as the following. The quantities were scaled up to make a master mix with an excess volume of 10%.

Master mix	
Quantiscript Reverse Transcriptase	1 µl
Quantiscript RT buffer 5X	4 µl
RT Primer Mix	1 µl

For negative control, 1µl RNase-free water was used instead of the Quantiscript Reverse Transcriptase. 14µl of RNA mix from step 1 were added to 6µl of the master mix, mixed and kept on ice. The reaction was then incubated at 42°C for 15min and incubated at 95°C for 3min to inactivate the Quantiscript Reverse Transcriptase. Finally, tubes were stored on ice ready to proceed with qRT-PCR, or stored at -20°C.

2.5.12- qRT-PCR

qRT-PCR was performed in a 15µl reaction mixture with Quantinova SYBR Green PCR Master Mix (Qiagen), 22.5ng of cDNA and appropriate concentration of each primer (Sigma). Forward and reverse primers for amplification of cDNA of target and house-keeping genes were used (Table 2.5). Primers were optimized using different concentrations (0.5-9µM) of forward and reverse primers with 10ng/µl of cDNA utilized as template and 2x buffer of Quantinova SYBR Green PCR Master Mix. To test PCR efficiency, a standard curve was generated with serial 10-fold dilution of a known amount (100ng/µl) of cDNA using the appropriate concentration of primers. PCR was performed in a Prime Pro 48 Real Time PCR machine

with the following thermocycling conditions; 95°C for 2min followed by 40 cycles of 95°C for 5s (denaturation) and 60°C for 10s (combined annealing/extension). Three biological replicates from 3 independent RNA extractions from each of the 3 strains were run in duplicate, unless otherwise stated, along with the corresponding no reverse transcriptase control for each of these samples and a no RNA control. Relative quantification of gene expression was performed using the Comparative CT Method (ΔΔCt) (Livak and Schmittgen, 2002) whereby target genes expression levels were normalized to the mean levels of control (*recA*) transcripts.

2.5.13- Reverse Transcription PCR (RT-PCR)

Following a similar approach to da Silva *et al.* (2017), RNA was extracted from 1ml cell suspensions of each strain standardised to A_{600} 0.65 (containing approximately 2 x 10^8 cells) using the RNeasy Mini kit (Qiagen) with enzymatic lysis and Proteinase K digestion. On-column DNA digestion was performed using the RNase Free DNase kit (Qiagen). RNA quality was assessed for genomic contamination and integrity using a NanoDrop Lite (Thermo Fisher Scientific) and running 1µl of RNA on a 1% agarose gel, respectively. One µg of cDNA was synthesised using the QuantiTect reverse transcription kit (Qiagen) with the initial genomic wipe-out step included. RT-PCR was performed in a 50 µl reaction mixture with One Taq 2x master mix (NEB), 20 ng of cDNA and 0.2µM of each primer (Sigma). For amplification of cDNA of *fHbp*, fHbp-for and fHbp-rev primers were used and for amplification of *recA*, recA-for and recA-rev primers were used (Table 2.5). To qualitatively check for different levels of expression, PCR

was performed in a C1000 Touch™ Thermal Cycler (BioRad) with the following thermo-cycling conditions; 94°C for 30s followed by 35 cycles of 94°C for 15s (denaturation), 56°C for 15s (annealing) and 68°C for 20s (extension). PCR products were visualised on a 1% agarose gel and imaged by Image lab v4.0.1.

2.5.13- RNA Sequencing (RNAseq)

As previously described in 2.5.11.1, RNA samples extracted from meningococcal cultures grown to OD_{600} 0.5 were sent for RNAseq at the DeepSeq facility at the University of Nottingham and analysed as follows. Prior to sequencing, RNA samples were analysed for integrity in Bioanalyser.

2.5.13.1- Quantification of RNA integrity using 2100 Bioanalyzer

The integrity of RNA was evaluated using a 2100 Bioanalyzer (Agilent Technologies, UK) and an Agilent RNA 6000 Nano Kit. The software and algorithm classify total RNA by calculating an RNA integrity number (RIN) from 1 to 10, with 1 corresponding to the most degraded RNA profile and 10 to the most intact (Schroeder *et al.*, 2006). The preparation of all samples was completed according to the Agilent RNA 6000 Nano Kit Guide (Manual Part Number G2938-90034, Edition 08/2006) in the following way. Each RNA chip contains an interconnected set of microchannels, used for separation of nucleic acid fragments based on their size as they are driven through it electrophoretically. All reagents were equilibrated at room

temperature for 30min before use. The electrodes of the 2100 Bioanalyzer were decontaminated by washing with RNase ZAP (Qiagen) for 1min followed by a wash with RNase-free water for 10s. A volume of 550µl of the Agilent Nano gel matrix was passed through the spin filter by centrifugation at 1,500 × *g* for 10min at room temperature. Aliquots of 65µl of the filtered gel matrix were transferred into microcentrifuge tubes and were stored at 4°C for up to 4 weeks. Prior to use, 1µl of the provided dye solution was added to the filtered 65µl gel aliquot and mixed by vortexing followed by a centrifugation step at 13,000 × *g* for 10min at room temperature and the gel-dye mix was used within one day. An RNA chip was placed on the chip priming station, and 9µl of the gel-dye mix was pipetted into the proper well. The plunger was positioned at the 1ml and the chip priming station was closed. The syringe plunger was pressed down until it was held by the clip, was then released after 30s and 5s later the plunger was pulled back to the 1ml position. The priming station was opened, and 9µl of the gel-dye mix was pipetted in each of the wells reserved for this step. A volume of 5µl of the Nano marker was added into the 12 sample wells and to the ladder well. The RNA samples and the ladder were heat-denatured at 70°C for 2 min to minimise secondary structures. From each RNA sample, 1µl containing 25-500ng RNA was added in each of the 12 sample wells and 1µl of the ladder into the ladder well. The chip was placed in an appropriate vortexer (IKA - Model MS3; Staufen, Germany) and was vortexed for 1min at 2,400 rpm. The chip was inserted in the Agilent 2100 Bioanalyzer within 5min and analysed. The analysis was performed via Agilent's 2100 expert software selecting for prokaryotic analysis. The RNA integrity number (RIN) was estimated for each sample. Only RNA samples with RIN index close to 10

or above 8.5 were selected for RNAseq analysis (Fleige and Pfaffl, 2006; Schroeder et al., 2006)

2.5.13.2- DS693 Library preparation, QC and Sequencing protocol

This protocol was performed at DeepSeq University of Nottingham facility. Briefly, RNA concentrations were measured using the Qubit Fluorometer and the Qubit RNA BR Assay Kit (ThermoFisher Scientific) and RNA integrity was assessed using the Agilent 4200 TapeStation and the Agilent RNA Screentape (Agilent). Ribodepletion was performed on 1 µg of total RNA, using the Ribo-Zero rRNA Removal Kit (Bacteria) (Illumina). Depleted RNA samples were cleaned-up using the RNeasy MinElute Clean-up Kit (Qiagen) and samples were assessed for ribodepletion efficiency using the Agilent TapeStation 4200 and the High Sensitivity RNA ScreenTape assay (Agilent). Indexed sequencing libraries were then prepared using the NEBNext Ultra Directional RNA Library Preparation Kit for Illumina (NEB) and NEBNext Multiplex Oligos for Illumina, Index Primers Set 1 (NEBNext). Libraries were quantified using the Qubit Fluorometer and the Qubit dsDNA HS Kit (ThermoFisher Scientific). Library fragment-length distributions were analyzed using the Agilent TapeStation 4200 and the Agilent High Sensitivity D1000 ScreenTape Assay (Agilent). Libraries were pooled in equimolar amounts and final library quantification performed using the KAPA Library Quantification Kit for Illumina (Roche). The library pool was sequenced on the Illumina NextSeq500 on a NextSeq500 Mid Output 150 cycle kit (Illumina), to generate over 10 million pairs of 75bp paired-end reads per sample

2.5.13.2- Read Alignment, Trimming, Mapping

The trimming pipeline was used to filter reads with low sequencing score as well as reads aligned to adaptor sequences. First, raw reads were trimmed against adaptors, and then reads were quality trimmed by skewer as described in Jiang *et al.* (2014). Trimmed reads were mapped onto the reference genome of Nm MC58, found on NCBI under the accession number NC_003112.2, by hisat2 mapping tool using default settings as found in (Kim *et al.*, 2015).

2.5.13.3- RNAseq Raw files Analysis

After trimming, generated raw files were then analysed using the platform PATRIC. On PATRIC, Tuxedo (Trapnell *et al.*, 2012) was run to process the RNAseq results. Tuxedo strategy uses Bowtie2, Cufflinks, and CuffDiff to align, assemble, and compare samples, respectively.

2.5.14- Bacterial Two-Hybrid Assay (BACTH)

The protein–protein interaction of MC58 FHbp and L91543 FHbp with SecA was investigated using the Bacterial Adenylate Cyclase Two-Hybrid System Kit (Euromedex) according to manufacturer's instructions. First, *fHbp* from MC58 and from L91543 was PCR-amplified with the primer pair BamHIfwd_MC58FHbp and EcoRIrev_MC58FHbp and primer pair BamHIfwd_L91543FHbp, and EcoRIrev_L91543FHbp, respectively, then the PCR products cloned separately into vector pUT18. The gene encoding SecA from MC58 was PCR-amplified with primer pair, PstIfwd_SecA and

SmaIreverse_SecA then cloned into vector pKT25. 25-50 ng of the appropriate prey, pKT25-based construct and the equivalent concentration of appropriate bait, pUT18-based construct were co-transformed into 100 µl of electrocompetent *E. coli* BTH101 cells and plated on MacConkey agar containing 0.5 mM IPTG and appropriate antibiotics. Bacteria expressing interacting hybrid proteins formed pink/purple colonies while cells expressing non-interacting proteins remained white/light pink. As a positive control, a co-transformant containing pKT25-zip and pUT18-zip constructs was used. Co-transformants containing one empty vector pKT25 and/or pUT18 were used as negative controls. Pink colonies were isolated and grown in LB broth and plasmid DNA extracted for verification by PCR and sequencing.

2.5.15- Construction of the transposon library in strain MC58

2.5.15.1- Modification of EZ::Tn5< KAN-2 > transposon to incorporate DUS

As described in da Silva *et al.* (2017), the transposon (Tn) from the EZ::Tn5<KAN-2> insertion kit (Epicentre) was modified to incorporate the DNA uptake sequence (DUS) known to facilitate the uptake of DNA in Neisseriaceae (Frye *et al.*, 2013). This was achieved by PCR amplification of the EZ::Tn5<KAN-2> Tn using forward primer HindIIIkan2for, which incorporates a *Hind*III site and anneals immediately downstream of the 5′ mosaic end (ME) of the Tn and the reverse primer EcoRIDUSkan2rev, which incorporates an *Eco*RI site and DUS element and anneals downstream of the stop codon and upstream of the 3′ ME. The PCR product was cloned into the *Eco*RI, *Hind*III sites of plasmid pMOD$_{TM}$-2<MCS>

(Epicentre). The ligation mixture was used to transform E. coli and transformants were selected by growth on kanamycin. Clones were verified by PCR using primers PCRFP and PCRRP (Epicentre) and by sequencing. Phosphorylated primers PCRFP and PCRRP (Sigma) were used to amplify the region encompassed by and including the MEs.

2.5.15.2- *In vitro* transposition and transformation of strain MC58

As performed in da Silva *et al.* (2017), approximately 1µg of *Dra*I digested and purified genomic DNA of strain MC58 was mixed with 0.6µg of phosphorylated PCR product, Ez-Tn5 reaction buffer and 4U of transposase (Epicentre) in a total volume of 40µL. The reaction was incubated at 37°C for 2h then stopped according to the manufacturer's instructions. The DNA was purified and 3′ overhangs were repaired by adding 5 U of T4 DNA polymerase to 1µg of the DNA, 100 µM of each dNTP and T4 DNA polymerase buffer to a total volume of 50µL. The reaction was incubated at 37°C for 5 min followed by heat inactivation at 75°C for 10min. The DNA was purified and 6 U of T4 ligase and ligase buffer were added to the DNA to a total volume of 50µL, and the reaction was incubated at 21°C for 2h. The DNA was again purified and 250ng used to transform Nm with selection on LB-Kanamycin.

2.6- Protein analysis assays

2.6.1- SDS-PAGE, Western immunoblotting and immuno-dot blotting

Whole cell lysates (WC) were prepared from broth or plate cultures adjusted to a wet weight of 10^9 cells/ml, fractionated by 12% or 16% (w/v) SDS-PAGE and immunoblotted as described previously (da Silva *et al.*, 2017).

For immunodot blotting, cell suspensions of the meningococcal strains were heat killed at 65°C for 30min and 5 µL were spotted onto a nitrocellulose membrane (Thermo Scientific), dried, then blocked in PBS containing 0.05% (*v*/v) Tween 20 (PBST) and 2% BSA (w/v) (Sigma) with gentle shaking for 1h. Three 2min washes in PBST were conducted then the membrane incubated in PBST containing 2% BSA (w/v) and 1 µg/ml of anti-FHbp antibody, JAR4, with gentle shaking for 3 to 4h. The washes were repeated, and the membrane incubated in anti-mouse alkaline-phosphatase conjugated secondary antibody solution (Invitrogen) for 1 to 3h, washed as before and then developed with 5-bromo-4-chloro-3-indolylphosphate, nitro-blue tetrazolium liquid substrate (Invitrogen).

When performing Western immunoblotting (WB), membranes were incubated in Tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20 (TBST) and 5% (w/v) non-fat dry milk. Membranes were incubated with primary antibody in TBST containing 1% (w/v) non-fat dry milk overnight at 4°C. Membranes were then washed for 60min with TBST at room temperature and then incubated for 2h at room temperature with secondary antibodies. Membranes were then washed with TBST for 30min and specific protein bands were detected by enhanced chemiluminescence (GE Healthcare, UK) or 3,3',5,5'-tetramethylbenzidine) (TMB) (Sigma). Band

intensity was quantified using a GS-800^{TM} calibrated densitometer (Bio-Rad) or ImageJ 1.x (Eliceiri et al., 2012) calibrated to perform Optimal Density (OD) based on a pallet of colours in greyscale. Immuno-dot blots were performed exactly as described previously (da Silva *et al.*, 2017).

2.6.2- Antibodies

Mouse anti-FHbp-Mabs, JAR4 and JAR5, were obtained from the National Institute for Biological Standards and Controls. JAR4 is IgG2a and JAR5 is IgG2b; both were isolated from mice immunized with recombinant FHbp derived from MC58 (Welsch *et al*., 2004). Mouse anti-FHbp polyclonal antibody was kindly provided by Professor Christoph Tang (University of Oxford) and rabbit anti-RecA antibody purchased from Abcam. Secondary antibodies included donkey anti-rabbit HRP-linked antibody, sheep anti-mouse HRP-linked antibody (GE Healthcare, UK) for Western immunoblotting and rat anti-mouse IgG H+L conjugated with FITC for FACs analysis (Thermo Fisher Scientific).

Table 2. 4- List of antibodies and dilutions used throughout this thesis

Antibodies and Dilutions	
Primary antibody	**Secondary antibody**
Mouse monoclonal Jar4 and Jar5 (NIBSC) diluted 1:10000 in 1% Milk	Sheep anti-mouse HPR-conjugated secondary (Cell Signalling Technology) diluted 1:1000 in 1% milk
Mouse anti-FHbp polyclonal diluted in 1:10000 in 1% milk	Rat anti-mouse IgG H+L conjugated with FITC (Thermo Fisher Scientific) diluted 1:100 in PBS-BSA
Rabbit Anti-RecA (Abcam) diluted in 1:5000 in 1% milk	Donkey anti-rabbit HRP-linked antibody (Cell Signalling Technology) diluted 1:10000 in 1% Milk
Mouse monoclonal IgG1 Factor H Antibody (OX24) conjugated with PE (Santa Cruz Biotechnology)	-

2.6.3- Palmitate Labelling of Lipidated Proteins

Bacterial cultures were grown in supplemented GC broth then after an initial doubling period, alkyne-labelled palmitic acid (Cayman Chemical) was added to a final concentration of 45μM. Bacteria were incubated for at least 4 more hours at 37°C.

2.6.4- Immuno-precipitation of FHbp from precipitated supernatant

Samples were immuno-precipitated with Protein G Mag Sepharose (GE Healthcare Life Sciences) and Mab JAR4. Following incubation of 100μl of precipitated sample with 5μg of JAR4 overnight at 4°C, samples were incubated for 1h with 100μl of magnetic bead slurry. Using a Magnetic Particle Concentrator (MPC), beads were washed twice with PBS and FHbp recovered following the addition of 100μl 0.1M glycine-HCl (pH 2.5 to 3.1). Buffer exchange from glycine-HCl buffer to click reaction buffer (100 mM Na-Phosphate Buffer, pH 7) was performed with Slide-A-Lyzer Dialysis cassettes (Sigma).

2.6.5 Click chemistry

The Click Chemistry labelling system “CuAAC Biomolecule Reaction Buffer Kit (THPTA based)” (Jena Bioscience) was used following manufacturer’s instructions (Ostberg *et al*., 2013). FHbp was coupled to biotin azide (Stratech) then samples fractionated on 10-20% (w/v) SDS-PAGE gels (Novex). WB was performed by incubating the membrane with Streptavidin HRP-linked protein in PBS-BSA buffer and developing with TMB (Sigma).

2.6.6- Harvesting cellular compartments of *N. meningitidis*

Periplasmic extracts were prepared using a method previously described (reference). Cells from overnight GC plate cultures were suspended to A_{600} 1.0 in 500µl buffer (50mM Tris-HCl pH 8.0) and pelleted at 3,500 × *g* for 2min. The pellet was resuspended in 200 µl of the same buffer and 20 µl of chloroform was added. After brief vortexing, tubes were incubated for 15min at room temperature. After centrifugation at 6,500 × *g* for 2min, the upper portion of the supernatant, containing the periplasmic proteins, was carefully aspirated and placed in a second tube. The pellet containing the remaining IM, OM and cytoplasmic proteins was partitioned following approaches adapted from two different research group (Clark *et al*., 1987; Rahman *et al*., 2000). The pellet was re-suspended in 500 µl buffer (50 mM Tris-HCl pH 8.0, 20% [w/v] sucrose) containing 1mg/ml lysozyme and incubated for 30 min at 4°C. After 2 cycles of freeze-thawing, the cells were subjected to sonication (2 bursts of 30s). Cellular debris were removed by centrifugation at 9,500 x g for 10min. Ultra-centrifugation at 100,000 x *g* for 60min enabled partitioning of the membranes in the pellet from cytoplasmic proteins in the supernatant. The IM was selectively solubilized by treatment with 200µl of 1% (w/v) sodium lauroyl sarcosinate in 10mM HEPES (N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid) pH 7.4 buffer. After centrifugation at 100,000 x *g* for 1h the supernatant containing solubilized IM proteins was separated from the pellet containing OM proteins. The pellet was washed with ethanol and resuspended in 200µl PBS.

2.6.7- β-Galactosidase Assay

Following the approach described (Chambonnier *et al*., 2016), to measure the level of protein-protein interaction between FHbp from MC58 or from L91543 with SecA, LB broth cultures were grown to A_{600} 0.6 then induced for 3 h with 0.5 mM IPTG. 5 µl of induced culture were mixed with 900 µl of Z buffer (0.06M Na_2HPO_4 $2H_2O$, 0.04M NaH_2PO_4, 0.01 M KCl, 2mM $MgSO_4.7H_2O$, 14.20 mM β-mercaptoethanol (pH 7), before addition of 20µl of 0.1% (w/v) SDS and 100µl of $CHCl_3$ to permeabilise the cells. Substrate solution was prepared by solubilising orthonitrophenyl-β-galactosidase (Sigma) in Z buffer without the β-mercaptoethanol to a final concentration of 4mg/ml. 40µl of substrate solution were added to 180 µl of Z buffer and 20 µl of permeabilised cells in a 96-well plate. The plate was incubated at room temperature for 20min minimum. Readings were taken on a microplate reader at A_{405} and A_{540} and β-galactosidase activity calculated using the equation below and expressed in Miller units.

$$\beta\text{-galactosidase activity} = \frac{1000 \times (A_{405} - A_{540})}{\text{Time (min)} \times \text{vol. of cells in assay (ml)} \times A_{600}}$$

2.7- Cell analysis assays

2.7.1- FACS analysis of surface FHbp with JAR4 and number of antibodies bound per cell (ABC) determined

FHbp surface expression was assessed using a MoFlo Astrios EQ, Cell Sorter (Beckman Coulter). The approach used was similar to that used previously (Biagini *et al*., 2016). Approximately 1 × 10^8 bacteria were suspended in PBS containing 1% (w/v) bovine serum albumin (PBS-BSA) and incubated with JAR4 in a final reaction volume of 100 µl, for 1 h at 37 °C. After two washes with PBS, JAR4 binding was detected using rat anti-Mouse IgG (H+L), FITC (Thermo Fisher) at a 1:50 dilution for an incubation period of 1 h. After the final 2 washes with PBS, samples were resuspended in 500 µl of PBS-BSA with 4% (w/v) formalin. The negative control consisted of cells incubated with the secondary antibody alone. Quantum™ Simply Cellular® anti-rat (Bang Laboratories) microspheres were used to determine the number of antibodies bound per cell according to the manufacturer's instructions. Median channel values for each population of microsphere were acquired for entry into the QuickCal® spreadsheet to generate a curve that enabled the acquisition of ABC for each isolate.

2.7.2- FACS analysis of surface FHbp binding to Factor H

FHbp surface binding to Factor H was assessed using an ImageStreamX MkII (Luminex Corporation). The approach used was inspired by the binding tests performed previously using ELISA (Giuntini *et al.,* 2011 and Costa *et al.,* 2014). Approximately 1 × 10^5 bacteria were suspended in PBS containing 1% (w/v) bovine serum albumin (PBS-BSA) and incubated with

Factor H (Biorad) in a final reaction concentration of 5 µg/mL for 1h at room temperature. After two washes with PBS, FH binding was detected using Factor H Antibody (OX24) PE at a 1:50 dilution for an incubation period of 1h. After the final 2 washes with PBS, samples were resuspended in 200µl of PBS-BSA with 4% (w/v) formalin. The negative control consisted of cells incubated with the Factor H antibody alone. Relative Mean of Fluorescence Intensity (rMFI) were acquired for each sample. Values consist of rMFI's acquired for total fluorescing cell incubated with Factor H and Factor H antibody. The values were normalised against the negative control which was made of the rMFI of cells incubated with secondary antibody only.

2.7.3- Serum Bactericidal Assay

Complement-mediated bactericidal activity with antibodies JAR4 and JAR5 was measured using approaches adapted from previous studies (Beernink *et al.*, 2011; Vu *et al.*, 2012; and Li *et al.*, 2016) JAR4 and JAR5 were used in combination, in place of serum antibodies, to generate bactericidal activity (Welsch *et al.*, 2008; Li *et al.*, 2016; and Huber *et al.*, 2017). 8µg of each antibody were used to ensure maximum killing (Beernink *et al.*, 2011). The complement source was obtained from lyophilized human sera (Sigma) reconstituted in sterile water as per manufacturer's instructions. Complement and antibodies were added to IgG-depleted human sera (Stratech) that had been heat inactivated for 30 min at 56 °C. The bactericidal activity *i.e.* the percentage of killing by the antibodies for each strain was then determined from the CFU counts between isolates after 60

min incubation in the reaction mixture compared with cfu in negative control wells at time zero.

2.7.4- Ultrastructural analysis of meningococci by electron microscopy

Following a similar approach to Rowlett *et al.* (2017), overnight cultures of meningococci grown on GC agar were diluted 100-fold and further grown in GC broth at the appropriate temperature until they reached OD_{600} 0.1 or OD_{600} 1. Cells were harvested by centrifugation at 6,000 × *g* for 5min, and bacteria from 2ml of culture medium were washed three times with 5ml phosphate-buffered saline (PBS; 0.1 M, pH 7.4) containing 50mM $MgCl_2$. Cells were fixed by reaction with glutaraldehyde (2.5% [v/v] in 0.1M PBS, pH 7.4) at 4°C, treated with osmium tetroxide (0.1% [w/v] in 0.1M PBS) at 4°C for 2h (Ryter and Kellenberger, 1958) and washed in this buffer at 4°C for 15 min. Then, cells were dehydrated in a graded ethanol series at 4°C (to reduce loss of lipids) as follows: 70% ethanol (2 times for 10min each), 90% ethanol (2 times for 10 min each), and 100% ethanol (3 times for 20min each). Cells were stained initially in this first step with a low concentration of uranyl acetate (4% [wt/vol] in 70% ethanol) for 1h. The dehydrated cells were embedded in Epon 812 resin by transferring them into propylene oxide (2 times for 10 min each) and exchanging them to a mixture of propylene oxide and resin (1:1) for 6h. Cells were then placed in 100% resin for 24h under vacuum, leaving the cap off to allow solvent evaporation. Finally, cells were blocked out in fresh resin and allowed to polymerize at 60°C for 48h. Blocks were cut on an ultramicrotome with glass knives, and sections 150nm thick were picked on copper grids and restained for 5 min each with

uranyl acetate (15% [wt/vol] in methanol) prior to transmission electron microscopy (TEM) imaging. The sections were examined in a JEOL 2100 electron microscope operated at 120 kV, equipped with an Orius camera (Gatan, Inc., Pleasanton, CA). Images were collected at magnifications ranging from ×50,000 to ×100,000.

2.7.5- Antibiotic susceptibility assays by Microbroth Dilution

Meningococcal strains were compared for their susceptibility to a panel of antibiotics by the Microbroth Dilution method. For each antibiotic, the concentration range was centered around the Minimum Inhibitory Concentration (MIC) value according to the Clinical and Laboratory Standards Institute (CLSI). The antibiotics purchased from Sigma that were tested included Tunicamycin, Ceftriaxone, Penicillin G, Chloramphenicol, Sulfanilamide, Globomycin, Ciprofloxacin and Rifampicin. Bacterial suspensions were standardised to A_{600} 0.4 then 5µl added to 95µl of Mueller Hinton broth containing doubling dilutions of antibiotic in a Thermo Scientific sterile 96 well plate (Nunclon Delta surface). Each experiment was performed in triplicate and negative controls included no bacterial suspension and no antibiotic. Each experiment was repeated at least 3 times. A gas permeable seal was placed over the plate and the plate incubated at 37°C in 5% CO_2 with gentle shaking for approximately 20 h. Readings were performed in infinite M200 pro plate reader (Life Science – Tecan). The first concentration to present OD < 1.0 was considered to be

the MIC as this is equivalent to visual assessment of the tested concentration that showed no turbidity by eye.

2.7.6- Assays with Human monocyte cell line THP-1, Human Umbilical Vein Endothelial Cells (HUVEC), and Human Corneal Epithelial Cells (HCEC)

2.7.6.1- Thawing and culturing of cells from storage in liquid nitrogen

Cells in cryotubes were retrieved out of liquid nitrogen and immediately placed in ice. Cryotubes were warmed in 37°C water bath. Cells were slowly pipetted up and into the 75 cm^2 (T75) flask with pre warmed media with 20% Foetal Bovine Serum (FBS, Gibco). T75 flask was placed into the incubator at 37°C. After 24h, the flasks were checked to verify if the cells have adhered. Fresh media with 10% FBS was then added to remove completely the DMSO that was used in the freezing media and any dead cells. From this point, the media with 10% FBS was changed every 2-3 days until about 80% confluent, when cells can then be split.

2.7.6.2- Splitting of cells once 80% confluent

Cells were checked and it was verified if they had formed a monolayer that was about 80% confluent. The media was taken off without disrupting the monolayer and the flask washed with about 5ml PBS. PBS was taken off and trypsin (0.5g/L) was added (1 in 9ml of PBS). The cells were put in the incubator and checked to see if they had un-adhered from the flask every

couple of minutes. Once the cells have un-adhered, the flask was gently tapped on the side to dissociate all the cells from the bottom of the flask. The cells were removed by pipetting up the PBS and washing off any of the cells attached to the flask. 1ml of FBS was added to inactivate the trypsin. The solution was added to a 50ml centrifuge tube and centrifuged for 5 mins at 1,000rpm. Without disrupting the pellet, the solution was taken off and re-suspended in 1ml media with 10% FBS. Cells were counted with the help of trypan blue in a haemocytometer. Then 20ml of media was transferred per T75 flask. Generally, flasks were inoculated with about 10,000 cells/cm^2 and incubated. Finally, the media was changed every 2-3 days until the cells were confluent and the process was repeated.

2.7.6.3- Adhesion and invasion assays with HUVECs cells and HCEC

Following a similar approach to Klee *et al.* (2000), bacteria from GC agar plates were resuspended in RPMI (Gibco) containing 10% (FBS; Gibco) to an OD600 of 0.1. Then, 1 ml of a 100-fold dilution was added to a confluent monolayer of human umbilical vein endothelial cells (HUVEC) in 2-cm^2 tissue culture wells (Costar).

2.7.6.4- Adhesion

After incubation for 1h at 37°C in 5% CO_2, the supernatant was removed (non-adherent bacteria) and the monolayer was washed three times with RPMI. The adherent bacteria were released by adding 1 ml of PBS–1% saponin and scraping the bottom of the wells with a micropipette tip. The

numbers of adherent and non-adherent bacteria were determined after plating them on supplemented GC agar. Adherence was calculated as the number of adherent bacteria divided by the total number of adherent plus non-adherent bacteria.

2.7.6.5- Invasion

The wells were washed every hour as described above for 6h and then filled with RPMI-FBS containing 25 ug of gentamicin per ml. After 4h of incubation to kill external bacteria, internalized bacteria were harvested and enumerated as described above. Invasion was calculated as the number of internalized bacteria divided by the total bacteria at 1 h after infection.

2.7.6.6- Intracellular survival assays

Following the approach used by LoVullo *et al.* (2015), the human monocyte cell line THP-1 grown in RPMI with 10% foetal bovine serum at 37°C in 5% CO_2 was treated with 100ng/ml phorbol myristate acetate (PMA; Invivogen) for 3 days prior to survival assays, to allow for differentiation, and then seeded in 12-well plates at a population of 1×10^6 cells per well. Prior to infection, the wells were washed with 1× PBS, and fresh medium was added. To each well, a suspension of bacteria at a multiplicity of infection (MOI) of 100 was added, and bacterial uptake was allowed to occur for 4 h. Afterwards, the wells were washed twice to remove any free bacteria, and then fresh medium was added that contained 25µg/ml gentamicin. After 6 or 24 h, the wells were washed twice and the THP-1 cells were lysed by the

addition of 0.5ml distilled water and agitation by pipetting. After addition of 0.5 ml 2× PBS, 10µl of each lysate, serially diluted in PBS, was then spotted onto supplemented GC agar for determinations of viable counts. These assays were performed with n=5, and results are reported as averages ± standard deviations of the mean. Data were compared for each time point using an un-paired T Test with Welsch's correction with GraphPad v6.

2.8- Biofilm formation assay

The biofilm formation plate assay approach was adapted from the published method by O'Toole *et al.* (1999) and performed for Nm as described by O'Dwyer *et al.* (2009). This assay is based on the ability of bacteria to form biofilms on polystyrene (Cellstar). A 16h plate of Nm was harvested into MH, adjusted to an OD_{600} 0.05 and inoculated into polystyrene 96-well plates (Cellstar). The plates were incubated statically for 24h and then rinsed with distilled water, baked for 1h at 60°C and stained with crystal violet (Pro-Lab Diagnostics). The crystal violet was solubilized by adding ethanol/acetone (80:20, v/v) and the biofilms were quantified by measuring A_{600}.

2.9- Human interleukins (IL-6 and IL-8) quantification

Quantification of human IL-6 and IL-8 was performed using Human IL-6 ELISA Ready-SET-Go! Kit (Invitrogen) and Human IL-8 ELISA Ready-SET-Go! Kit (Invitrogen), respectively.

As per manufacturer's instructions, corning costar 9018 (or Nunc Maxisorp) ELISA plate was coated with 100µL/well of capture antibody in coating buffer. After sealing the plate, it was incubated overnight at 4°C. Wells were aspirated and washed 3 times with >250 µL/well wash buffer, allowing time for soaking (~ 1min) during each wash step. Plate was blotted on absorbent paper to remove any residual buffer after every wash. Wells were blocked with 200µL/well of 1x assay diluent and incubated at room temperature for 1h. Next, 100µL/well of top standard concentration was added to the appropriate wells and 2-fold serial dilutions of the top standards was performed to make the standard curve for a total of 8 points. 100 µL/well of testing samples was added to the appropriate wells. The plate was sealed and incubated at room temperature for 2h. Wells were aspirated and washed for a total of 3-5 washes as described above. 100 µL/well of detection antibody diluted in 1X assay diluent was added. The plate as sealed and incubated at room temperature for another 1h. Wells were aspirated/washed for a total of 3-5 washes. Then, 100µL/well of Avidin-HRP* diluted in 1X assay diluent was added and the plate sealed and incubated at room temperature for 30min. Wells were aspirated/washed again for a total of 3-5 washes. Finally, wells were aspirated, washed and soaked with wash buffer for 1 to 2min prior to aspiration. This was repeated for a total of 5-7 washes. 100µL/well of substrate solution was added to each

well and the plate was incubated at room temperature for 15min. 50 µL of stop solution was added to each well. And reading of the plate was done at 450 nm and 570nm. Values of 570 nm were then subtracted from those of 450 nm to analyze the data.

2.10- *G. mellonella* larvae

G. mellonella larvae were acquired from UK Waxworms Limited and kept at 4°C in darkness with a non-restricted diet since it has been reported that food deprivation of *G. mellonella* larvae leads to reductions in cellular and immune responses (Banville *et al.*, *2012*). Larvae were used within 5 days of receipt. Larvae of approximately 250 to 350mg were selected for the experiments.

2.10.1- Infection of *G. mellonella* larvae

Similar to the approach used by Insua *et al.* (2013), bacteria grown overnight on GC agar plates were resuspended in PBS to an optical density at 600 nm (OD_{600}) of 1, which corresponds to approximately 1×10^9 CFU/ml. After surface disinfection using ethanol (70% [v/v]), larvae were injected with 10 µl of bacterial suspension, containing approximately 1×10^7 CFU/ml, into the last right proleg by use of a Hamilton syringe with a 30-gauge needle. A group of 10 larvae were injected with 10µl of PBS in parallel to ensure that death was not due to injection trauma. Larvae were placed in 9.2-cm petri dishes and kept at 37°C in the dark. Insects were considered dead when they did not respond to physical stimuli. Larvae were examined for pigmentation, and time of death was recorded. Assays were allowed to

proceed for 4 days with no pupa formation being seen. Five independent experiments were performed.

2.11- Bioinformatics

This thesis used among others, Fiji (ImageJ), Phyre2, DOLOP, LipoP, SignalP 5.0, Bioedit v7.2.5, SnapGene v4.3.11, Image Lab v4.0.1, FlowJo v10, Quickcal v2.3, and GraphPad v6.0 to analyse data and generate nucleotide comparisons according to manufacturer's instructions. An appropriate explanation of the usage is given when the software is mentioned throughout the thesis.

2.12- Statistics

Data are shown as mean ± SEM. Unpaired T test with Welsh's corrections was used for two groups comparisons. Multiple comparisons among groups were performed by one-way ANOVA followed by Dunnett's test. A value of $p \leq 0.05$ was considered statistically significant. Post hoc tests were only run if P achieved $P < 0.05$ and there was no significant variance in homogeneity. All statistical analysis tests were performed in GraphPad Prism v6 for Windows.

2.13- Data availability

The FACS data discussed in this thesis have been deposited in FlowRepository (Spidlen *et al.*, 2012) and are temporarily available until article publication through accession numbers (after which the reader will be able to access this raw data by means stated in the paper);

FR-FCM-ZYTU,
http://flowrepository.org/id/RvFrLUcAM69ZrTziXZJn622NsvQgwRuVqoRC28XA9wBxR2KZsAJ2yym5ToAfMt6p,

FR-FCM-ZYTV,
http://flowrepository.org/id/RvFryhGbJHv5UMqqZ1bEfLEz6aO8QEV0EsQtjKdcSjlMt1rCtFIiT9HmOrMZYBms and

FR-FCM-ZYUZ,
http://flowrepository.org/id/RvFraaVCkdcJJYpYJOc4CFQNbETeVGwTy1bcNwXpA3YotemW9trGUDNKJFgxXnKi.

The closed genome sequence of L91543 is available under accession number CP016684.

Table 2. 5- List of primers

PCR primer pairs (forward, fwd; reverse, rev) used for PCR, qRT-PCR, Bacterial Two-Hybrid, Site-Directed Mutagenesis, and SOEing. Restriction sites are underlined.

Primer name	Primer Sequence
PCR primers	
PacI-fHbp-for	5'-GCGCAATTAATTAATTGCTTCTTTGACCTGCC-3'
PmeI-fHbp-rev	5'-ACCTGTTTAAACAATGGTTATTGCTTGGCG-3'
Transposon library primers (DUS element in bold)	
HindIIIkan2for	5'-CGCCAAGCTTCAACCATCATCATCGATGAATTGTGTCTCAAAA-3'
EcoRIDUSkan2rev	5'-ATCGGAATTC**ATGCCGTCTGAA**GCGTAATGCTCTGCAGT-3'
PCRFP (Epicentre)	5'- ATTCAGGCTGCGCAACTGT-3'
PCRRP (Epidentre)	5'- GTCAGTGAGCGAGGAAGCGGAAG-3'
Truncated FHbp primers	
BamHI_SIfHbpfor	5'-ATCTAGGATCCATGCCGTCTGAACCGCCG-3
XhoI_SfHbprev	5'- AATCACTCGAGTTTGTATACTTGGAACTCTCCT -3'
PacI_SfHbpfor	5'-

	AGCTCTTAATTAAATGCCGTCTGAAATGCCGTCTGAACCGCCG-3’
PmeI_SfHbprev	5’-TGCAGGTTTAAACACCCTTTGTTAGCAGCCGGATCTCA-3’
Sequencing primers	
pGCC4-fwd	5’-AGACATCCGCCAACAAGAAC-3’
pGCC4-rev	5’-CTAGGCACCCCAGGCTTTACA-3’
T25F	5’-GTGACCAGCGGCGATTCGGTGACCGATTAC-3’
T25R	5’-TGGCGAAAGGGGGATGTGCTGCAAGGCGAT-3’
T18F	5’-ATGCTTCCGGCTCGTATGTTGTG-3’
T18R	5’-TTTCCACAACAAGTCGATGCGTT-3’
RT-PCR primers	
RTfHbpFwd	5’- GTTTCGCAACCATCTTCCCG-3’
RTfHbpRev	5’- GACTTTATCCGTCAAATCGA-3’
qRT-PCR Primers	
fHbp-for	5’-CAGTCTTTGACGCTGGATCA-3’
fHbp-rev	5’-TCGATTTGGCGGATAAAGTC-3’
recA-for	5’- GAAGAGGTATTGGCAACGA-3’
recA-rev	5’- CGGATTTGTTGATGATGTCG-3’
ppiA-fwd	5’- GCGGCAACCCATGTTTTGAT-3’
ppiA-rev	5’- TGCCAAGTCCTCGGTCAATC-3’
surA-fwd	5’- CGACGATGCGTTCAACCATC-3’

surA-rev	5’- TACGTCGTCGCTGAATACGG-3’
dsbA-fwd	5’- TGCTCGGTTTGGCTAGGATG-3’
dsbA-rev	5’- GCAGCTTCGGGGGAATCATA-3’
misR-fwd	5’- TATGTCCCCAAACCCTGCAC-3’
misR-rev	5’- CTGTTGGGTGCGTTGTTCTG-3’

Primer pairs used for Bacterial Two-Hybrid

Primer Name	Primer Sequence
	5’-GCGAGGATCCATGACTAGGAGTAAACCTGTGAATC-3’
EcoRIrev_MC58FHbp	5’-GATCGAATTCTTATTGCTTGGCGGCAGGCCGATATG-3’
BamHIfwd_L91543FHbp	5’-GCGAGGATCCATGCCGTCTGAACCGTTGTTCGGACGGC-3’
EcoRIrev_L91543FHbp	5’-GATCGAATTCTTACTGCTTGGCGGCAAGACCGATATGG-3’
PstIfwd_SecA	5’-CGATCTGCAGATGCTGACAAACATTGCCAAGAAAATC-3’
SmaIrev_SecA	5’-CGACCCGGGTTAAGCCAGTTTGCCGTGGCATTG-3’

Primer pairs used for Site-Directed Mutagenesis (incorporated mutations underlined)

Primer Name	Primer Sequence
SP1_Fwd	5’-CTTCTGCTGCCTTTCTCTGACCG-3’
SP1_Rev	5’-GCAGTTCGGTTCACAGGT-3’
SP2_Fwd	5’-TTCTCTGACCACTGCCCTGATTC-3’

SP2_Rev	5’-AAGCAGCAGAAGGCAGTT-3’

Primer pairs used for SOEing (in bold regions that will bind to *kan* gene, underlined the regions that will bind to HA of interest, and in italics the regions that over-lap)

Primer Name	Primer Sequence
HA1_FHbp_Fwd	5’-GATAGAATTCCGAGTATGCAGCTTTG-3’
HA1_FHbp_Rev	5’-***GATGATGGTTG****CCATTGTGAA*AATGCCGTCC-3’
Kan_Fwd	5’-*TTCACAATGG****CAACCATCATC*****GATG**-3’
Kan_Rev	5’-*AAACCT****TTCAGACGGCAT*****GTAATGCTCTGCC**-3’
HA2_FHbp_Fwd	5’-***ATGCCGTCTGAA****AGGTTT*ACTCCTAGTCATACG-3’
HA2_FHbp_Rev	5’-CTTAGGATCCCCACGGCGCATACAAATTC-3’
HA1_0313_Fwd	5’-GATAGAATTCAGGCGCAGTTTACCTACTTG-3’
HA1_0313_Rev	5’-***GATGATGGTT****GTATCAATC*GGCGGATTGTATC-3’
0313_Kan_Fwd	5’-*GATACAATCCGCCGATTGATA****CAACCATCATC*****GATG**-3’
0313_Kan_Rev	5’-*AACAGCAA****TTCAGACGGCAT*****GTAATGCTCTGCC**-3’
HA2_0313_Fwd	5’-***ATGCCGTCTGAA****TTGCTGTT*CCTTTTCGGAGG-3’
HA2_0313_Rev	5’-CTTAGGATCCGAACGGCTTATGGCTTTGGGAC-3’

Primer pairs used for amplification of disrupted *Int* region of MC58Lnt

Primer Name	Primer Sequence

Int_for	5’-AGAAGGCCGGCCGCACGCGCAACACTGGGA--3’
Int_rev	5’-TCAGTTAATTAAGGACGGGCTGTGCAGGTA-3’

Chapter 3

Role of Lnt in modification of the lipoprotein FHbp

3.1- Introduction

Lipoproteins are a class of diverse proteins that provide the tertiary structure to the cell envelope of bacteria and some are important immune modulators (Sutcliffe and Russell, 1995, Kovacs-Simon *et al.*, 2011). This results chapter focuses on the meningococcal lipoprotein, Factor H Binding Protein (FHbp), and its lipidation by the enzyme Lnt. FHbp is a component of two current vaccines against Nm group B. It is one of the antigens in the multicomponent vaccine Bexsero (GSK), and the sole antigen of Trumenba sold by Pfizer. However, there is variation in the level of expression of this antigen between strains, which may limit the effectiveness of these vaccines against low-expressing strains (Beernink *et al.*, 2010; De Angelis *et al*., 2016). Elucidating the molecular pathway for the lipidation and transport of FHbp to the cell surface and investigating possible regulation of this pathway will help in evaluating the performance of FHbp-based vaccines and identify potential new drug targets.

In this chapter, a random transposon library[10] (Tn) was constructed in strain MC58 and mutants screened for those with reduced levels of FHbp surface expression using the anti-FHbp monoclonal antibody, JAR4. A mutant was identified which exhibited strongly reduced JAR4-binding and the transposon was subsequently localised to the *lnt* gene.

3.2- Transposon Library construction

Tn libraries are powerful molecular tools that enable the investigation of genes and their function. In order to identify potential genes that could affect expression of FHbp, a random Tn library was constructed in Nm strain MC58 (performed initially for my undergraduate project when the first 100 mutants were tested and subsequently repeated during my PhD).

Initially, direct transposon mutagenesis by EZ::Tn5 (Epicentre) was attempted in strain MC58, according to manufacturer's instructions (Fig. 3.1). This approach however did not generate any Kanamycin-resistant transformants (Fig. 3.1).

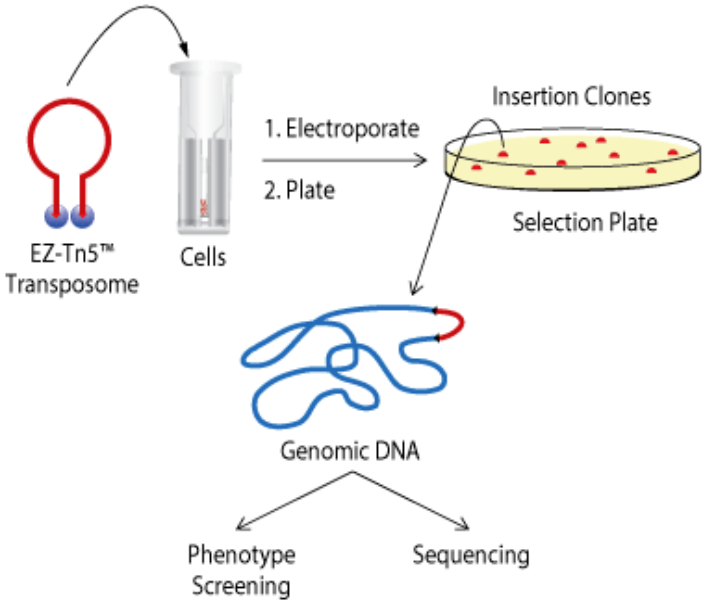


Figure 3. 1- Direct transposon mutagenesis by EZ::Tn5 (Epicentre)

An EZ-Tn5 Transposome Complex can be electroporated into living cells where it randomly inserts the transposon component into the host's genomic DNA.

After attempting direct transposon mutagenesis, *in vitro* transposition was the next logical step to be tried. The *in vitro* transposition kit (Epicentre) was used to incorporate the mobile element (Tn) into genomic MC58 DNA (Fig 3.2). First, genomic DNA was digested with *Dra*I and fragments around 6kb were excised from an agarose gel 1x following electrophoresis and purified.

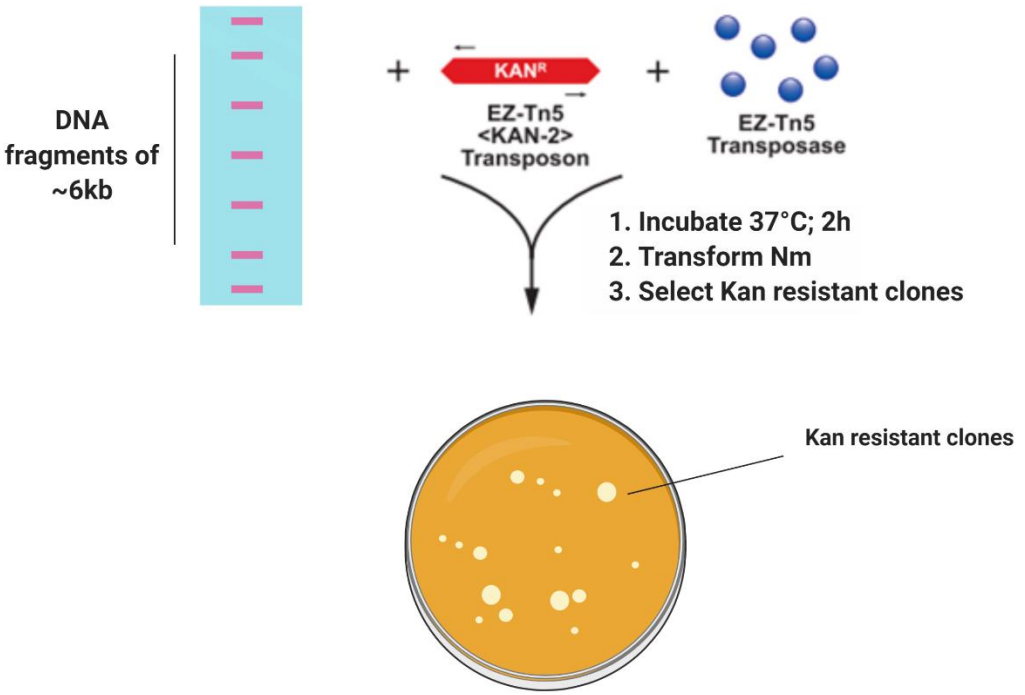


Figure 3. 2- - In vitro transposition with EZ-Tn5<Kan-2> transposon

In vitro transposition consists of inserting EZ-Tn5<Kan-2> transposon into random pieces of genomic DNA (gDNA) (previously digested with a restriction enzyme that ideally will not cut the gDNA smaller than pieces of 6kb) and then using these modified pieces of DNA to transform bacteria. A representation of the fragments of MC58 gDNA of up to 6kb that were generated after digestion with *Dra*I enzyme are shown. The fragments of MC58 gDNA were incubated with the transposome (transposon and transposase) according to the manufacturer's instructions (Epicentre) as shown. This reaction allows the random insertion of Tn into MC58 gDNA. Since the Tn is then flanked by Nm DNA sequences, this now, modified with Tn, gDNA can be used to transform Nm. Mutants would be expected to arise from homologous recombination[3] after transformation and selection for Kanamycin (schematic figure adapted from Epicentre Manufacturer's manual).

Upon failure to transform strain MC58 with this approach, the plasmid pMOD$_{TM}$-2<MCS> bearing the EZ::Tn5<KAN-2> transposon was modified to include neisserial DNA uptake sequence (DUS). DUS elements are known to help with endogenous DNA exchange between the genus *Neisseria.* They are short inverted repeated sequences that can form hairpin structures and are well conserved in *Neisseria* (Spencer-Smith *et al.*, 2016). We reasoned that the inclusion of a DUS element, in this case ATGCCGTCTGAA, would greatly increase the efficiency of transformation.

To incorporate DUS, the approach used included amplification of the *kan* resistance gene with primers *Hind*III*kan2*for, which incorporates a *Hind*III site and anneals immediately downstream of the 5′ mosaic end (ME) of the Tn and the reverse primer *Eco*RIDUS*kan2*rev (Table 2.5), which incorporates an *Eco*RI site and DUS element and anneals downstream of the stop codon and upstream of the 3′ ME (Fig 3.3a and b). After successful amplification of the *kan* gene, the PCR product was digested with the enzymes *Eco*RI and *Hind*III, gene cleaned and cloned into the vector pMOD$_{TM}$-2<MCS> vector at the corresponding restriction sites (Fig 3.3c). The construct was transformed into *E. coli* and mutants were selected on kan. Successful transformants were selected and plasmid extraction was performed. To verify the correct incorporation of DUS, plasmids were sent for sequencing (appendix III). The resulting plasmid was designated pMOD$_{TM}$-2<Tn5KAN-2DUS>.

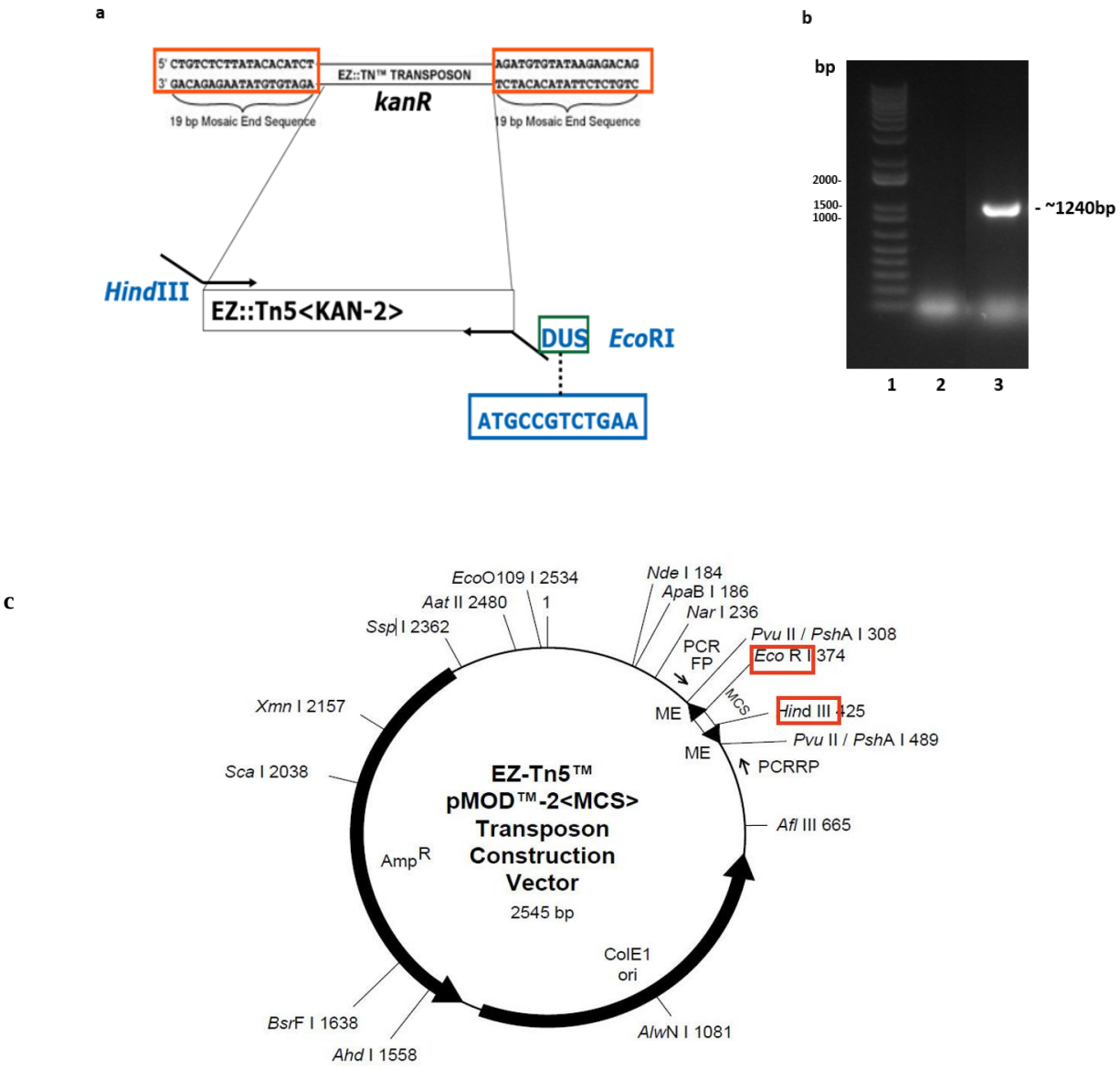


Figure 3. 3- Insertion of a meningococcal DUS into the EZ::Tn5<KAN-2> transposon

a. Schematic figure of the modified EZ::Tn5<KAN-2> construct. **b.** Amplified *kan* gene. The final product contained the DUS element and the restriction sites *Hind*III and *Eco*RI. Lane 1, ladder; lane 2, PCR reaction negative control; lane 3, modified EZ::Tn5<KAN-2> containing DUS (1240bp) **c.** pMOD$_{TM}$-2<MCS> vector map.

Following successful modification of EZ::Tn5<KAN-2> to include a DUS element, phosphorylated primers PCRFP and PCRRP (Epicentre, Table 2.5) were used to amplify the region from pMOD$_{TM}$-2<Tn5KAN-2DUS> where the DUS was inserted plus the areas of the mosaic ends (ME), important for correct transposition reaction (Fig 3.4). MC58 genomic DNA was then extracted and digested with *Dra*I (Fig 3.4a). An *in vitro* transposition reaction followed by T4 DNA polymerase and T4 ligase-mediated repair of single stranded DNA (Fig 3.4b). This entire process should insert Tn5 randomly into digested chromosomal DNA. The final reaction was gene cleaned and the DNA used for transformation of Nm MC58. Mutants were selected on Kan and several hundred transformants obtained and stored for subsequent screening.

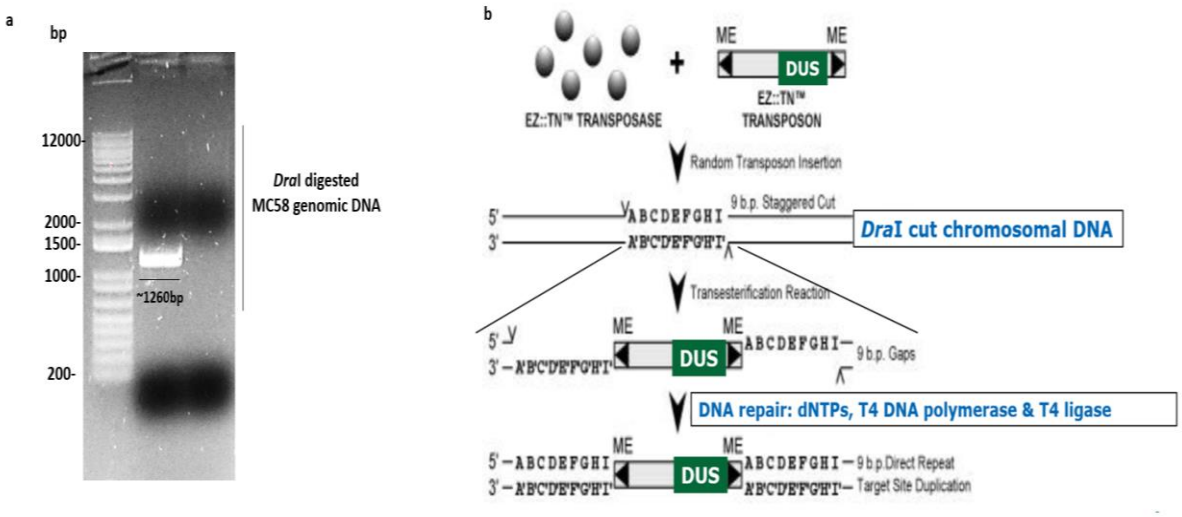


Figure 3. 4- *In vitro* transposition

a. Schematic figure of the steps followed to perform *in vitro* transposition. After successful random incorporation of the modified Tn5 into MC58 chromosomal DNA, single stranded DNA was repaired with T4 DNA polymerase and T4 DNA ligase. **b.** Lane 1, ladder; lane 2, PCR product containing modified EZ::Tn5<KAN-2> plus ME; lane 3, *Dra*I cut chromosomal DNA (faint smear of DNA after digest is shown).

Following successful generation of Tn mutants, initially 100 mutants were screened by immuno-dot blot for reduced levels of surface FHbp. One mutant showed less binding to the monoclonal antibody JAR4 (Fig 3.5).

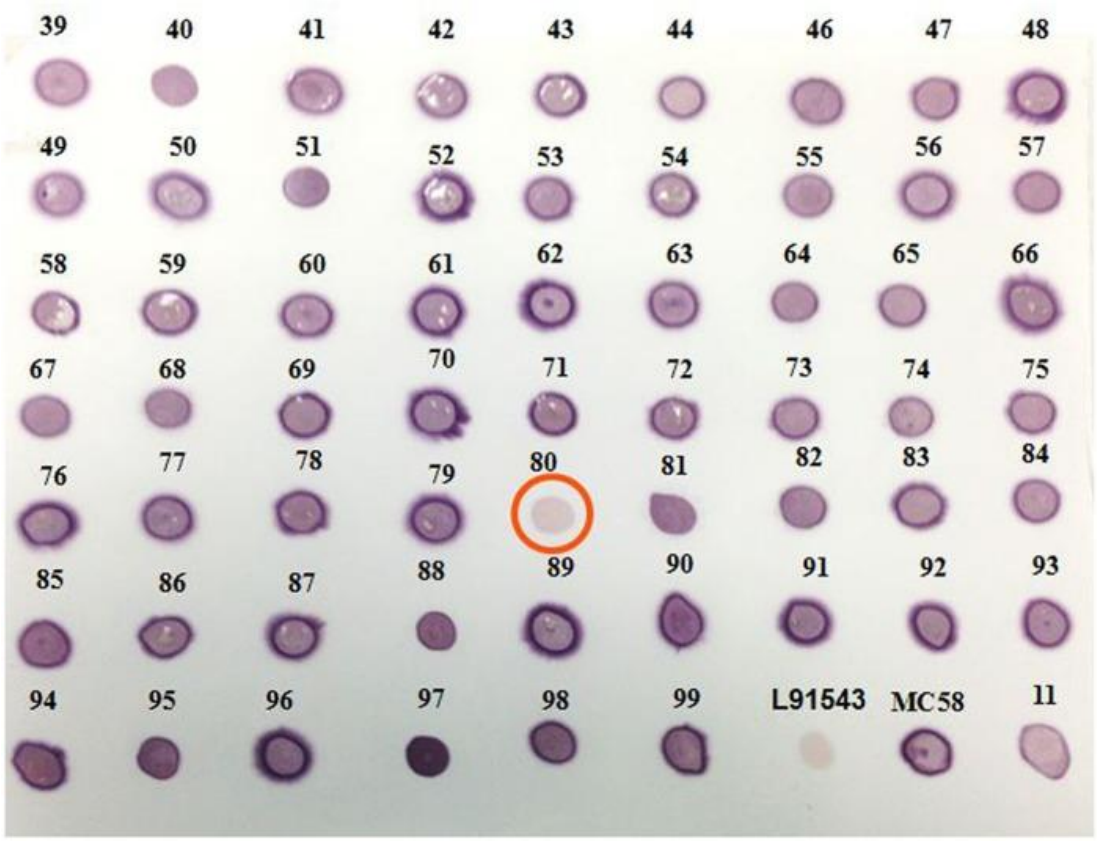


Figure 3. 5- Whole cell immuno-dot blot of MC58 Tn mutants using JAR4 monoclonal antibody

WC immuno-dot blot of MC58 Tn mutants were screened using JAR4 monoclonal antibody. Strains MC58 and L91543 were included as positive and negative control strains, respectively, for reactivity to JAR4. Red circle shows the mutant 80 that presented reduced binding to JAR4.

Strain L91543 was used as negative control for this experiment. L91543 is known for having poor expression of FHbp on its surface (Newcombe *et al.*, 2014). It expresses the same FHbp variant, of the same subfamily as of MC58; variant 1, subfamily B (Newcombe *et al.*, 2014) with 95 and 93% similarities at the nucleotide and amino acid levels, respectively (Karlyshev *et al.*, 2015).

This mutant was re-tested at OD_{600} 0.5 and the reduced binding to JAR4 confirmed at this lower OD_{600} (Fig 3.6).

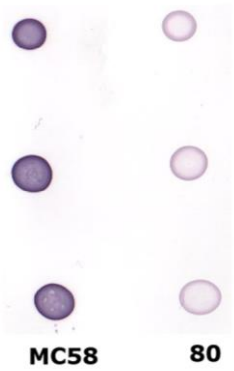


Figure 3. 6- Whole cell immuno-dot blot of MC58 Tn mutant number 80 using JAR4 monoclonal antibody at OD_{600} 0.5

Standardised bacterial suspensions were probed with anti-FHbp antibody, JAR4.

Tn mutant number 80 was subjected to whole genome sequencing (WGS) (kindly performed by Prof Andrey Karlyshev) and it was found to have the Tn inserted in the middle of the gene NMB0713, annotated as the *lnt* gene. This mutant was subsequently called MC58Lnt. Moreover, WGS confirmed the insertion of only one transposon.

3.2- Disruption of Lnt strongly reduces FHbp cell surface exposure

To confirm that the reduction in FHbp surface expression found in MC58Lnt was solely caused by *lnt* disruption, *lnt* was cloned into the complementation vector pGCC4 (Mehr and Seifert, 1998) in reverse orientation such that *lnt*

expression was under the control of its own putative promoter, and transformed into MC58Lnt. The complemented strain was made by Dr Ruth Griffin and named MC58LntC, and fluorescence microscopy (performed by Dr Collins) of MC58Lnt confirmed FHbp was no longer present on cell (Fig 3.7). Full restoration of surface display was seen in the complemented strain. From these results it can be concluded that disruption of *lnt* was responsible for the poor surface localisation of FHbp on strain MC58Lnt.

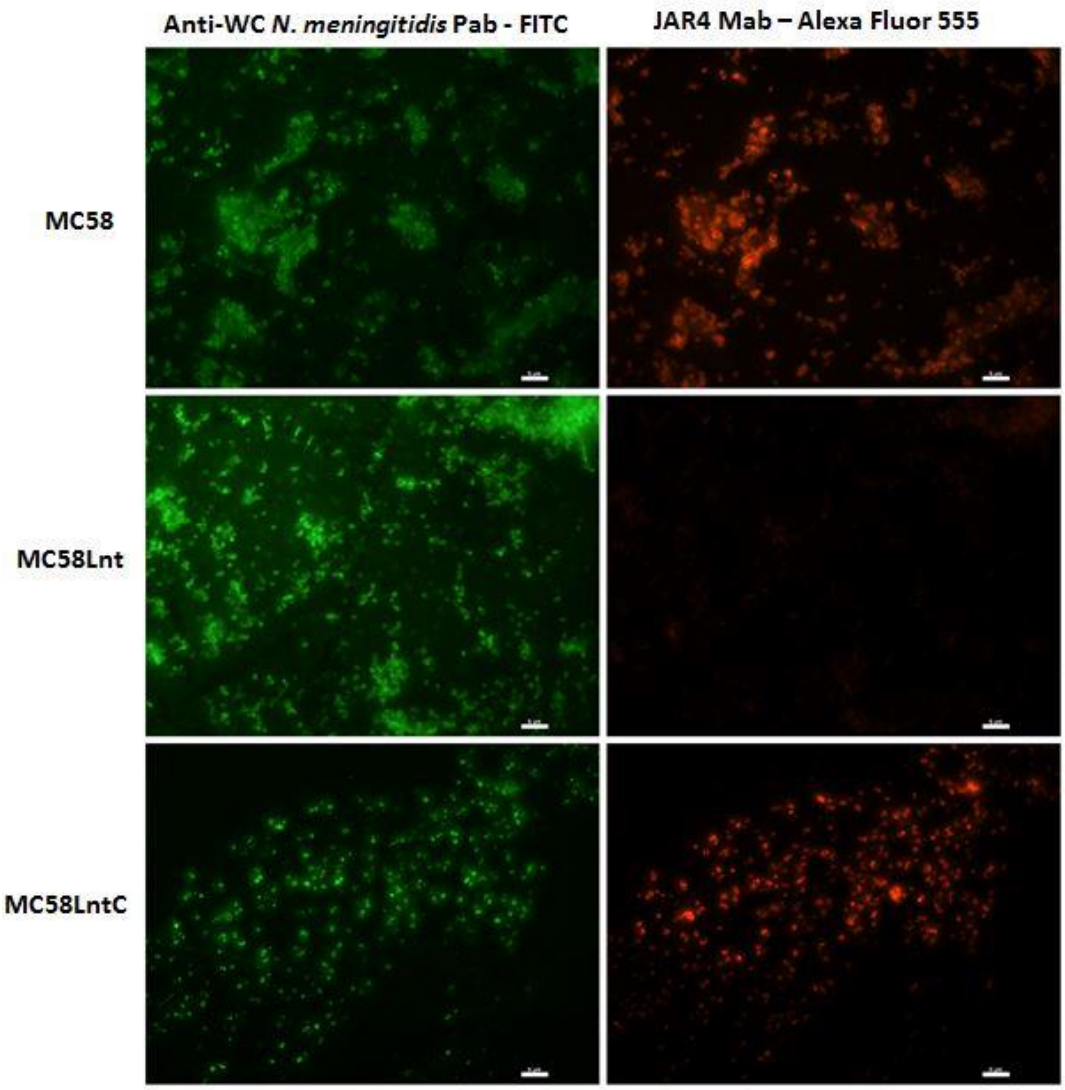
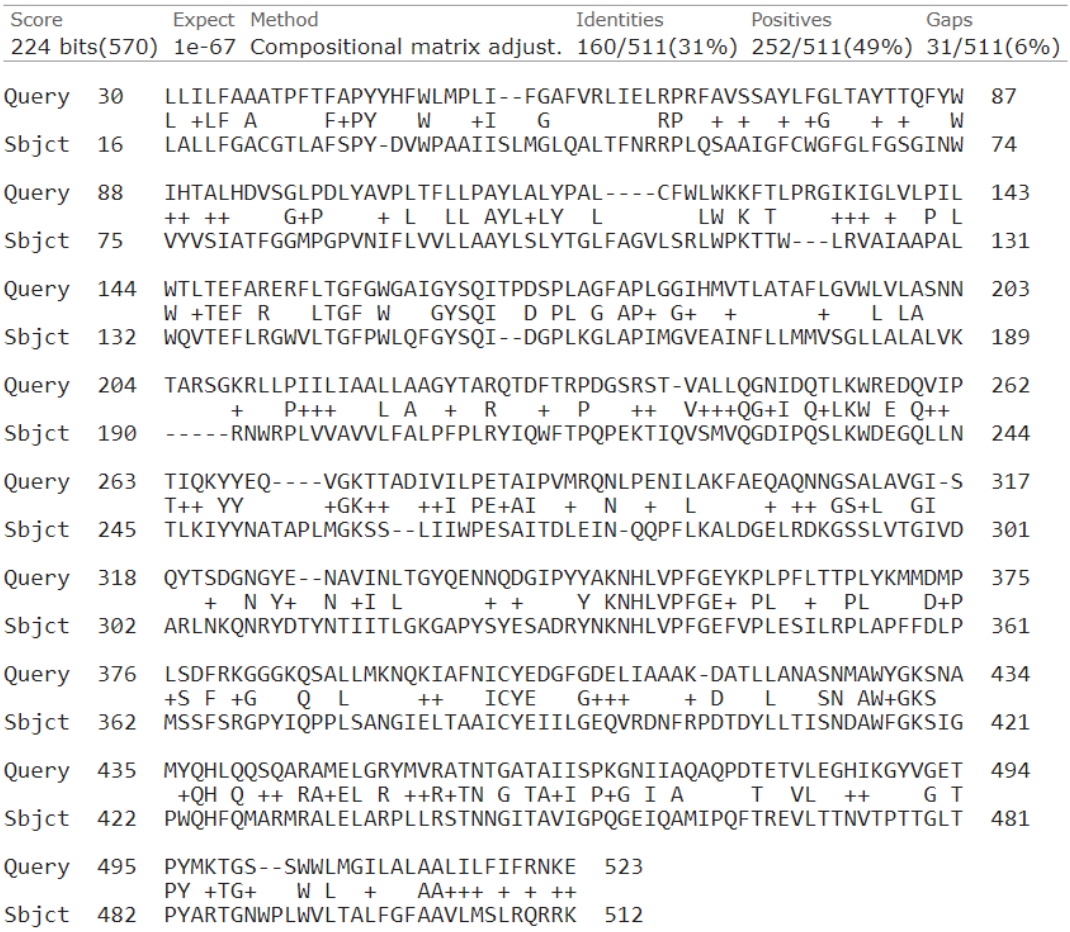


Figure 3. 7- Immunofluorescence microscopy of strains MC58, MC58Lnt and MC58LntC

To confirm the presence of meningococcal cells, cells were incubated with FITC-labelled rabbit polyclonal IgG (Pab) raised against WC Nm (left panels). To compare FHbp cell surface expression between the strains, cells were also incubated with anti-FHbp antibody JAR4 that was detected by Alexa Fluor 555 labelled donkey anti-mouse IgG secondary antibody (right panels). The data confirm that the absence of surface FHbp on MC58Lnt can is restored upon complementation of *lnt*.

3.3- Further *in silico* studies of Neisserial Lnt

Blast analysis revealed a 31% similarity at the amino acid level to Lnt found in *E. coli* MG1655 (Fig 3.8). Phyre2 is free web-based service for protein structure prediction (Kelley *et al.*, 2015). A Phyre2 search using normal mode was able to structurally model the meningococcal Lnt based to Lnt of strain PAO1 of *Pseudomonas aeruginosa* with a 100% confidence and 93% coverage (Fig 3.9). From the literature, studies mainly in *E. coli* show that Lnt is a transmembrane enzyme responsible for adding a third acyl group to lipoproteins (Gupta *et al.,* 1993; Buddelmeijer and Young, 2010). After a preprolipoprotein has undergone di-acyl modification and cleavage of the SP by Lgt and LspA, respectively, the apolipoprotein undergoes its final modification with the addition of an acyl chain to the N-terminal cysteine, generating a triacylated lipoprotein (Sankaran and Wu, 1994, Tokunaga *et al.,* 1982). Lnt was thought to be essential for gram-negative bacteria and is typically found absent in gram-positive bacteria (Narita and Tokuda, 2017).



Score	Expect	Method	Identities	Positives	Gaps
224 bits(570)	1e-67	Compositional matrix adjust.	160/511(31%)	252/511(49%)	31/511(6%)

```
Query  30   LLILFAAATPFTFAPYYHFWLMPLI--FGAFVRLIELRPRFAVSSAYLFGLTAYTTQFYW  87
            L +LF A     F+PY   W   +I   G        RP  + +  + +G   + +   W
Sbjct  16   LALLFGACGTLAFSPY-DVWPAAIISLMGLQALTFNRRPLQSAAIGFCWGFGLFGSGINW  74

Query  88   IHTALHDVSGLPDLYAVPLTFLLPAYLALYPAL----CFWLWKKFTLPRGIKIGLVLPIL  143
            ++ ++    G+P    + L  LL AYL+LY  L       LW K T    +++ +  P L
Sbjct  75   VYVSIATFGGMPGPVNIFLVVLLAAYLSLYTGLFAGVLSRLWPKTTW---LRVAIAAPAL  131

Query  144  WTLTEFARERFLTGFGWGAIGYSQITPDSPLAGFAPLGGIHMVTLATAFLGVWLVLASNN  203
            W +TEF R   LTGF W   GYSQI  D PL G AP+ G+  +      +   L LA
Sbjct  132  WQVTEFLRGWVLTGFPWLQFGYSQI--DGPLKGLAPIMGVEAINFLLMMVSGLLALALVK  189

Query  204  TARSGKRLLPIILIAALLAAGYTARQTDFTRPDGSRST-VALLQGNIDQTLKWREDQVIP  262
                 +   P+++   L A  +  R   +  P   ++  V+++QG+I Q+LKW E Q++
Sbjct  190  -----RNWRPLVVAVVLFALPFPLRYIQWFTPQPEKTIQVSMVQGDIPQSLKWDEGQLLN  244

Query  263  TIQKYYEQ----VGKTTADIVILPETAIPVMRQNLPENILAKFAEQAQNNGSALAVGI-S  317
            T++ YY      +GK++  ++I PE+AI  +  N  +  L     + ++ GS+L  GI
Sbjct  245  TLKIYYNATAPLMGKSS--LIIWPESAITDLEIN-QQPFLKALDGELRDKGSSLVTGIVD  301

Query  318  QYTSDGNGYE--NAVINLTGYQENNQDGIPYYAKNHLVPFGEYKPLPFLTTPLYKMMDMP  375
               +  N Y+  N +I L      + +    Y KNHLVPFGE+ PL  +  PL    D+P
Sbjct  302  ARLNKQNRYDTYNTIITLGKGAPYSYESADRYNKNHLVPFGEFVPLESILRPLAPFFDLP  361

Query  376  LSDFRKGGGKQSALLMKNQKIAFNICYEDGFGDELIAAAK-DATLLANASNMAWYGKSNA  434
            +S F +G   Q  L     ++   ICYE   G+++    + D   L   SN AW+GKS
Sbjct  362  MSSFSRGPYIQPPLSANGIELTAAICYEIILGEQVRDNFRPDTDYLLTISNDAWFGKSIG  421

Query  435  MYQHLQQSQARAMELGRYMVRATNTGATAIISPKGNIIAQAQPDTETVLEGHIKGYVGET  494
             +QH Q ++ RA+EL R ++R+TN G TA+I P+G I A     T  VL  ++    G T
Sbjct  422  PWQHFQMARMRALELARPLLRSTNNGITAVIGPQGEIQAMIPQFTREVLTTNVTPTTGLT  481

Query  495  PYMKTGS--SWWLMGILALAALILFIFRNKE  523
            PY +TG+   W L  +   AA+++ + + ++
Sbjct  482  PYARTGNWPLWVLTALFGFAAVLMSLRQRRK  512
```

Figure 3. 8- Blastp screenshot of MC58 Lnt (NMB0713) against *E. coli* MG1655 Lnt

Screenshot of the alignment between NMB0713 (query) and *E. coli* MG1655 (Subject) Lnt. It shows that of the 94% of the amino acids of NMB0713 (query cover), 31% are identical to the amino acids of Apolipoprotein N-acyltransferase (Lnt) found in *E. coli* MG1655.

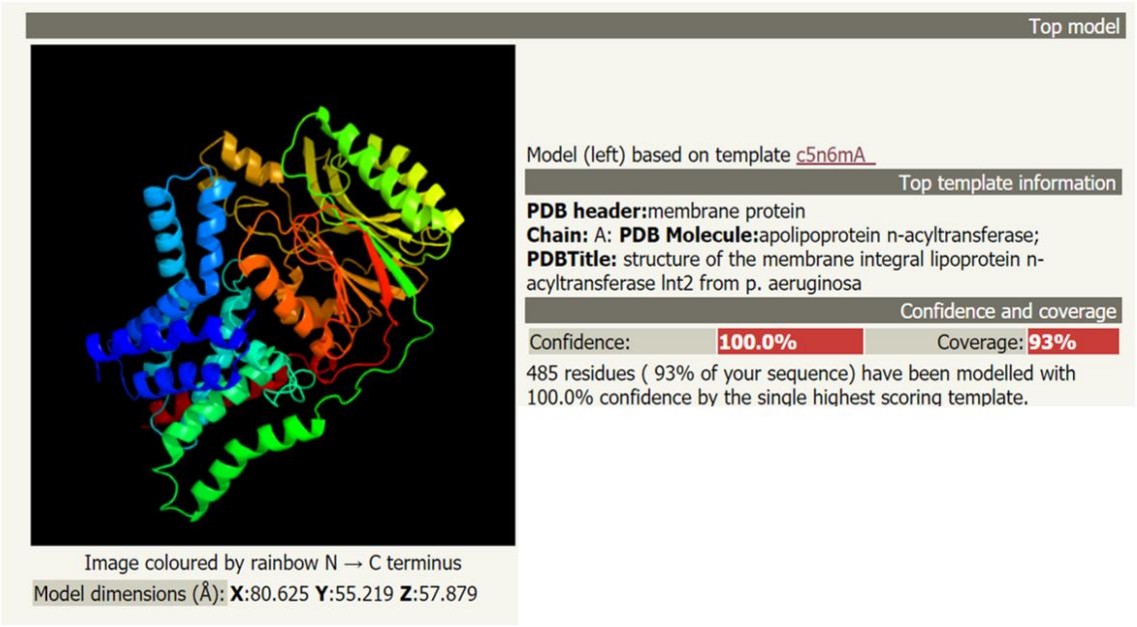


Figure 3. 9- Phyre2 search results screenshot of NMB0713 amino acid sequence

Phyre2 search using the normal mode was able to structurally model, with 100% confidence and a coverage of 93%, the Neisserial Lnt protein based on the structure of *Pseudomonas aeruginosa* strain PAO1 Lnt (unique identifier on Protein Data Bank 5NM6).

3.4- FHbp is not fully mature in MC58Lnt

Next, we proceeded to investigate the role of Lnt in relation to FHbp. From *in silico* investigations (Figs 3.8 and 3.9), we hypothesised Lnt would have a similar role to the one described for *E. coli*.

3.4.1- Construction of pGCC4S*fHbpHis*

Adopting the approach of Kurokawa *et al*. (2012) and LoVullo *et al.* (2015) we investigated whether Lnt was responsible for adding a fatty acid to the lipoprotein FHbp. These two research groups showed that by expressing truncated lipoproteins, corresponding to the first hundred or so amino acids, the molecular weight difference corresponding to the presence of one fatty acid could be determined between a Lnt mutant and the parental strain by SDS-PAGE.

A truncated version of the FHbp protein, incorporating the signal peptide and the first 100 amino acids (from the cysteine at +1), fused to a 6x His tag was expressed in Nm MC58. Initially, a fragment of *fHbp* encoding 120 amino acids of the original MC58 FHbp was amplified with BamHI_SIfHbpfor and XhoI_SfHbprev and cloned into the vector pET-28b(+) in the restriction sites *Bam*HI and *Xho*I (Fig 3.10), generating pET28b_S*fHbpHis*, totalling 528 bp for the *fHbp* fragment plus His tag.

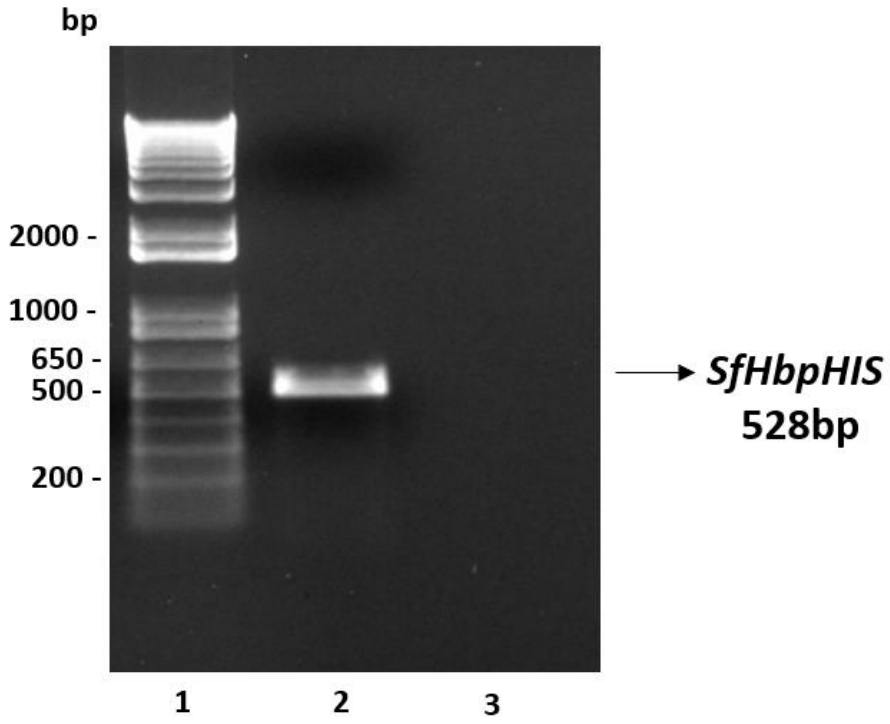


Figure 3. 10- PCR verification of the S*fHbpHis* after cloning into pET-28b(+) and transformation into *E. coli* JM109

The reaction performed with the primers BamHI_SIfHbpfor and XhoI_SfHbprev generated a PCR product of 528bp. Ladder, PCR product of S*fhbpHIS* gene amplification, negative control (MC58 genomic DNA) correspond to lanes 1-3.

Next, the primers PacI_SfHbpfor and PmeI_SfHbprev were used to amplify the S*fHbp*His fragment of pET28b_S*fHbp*His. As the name suggests, *SfHbpHis* contains the sequence to encode the His tag from pET-28b(+), important for subsequent differentiation of the different sized FHbp. The fragment S*fHbp*His, now containing the restriction sites *Pac*I and *Pme*I, was then cloned into pGCC4. This construct was called pGCC4S*fHbp*His. After transforming MC58 and MC58Lnt with sequenced plasmid DNA (Appendix III), transformants were checked by PCR with primers pGCC4 forward and pGCC4 reverse (Table 2.5). As expected, a PCR product of 748 bp was obtained from complemented strains, whilst a product of 220 bp was obtained from amplification of the plasmid backbone (Fig 3.11). Correct transformants were named MC58_SFHbpHis and MC58Lnt_SFHbpHis. A schematic diagram summarising the approach is shown in Fig. 3.12.

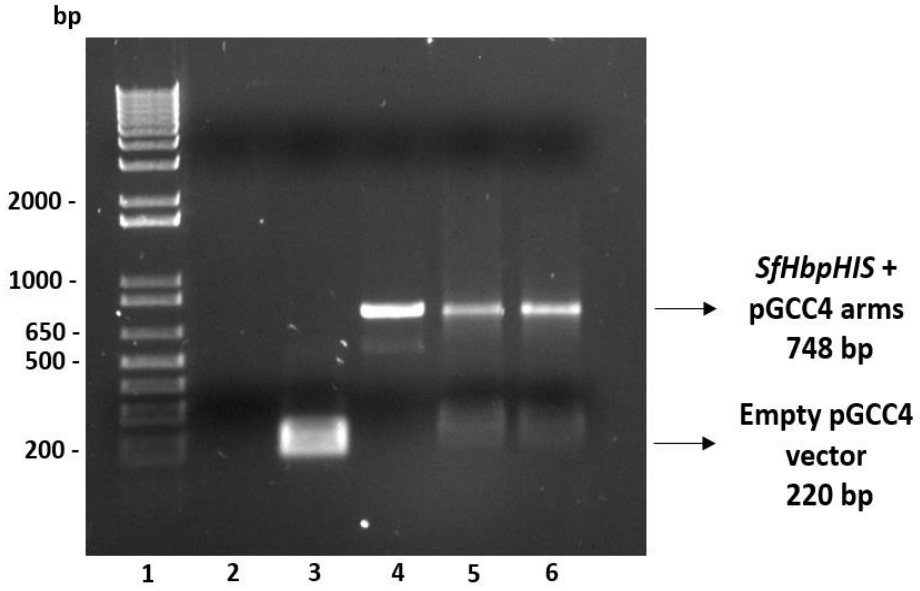


Figure 3. 11- PCR verification of the MC58_SFHbpHis and MC58Lnt_SFHbpHis strains using primers pGCC4for and pGCC4rev

Lanes 1-6 correspond to Ladder, PCR product from MC58 (negative control), pGCC4 vector with no insert (220 bp), pGCC4 with insert (748 bp, insertion also confirmed by sequencing, see appendix III), MC58_SFHbpHis and MC58Lnt_SFHbpHis after transformation with pGCC4S*fHbp*His.

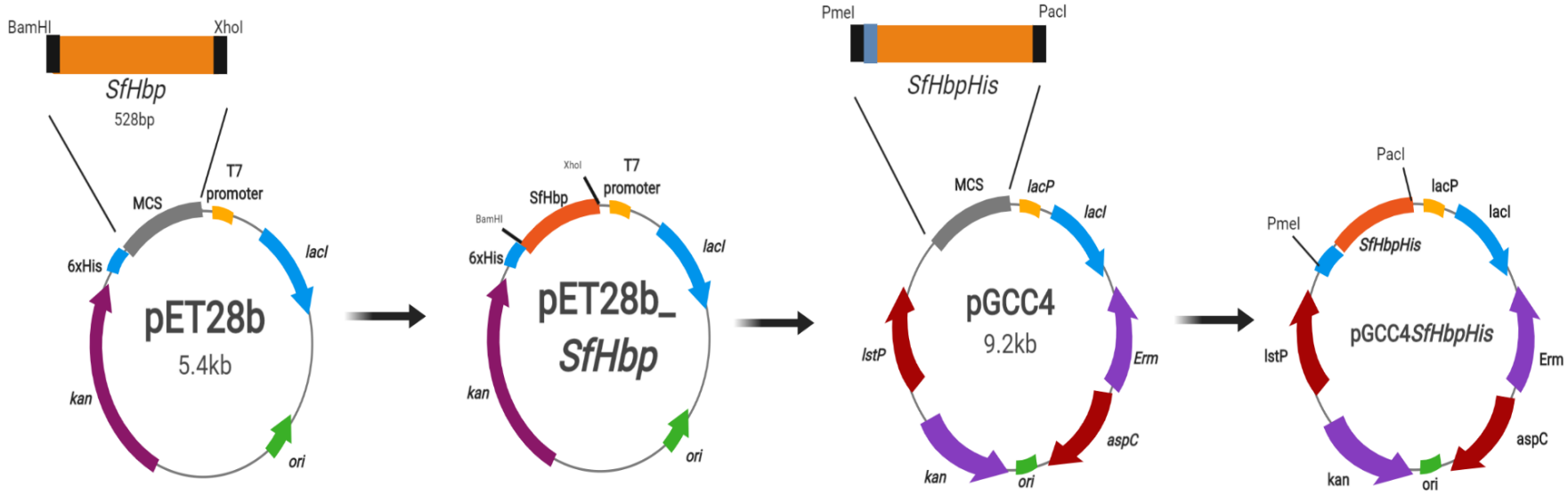


Figure 3. 12-Schematic diagram of the strategy used to generate shortened FHbp tagged with 6x His tag

This strategy was based on the approach used by Kurokawa *et al.* (2012) and LoVullo *et al.* (2015). Sfhbp was first cloned into pET28b, then SfHbpHis was cloned into pGCC4 to allow for transformation into Nm. DNA fragments not in scale.

3.4.2- Strains MC58_SFHbpHis and MC58Lnt_SFHbpHis express truncated His-tagged FHbps with different molecular weights

After confirmation of recombinant strains, MC58_SFHbpHis and MC58Lnt_SFHbpHis, WB was performed to detect any difference in mobility between the truncated His-tagged FHbp expressed by these strains. Analysis by SDS-PAGE of whole cell extracts and detection of FHbp by western immunoblotting with anti-His antibody showed the mobility of the truncated protein was greater in MC58Lnt_SFHbpHis of the size expected for one fatty acid difference (Fig 3.13). A similar change in mobility was observed by Kurokawa *et al.* (2012) in their Lnt mutant of *Staphylococcus aureus,* which was subsequently confirmed MALDI-TOF MS analysis. Since there are no other Lnt homologues in the genome of MC58, the observed size difference suggest that Lnt is responsible for triacylating FHbp in Nm strain MC58 and is also likely responsible for triacylating all other lipoproteins in this strain.

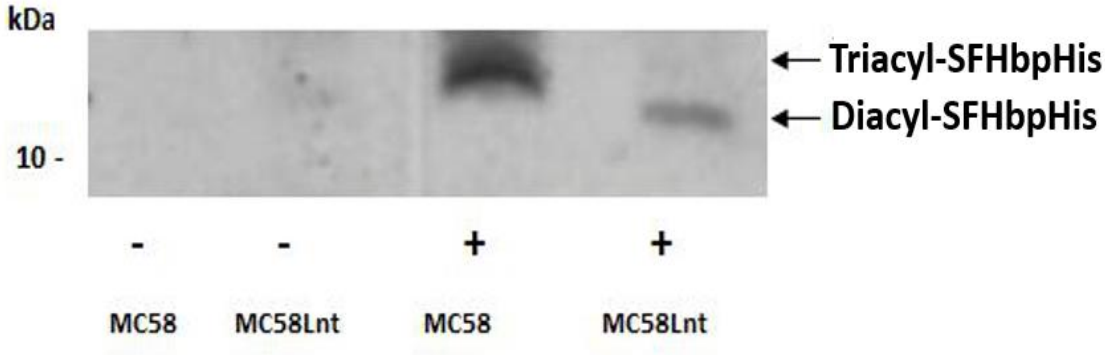


Figure 3. 13- WB of His-tagged truncated FHbp in MC58_SFHbpHis and MC58Lnt_SFHbpHis

Representative immunoblot of 3 experiments showing the electrophoretic mobility of His-tagged FHbp in cell lysates from strains MC58_SFHbpHis and MC58Lnt_SFHbpHis probed with anti-His antibody (lanes 3 and 4). Their respective parental strains were included as negative controls (lanes 1 and 2).

3.5- Low levels of diacylated FHbp in MC58Lnt are localised in the cell surface

To investigate the effect Lnt disruption has on FHbp surface display, WC immuno-dot blots were used to quantify and assess the levels of expression of FHbp on the surface of MC58Lnt. Cell suspensions from freshly grown plate were standardised to OD_{600} 0.5 in PBS. The immuno-dot blots not only confirmed the reduction of FHbp on the surface of MC58Lnt as shown by immunofluorescence experiments (Fig 3.14) but indicated that a small amount of FHbp localises to the surface when compared L91543.

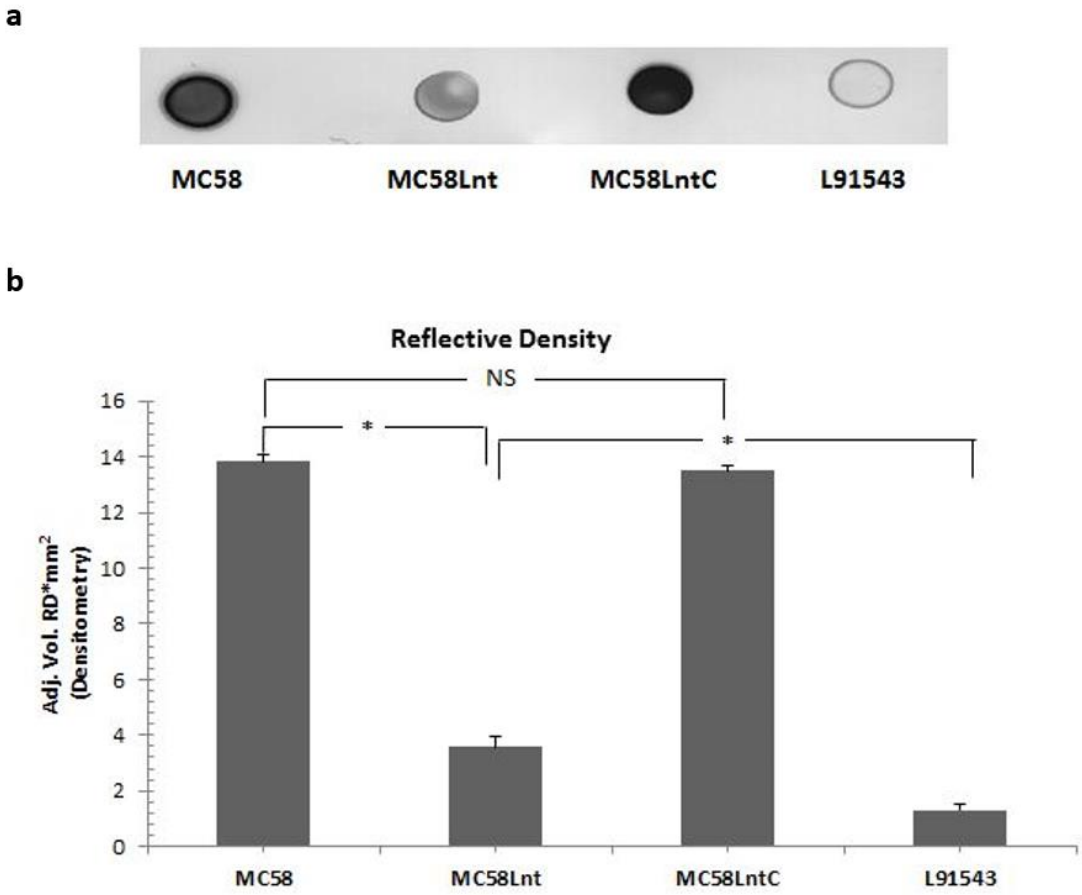


Figure 3. 14- Low levels of diacylated FHbp in MC58Lnt are localised in the cell surface

a. The binding of anti-FHbp antibody, JAR4, to standardized WC suspensions of strains MC58, MC58Lnt, MC58LntC and L91543 in an immuno-dot blot assay to compare the level of surface expression of FHbp between these strains. The image is representative of multiple independent experiments (*n* = 6). **b.** The reflective density of the dots was measured by a GS-800™ calibrated densitometer. All columns represent mean ± SEM. *p ≤ 0.05, significantly different as indicated; NS, not significant.

This result indicates MC58Lnt is displaying diacylated FHbp on its surface at a low level when compared to MC58 but of a greater level compared to

L91543. Thus, FHbp in MC58Lnt can localise to the surface despite not being fully mature.

3.6- Disruption of Lnt causes major reduction in total cellular levels of FHbp

Following the evaluation of FHbp display on the cell surface in MC58Lnt, the aim was to assess the total level of FHbp in whole cells. This would reveal if the lack of FHbp on the surface was due to poor surface localisation alone or due to changes in FHbp expression levels.

WC extracts of MC58, MC58Lnt and MC58LntC were fractioned by SDS-PAGE and immunoblotted with JAR4 for detection of FHbp. A surprising 10-fold reduction in the WC level of FHbp in MC58Lnt was observed when compared to MC58 and MC58LntC (Fig 3.15). The housekeeping, constitutively expressed, protein RecA (Loh *et al.,* 2013) was used as a loading control. A consistent level of RecA was observed for all three strains.

It is unlikely that the reduction observed is due to the inability of JAR4 to bind diacylated FHbp. It has been shown that the specificity of JAR4 is an N-terminal epitope including the amino acid (AA) residues DHK at positions 25 to 27 (Beernink and Granoff, 2009). Furthermore, we show in the following chapters that JAR4 not only binds to non-acylated FHbp's but also to the non-acylated FHbp that retains its signal peptide. Therefore, conclusively, Lnt disruption results in a reduction in total cellular FHbp.

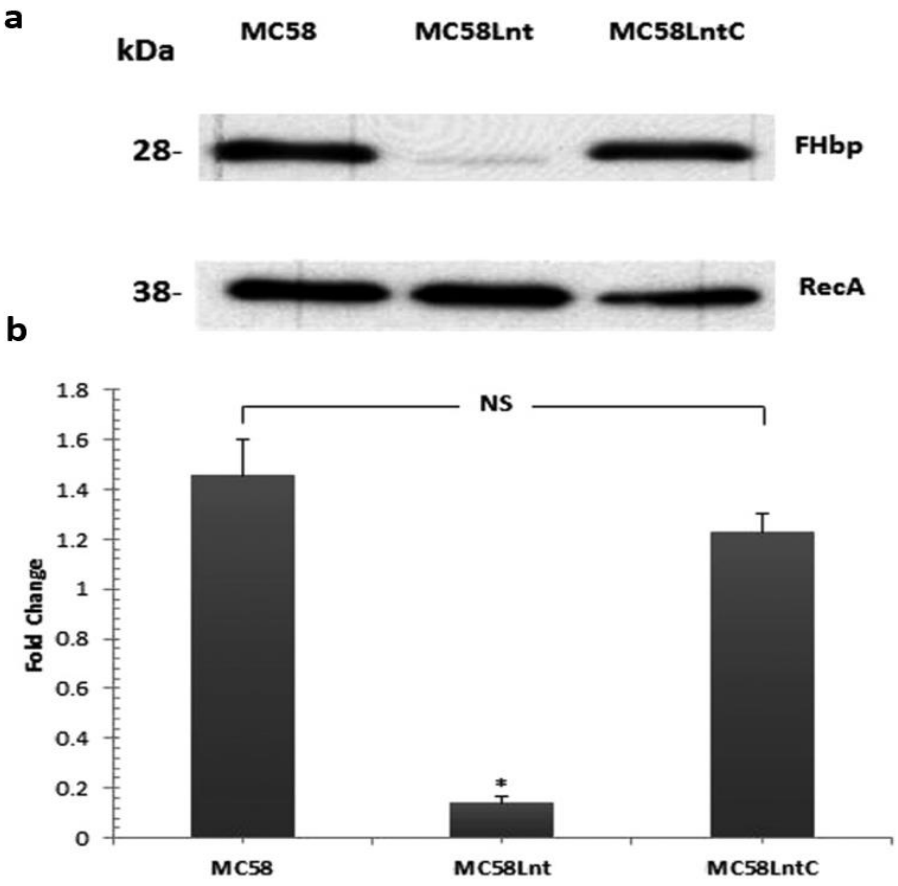


Figure 3. 15- Disruption of Lnt causes a significant reduction in total cellular levels of FHbp

a. Western immunoblot of WC lysates from strains MC58, MC58Lnt and MC58LntC probed with anti-FHbp antibody, JAR4. Equal protein loading was confirmed by the determination of RecA protein in each sample. The image is representative of multiple independent experiments (n = 8). **b.** The expression of FHbp was determined in MC58, MC58Lnt and MC58LntC by densitometry and normalized to RecA protein. The reflective density was measured by a GS-800™ calibrated densitometer. All columns represent mean ± SEM, *p ≤ 0.05, significantly different from strain MC58; NS, not significant.

3.7- Disruption of Lnt causes a reduction in *fHbp* transcription

The levels of *fHbp* mRNA were checked to see if the reduction in FHbp protein levels could be attributed to transcriptional regulation. Total mRNA was extracted from MC58, MC58Lnt and MC58LntC, and qRT-PR performed with the *fHbp*-specific primers fHbp-for and fHbp-rev (shown in table 2.5 in materials and methods).

3.7.1- RNA extractions

Total mRNA was extracted from 1ml of cell suspensions standardised to A_{600} 0.65 (containing approximately 2 x 10^8 cells) of strains MC58, MC58Lnt, and MC58LntC using the RNeasy Mini kit (Qiagen). The quality of the extracted RNA was assessed through agarose gel electrophoresis and recording the absorbance values on Nanovue spectrometer at 260/280 and 260/230 to check RNA purity. A representative gel of the extractions is shown below confirming that the RNA was of high quality (Fig 3.16). Degraded RNA would have a smeared appearance and lack the sharp rRNA bands.

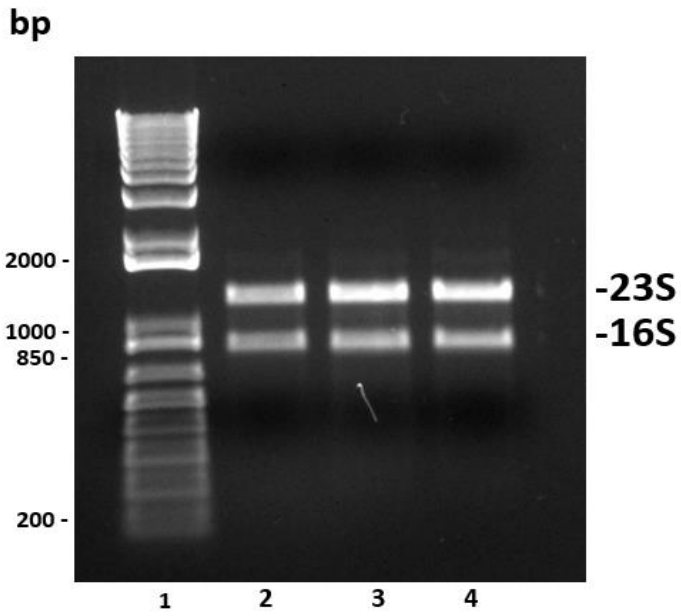


Figure 3. 16- High quality total RNA extractions from MC58, MC58Lnt and MC58LntC

Agarose gel electrophoresis was performed to determine the integrity and overall quality of total RNA extracted by inspection of the 23S (higher band) and 16S (lower band) ribosomal RNA bands (extractions 1-3 are shown). Lanes 1-4 correspond to Ladder, MC58 RNA extract, MC58Lnt RNA extract, MC58LntC RNA extract.

3.7.2- Primer optimisation and qPCR reaction efficiency

One µg of cDNA was synthesised using the QuantiTect reverse transcription kit (Qiagen) in a two-step procedure as described in section 2.5.11.2 of the Material and Methods chapter. Primers for *fHbp* and *recA* amplification were designed to amplify 155bp and 160bp regions, respectively. The primer concentrations were optimized using different ratios of the primers in a series of experiments. Fig 3.17 shows the amplification plot (cycle threshold, CT versus fluorescence, ΔR) of *fHbp* (gene of interest) and *recA* (house-keeping gene) respectively for all primer ratios used. The CT is defined as the amplification cycle number in which the curve crosses a specified threshold. The ΔR is the normalized fluorescence signals which are generated in the reaction. The mixture of 7µM forward and 7µM reverse primer produced an amplification plot with both the lowest cycle threshold (CT) value and highest fluorescence (ΔR). As a result, all experiments were performed using 7µM of forward and 7µM of reverse primer for both *fHbp* and *recA*.

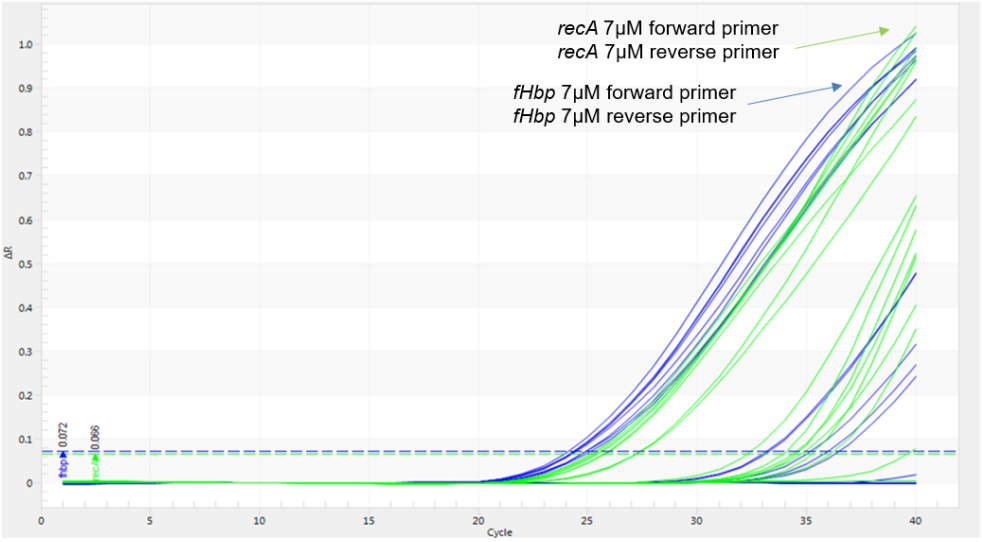


Figure 3. 17- Optimization of *fHbp* and *recA* primer concentrations

Primer concentration dependent amplification plots of *fHbp* (blue) and *recA* (green). Forward and reverse primers were tested at different concentrations (5-9 µM) using fixed 10 ng/µl of MC58 strain cDNA. The amplification blot indicated that the 7µM of forward and 7µM of reverse primer were the optimal primer concentrations for both *fHbp* and *recA* because this concentration produced an amplification plot with both the lowest cycle threshold (CT) value and highest fluorescence (ΔR).

Following primer optimisation, to calculate relative transcriptional levels of the genes of interest using the delta delta CT(ΔΔCT) method (Livak and Schmittgen, 2001), the reaction efficiency of qPCR was calculated. MC58 cDNA was used as a template to perform standard curves for *fHbp* and *recA* in order to examine the PCR efficiency and linearity of template amplification. As shown in Figure 3.18, the slopes of the standard curves were -3.416 and -3.5408 for *fHbp* and *recA*, respectively, revealing efficiencies of 96.45% and 91.64% for PCR amplification of *fHbp* and *recA*, respectively. Efficiencies between 90% and 110% are broadly accepted by this method without the need of any mathematical correction. The efficiency was calculated according to the following equation: E= (10 — 1/slope — 1) x 100. Moreover, the correlation coefficients were 0.9913 and 0.9964 for *fHbp* and *recA* respectively, confirming the accuracy of the amplification efficiency.

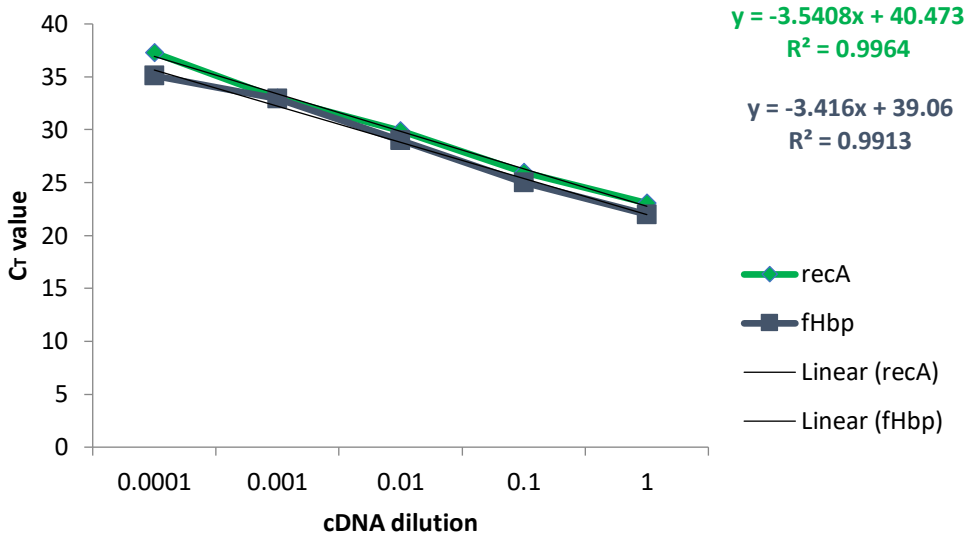


Figure 3. 18- qRT-PCR reaction efficiency standard curves for *recA* and *fHbp*

Serial diluted qPCR reactions with a cDNA starting concentration of 10ng/µl were performed for the genes *fHbp* (blue) and *recA* (green). *fHbp* presented a slope of -3.416 and *recA* a slope of -3.54, representing efficiencies of 96.45% and 91.64%, respectively.

3.7.2- Lnt disruption affects transcription of *fHbp*

After successful optimisation of the technique, qRT-PCR was performed to assess the levels of transcripts in MC58Lnt compared to MC58 and MC58LntC. A reduction of 53% in the *fHbp* transcript level was observed for strain MC58Lnt (Fig 3.19). This down regulation of transcription partly explains the 10-fold reduction in total FHbp protein levels. However, the immuno-dot blot data would suggest that Nm is able to shunt some of the diacylated FHbp to the cell surface, presumably to combat periplasmic stress encountered from toxic accumulation of mislocalised FHbp in the periplasm. Further investigation into the mechanisms Nm might be employing to prevent the toxic accumulation of FHbp is made in the Chapter 4.

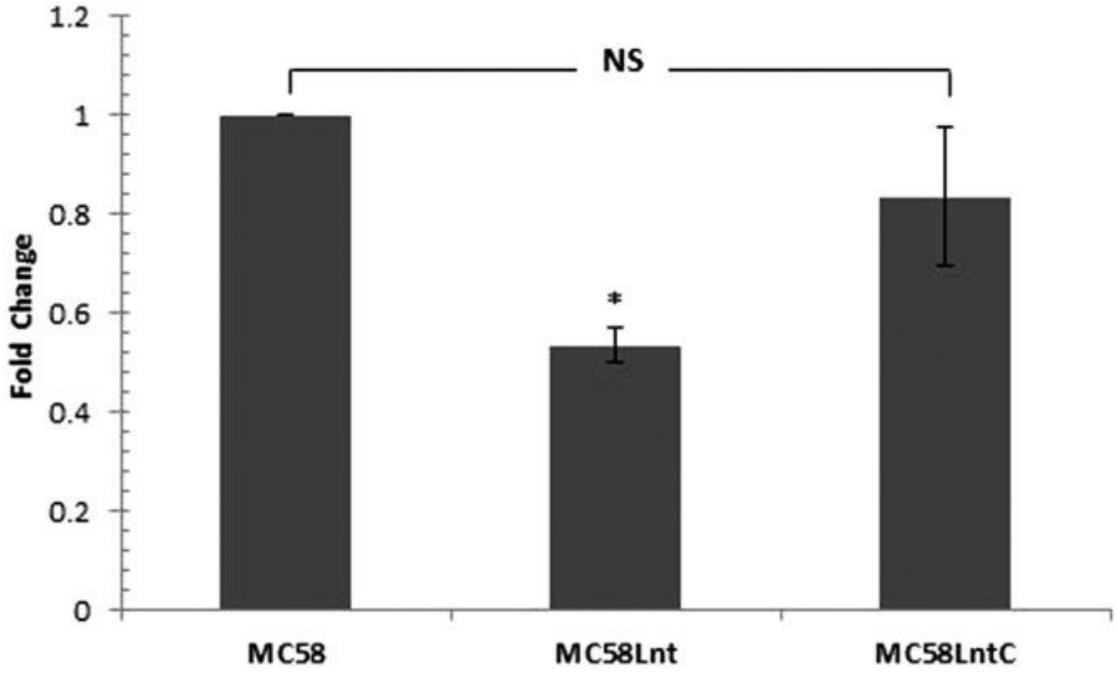


Figure 3. 19- Lnt disruption affects transcription of *fHbp*

Determination of *fHbp* mRNA levels in bacterial strains MC58, MC58Lnt and MC58LntC by qRT-PCR analysis. The data was obtained from six independent biological replicates ($n = 6$) with each including two technical replicates and normalized against the house-keeping gene, *recA*. All columns represent mean ± SEM, *$p \leq 0.05$, significantly different from strain MC58; NS, not significant.

3.8- Discussion

This chapter has provided evidence for the role of meningococcal Lnt in adding a third acyl group to the lipoprotein FHbp. A Tn library in strain MC58 was created to screen for possible genes whose products could affect the surface localisation of the vaccine antigen FHbp. In one mutant, the transposon was located in the *lnt* gene resulting in a reduction of FHbp levels on the surface of strain MC58.

The activity of Lnt was first studied in *E. coli*, which is considered the model organism for gram-negative microorganisms. Lnt activity occurs in the periplasm after translocation by the Sec Translocon, whereby preprolipoprotein from the cytoplasm is inserted in the IM. Addition of a diacyl group by the enzyme Lgt to the prolipoprotein at the cysteine +1 of the lipobox occurs, followed by removal of the signal peptide by LspA. Lnt then adds a third acyl to the apolipoprotein (Kovacs-Simon *et al.*, 2011).

A preprolipoprotein will have a cleavable SP at its N-terminus with the lipobox sequence ($[LVI]^{(-3)}[ASTVI]^{(-2)}[GAS]^{(-1)}C^{(+1)}$]. Lipoproteins that contain a Lol avoidance signal, such as an aspartic acid at position +2 in *E. coli* or a lysine and a serine at positions +3 and +4 in *Pseudomonas* spp. will be inserted in the IM and not follow to the next step after Lnt (Seydel *et al.*, 1999; Terada *et al.,* 2001, Hara *et al.*, 2003, Narita and Tokuda, 2007). On the other hand, lipoproteins that do not have this signal will be taken up by the machinery Lol for sorting to the OM after lipidation and SP cleavage described above (Tokuda, 2009).

The Lol apparatus in *E. coli* comprises an ABC transporter, LolCDE; a chaperone carrier, LolA; and an OM lipoprotein receptor, LolB. LolCDE releases mature lipoproteins from the IM after modification by Lnt, LolA shuttles them to the OM and LolB finalises the process of inserting lipoproteins into the OM (Fig 1.16). Most lipoproteins are thought to face the periplasm after their attachment to the OM while some are then flipped to face the exterior of the cell (Hooda and Moraes, 2018). One example of the latter is FHbp. The *E. coli* model is the one traditionally accepted for gram-negative bacteria. Based on this model, Lnt is essential. Lnt mutants undergo a process similar to a lipoprotein jamming in the IM. The highly abundant Braun's lipoprotein, Lpp, for instance, accumulates in the periplasm and cross-links with peptidoglycan causing lethality to the cells (Yakushi *et al.,* 1997, Robichon *et al.*, 2005).

In this chapter, following the approach of Kurokawa *et al.* (2012) and LoVullo *et al.* (2015), a truncated version of FHbp with intact SP was expressed in MC58 and MC58Lnt to discern whether or not FHbp becomes diacylated when Lnt is disrupted (Fig 3.13). We thus have provided strong evidence to suggest that similar to how it happens in *E. coli,* Lnt is adding a third acyl group to lipoproteins (Fig 3.13). Interestingly, Lnt in the MC58 strain is not essential as reported for *E. coli.* Similar observations have been made for Lnt mutants in *Francisella tularensis, Francisella novicida, and* Ng (LoVullo *et al*., 2015). Unlike these observations, our Lnt mutant seems to export diacylated lipoproteins less efficiently. LoVullo *et al.* (2015), showed that for *F. tularensis* the Lol system lacks a gene encoding LolE. LolE and LolC from the membrane component of the ABC transporter complex and

function as a heterodimer. LoVullo *et al.* (2015) showed LolC contains features of LolE, so they renamed LolC, “LolF”, and proposed LolF forms a homodimer in the IM. Bioinformatics studies revealed the lack of LolE was shown to not be unique to *F. tularensis* (LoVullo *et al.*, 2015). LolF could then act with LolD as a transporter with affinity for both di- and triacylated lipoproteins. To test this view, LoVullo and coworkers (2015) knocked out Lnt in Ng, which has the same genomic organisation for LolFD as *F. tularensis*. They showed that LolFD works equally well to sort di- or triacylated proteins to the OM.

Nm has a highly similar *lolF* gene to the one found in Ng and certain other gram-negative bacteria. Our immuno-dot blot observations show that MC58Lnt managed to export diacylated FHbp to the OM, but this appears to be at lower efficiency to that observed for Ng according to studies performed by LoVullo *et al.* (2015) (Fig 3.14 and 3.20). To test if the efficiency of export was low or if FHbp expression per se is affected in MC58Lnt mRNA and protein levels of FHbp were investigated (Fig 3.15 and 3.19). MC58Lnt showed just over 50% decrease in the transcriptional level and a 10-fold reduction in total cellular FHbp compared to MC58 indicating more than one level of downregulation of FHbp. Thus, despite being able to sort some diacylated FHbp to the OM for subsequent surface display by LolFD, we believe that MC58Lnt has a less efficient LolFD. The accumulation of the remaining diacylated FHbp (as well as of other lipoproteins) in the IM may trigger proteolysis accounting for the dramatic loss of FHbp protein level. Systems such as Cpx and sigmaE, have been shown to combat membrane stress in *E. coli* (MacRitchie *et al.,* 2008, Ruiz

and Silhavy, 2005), and similar mechanisms could be acting to prevent the accumulation of mislocalised FHbp in the IM and alleviate membrane stress.

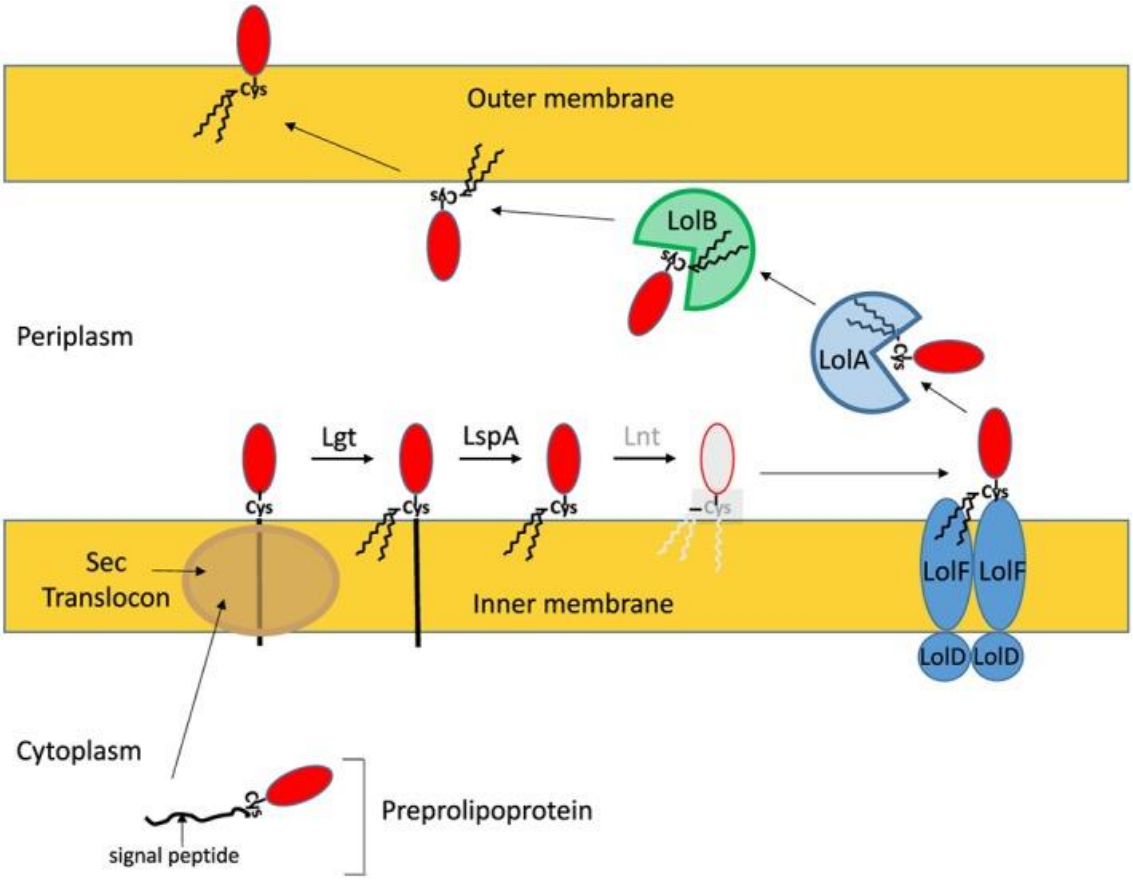


Figure 3. 20- Model for lipoprotein processing and sorting of tri- and diacylated lipoproteins in *N. meningitidis*

The SP at the N-terminus of the preprolipoprotein signals for its translocation across the IM by the Sec apparatus. Once in the periplasm, Lgt adds a diacylglyceride to the conserved cysteine, the last amino acid of the lipobox at the C terminus of the signal peptide. LspA cleaves the signal peptide exposing the diacylated cysteine which becomes the +1 residue to which Lnt adds the third acyl chain. Both the fully mature lipoprotein and the apolipoprotein resulting from mutation of Lnt are recognized by the LolFD transporter complex (LoVullo *et al.,* 2015). The LolA chaperone receives the lipoprotein or apolipoprotein and delivers it to the OM anchored lipoprotein LolB, which inserts it into the OM.

This chapter has raised some interesting questions including: how is the meningococcus responding to the membrane stress caused by lipoprotein jamming in the IM? Do microorganisms have the ability of switching the lipidation status of their lipoproteins? How does the host respond to a gram-negative microorganism expressing diacylated lipoproteins? The next chapter will address some of these questions.

Chapter 4

Investigating the impact of Lnt disruption on the cell and the potential of Lnt as a novel drug target

4.1- Introduction

Lnt is known as the enzyme responsible for adding the third acyl group to lipoproteins. As reviewed in the Introduction, Lnt acts on lipoproteins after prior di-acylation and cleavage of the signal peptide by Lgt and LspA, respectively.

Lnt disruption may affect protein levels and localisation globally in the meningococcus, acting in particular on the lipoproteome, as discussed in the previous chapter. Therefore, Lnt disruption might influence the abundance of several lipoproteins that would normally be triacylated by this enzyme, some of which may be surface antigens, hence not only impacting upon the neisserial cell envelope but also influencing host-microbe interactions.

Work described in this chapter set out to investigate the impact of an Lnt disruption on meningococcal cell structure, gene expression and interaction with host cells using Transmission Electronic Microscopic (TEM), RNAseq and *in vitro* cellular assays, respectively. Other approaches were used to investigate Lnt as a potential drug target including the first use of the invertebrate *in vivo Galleria mellonella* model with Nm.

4.2- Cellular ultrastructure of MC58Lnt

Lipoproteins are important for the integrity of the cell envelope and for maintaining homeostasis of the cell (Narita and Tokuda, 2017). An intact envelope is crucial for the cell's healthy functioning. Since Lnt disruption may be causing disturbances in the cell envelope and affecting the cell's homeostasis we decided to initially investigate the effects of Lnt disruption on the ultrastructure of the neisserial cells by TEM (Fig 4.1 and 2).

Experiments were performed on thin sections of MC58 and MC58Lnt cells grown to early exponential (OD_{600} 0.1) and stationary phase (OD_{600} 1). MC58 cells, grown to early exponential phase in supplemented GC medium, were observed with a normal cell shape and undamaged membrane structure with an intact, slightly wavy cell envelope (Fig 4.1a, top). At stationary phase, no detachment of the inner membrane from the outer membrane was observed for these cells. The mutant MC58Lnt, however, showed subtle ultrastructural differences but no alteration in shape and size as observed for an Lnt mutant of *Acinetobacter Baylyi* (Gwin *et al.*, 2018). At OD_{600} 0.1 (Fig. 4.1b) it was possible to observe some slight perturbations of the envelope, especially on one of the poles, that seemed to be more pronounced in stationary phase cells, as showed in Fig 4.2b and c. In particular, it was possible to consistently observe that in stationary phase cells, around the area where the periplasm would be expected to be visible, there was less stain in one of the poles, suggesting less protein in that area or possibly less peptidoglycan.

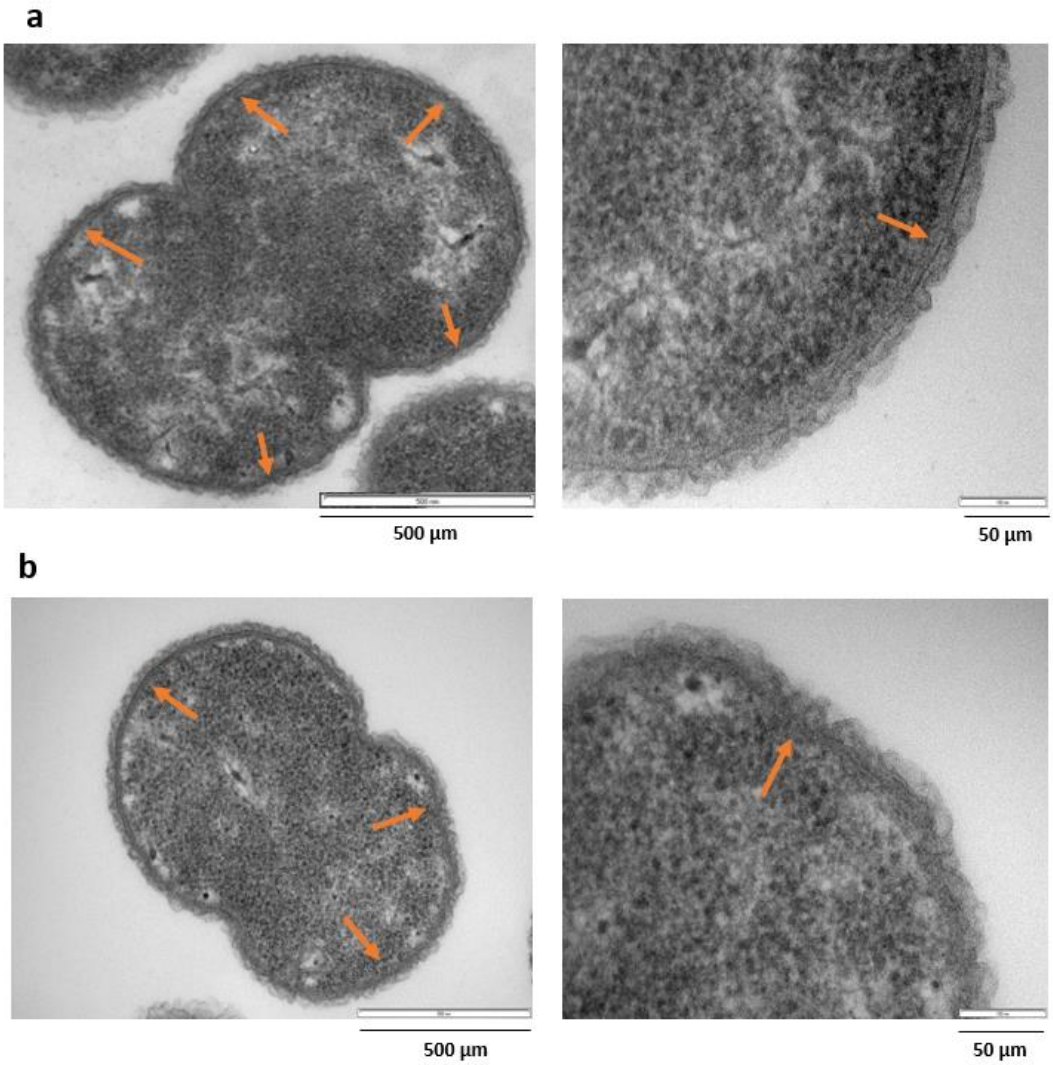


Figure 4. 1- Visualization of MC58 and MC58Lnt cellular ultrastructure at initial exponential phase by TEM

TEM of thin sections of **a.** MC58 and **b.** MC58Lnt grown in supplemented GC medium to initial exponential phase (OD_{600} 0.1). All specimens were fixed, embedded, ultrathin sectioned, and post-stained before imaging on a JEOL 2100 electron microscope. Bars, 500µm (left) and 50µm (center and right). Orange arrows are pointing to the periplasm.

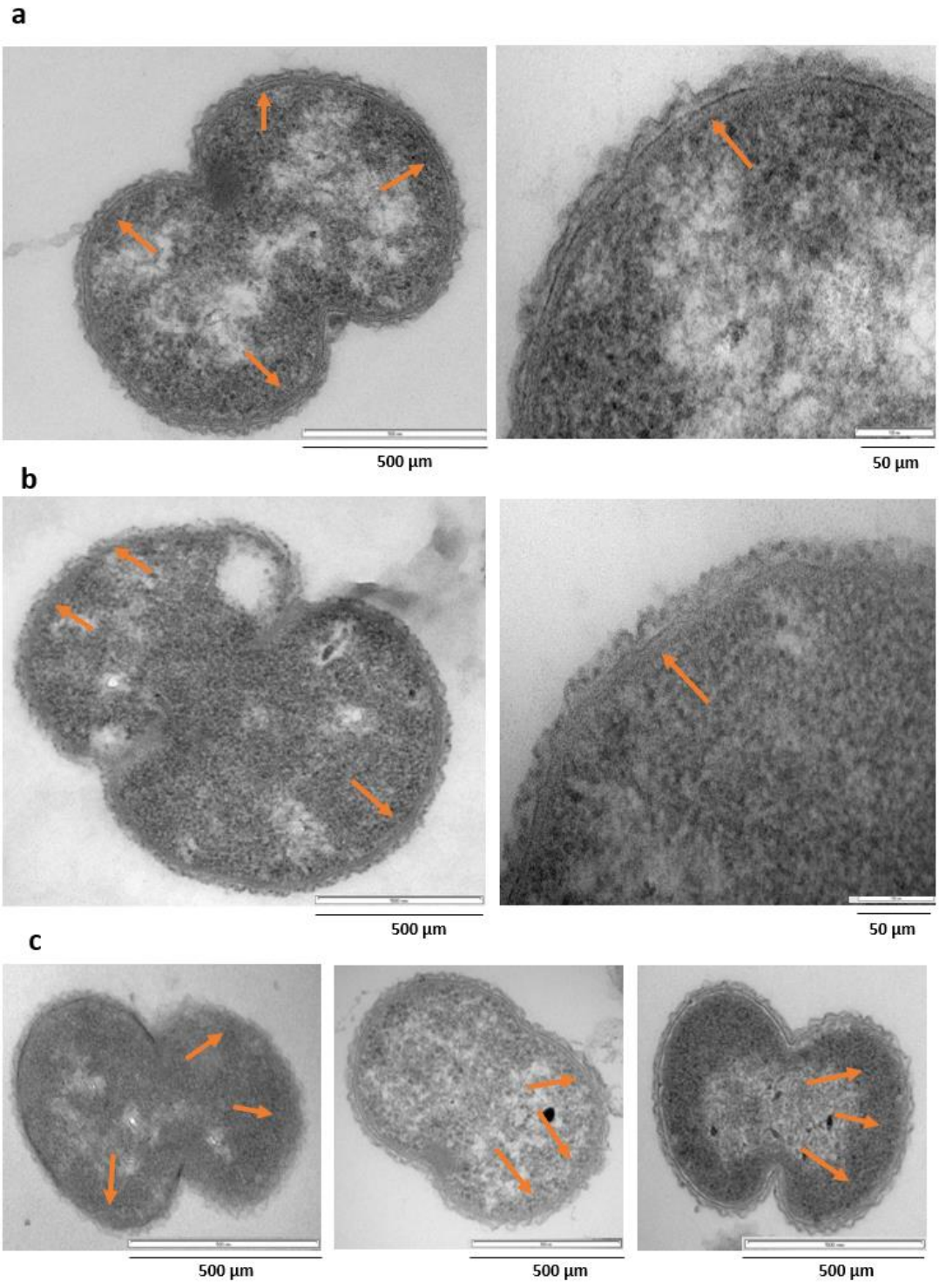


Figure 4. 2- Visualization of MC58 and MC58Lnt cellular ultrastructure at stationary phase by TEM

TEM of thin sections of **a.** MC58 and **b.** MC58Lnt grown in supplemented GC medium to initial exponential phase (OD_{600} 0.1). **c.** More examples of

MC58Lnt cellular ultrastructure. All specimens were fixed, embedded, ultrathin sectioned, and poststained before imaging on a JEOL 2100 electron microscope. Bars, 500μm (left) and 50μm (center and right). Orange arrows are pointing to the periplasm.

4.3- The gene expression profile of *N. meningitidis* MC58 changes in response to Lnt disruption

Next, the global expression of genes of MC58Lnt using RNAseq was examined in order to determine whether Lnt expression has any effects on global gene expression. At exponential phase (OD_{600} 0.5) total RNA was extracted from cultures of the WT and mutant. Exponential phase was chosen because it represents the phase when bacteria are metabolically active, synthetizing proteins and dividing (Reeve *et al.*, 1984; Gefen *et al.*, 2014). Thus, at this phase of growth, the impact of Lnt on genes involved in metabolism and other vital processes could be investigated. MC58 and MC58Lnt were grown at 37°C in supplemented GC broth. The quality of the RNA was assessed by Bioanalyser (Fig 4.3). RIN above 9.2 was considered acceptable for RNAseq. Two total RNA extractions of MC58 and three of strain MC58Lnt were prepared for sequencing at the University of Nottingham Deepseq facility.

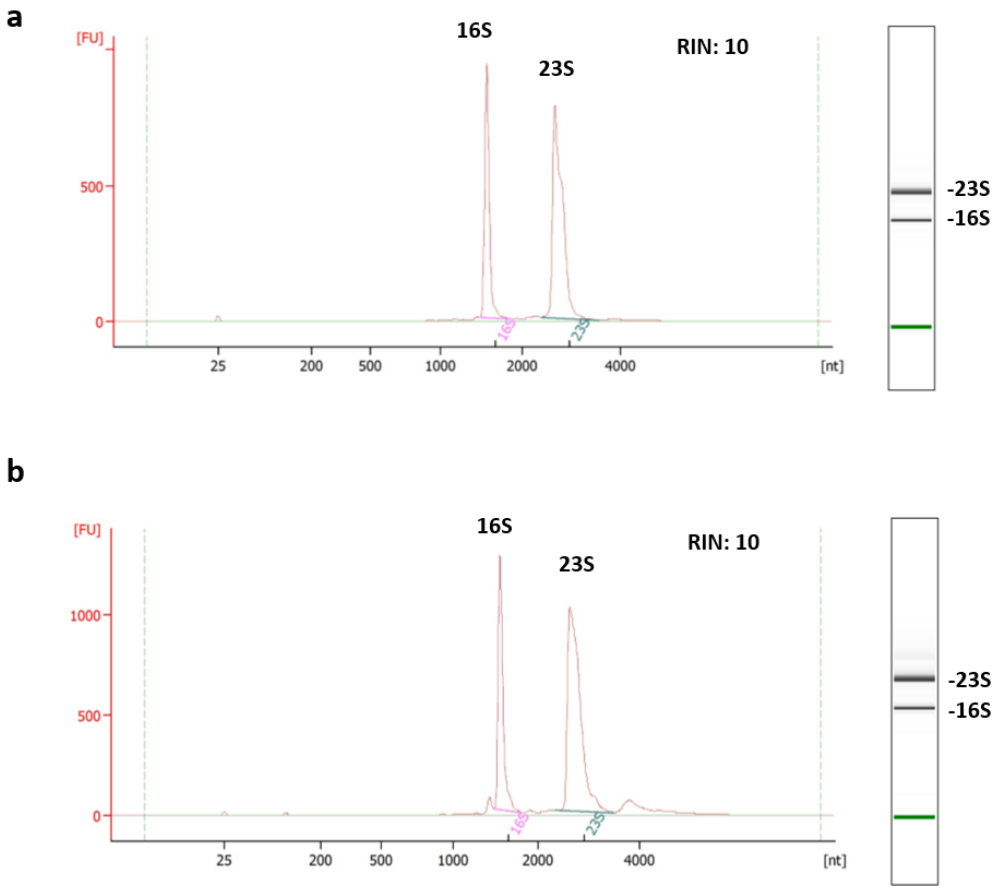


Figure 4. 3- Electropherograms and calculated RIN values of total RNA samples

a. MC58 and **b.** MC58Lnt. Total RNA was analysed using an Agilent RNA 6000 Nano Assay and 2100 Bioanalyzer (Agilent Technologies). The two peaks represent the 16S and 23S ribosomal RNAs. Data are representative of an individual RNA isolation for each strain.

Principal component analysis (PCA) was performed with the provided RNA samples (Fig 4.4). It shows homogeneity of the MC58 samples, validating the set of samples analysed (n=2) and confirming biological similarity. The plot shows a more heterogenous set of samples for MC58Lnt, indicating

that ideally more samples should be analysed (n=3). This plot illustrates how Lnt disruption affects gene expression and how the cells may be responding to this mutation expressing different genes. It suggests significant differences between MC58Lnt and WT samples.

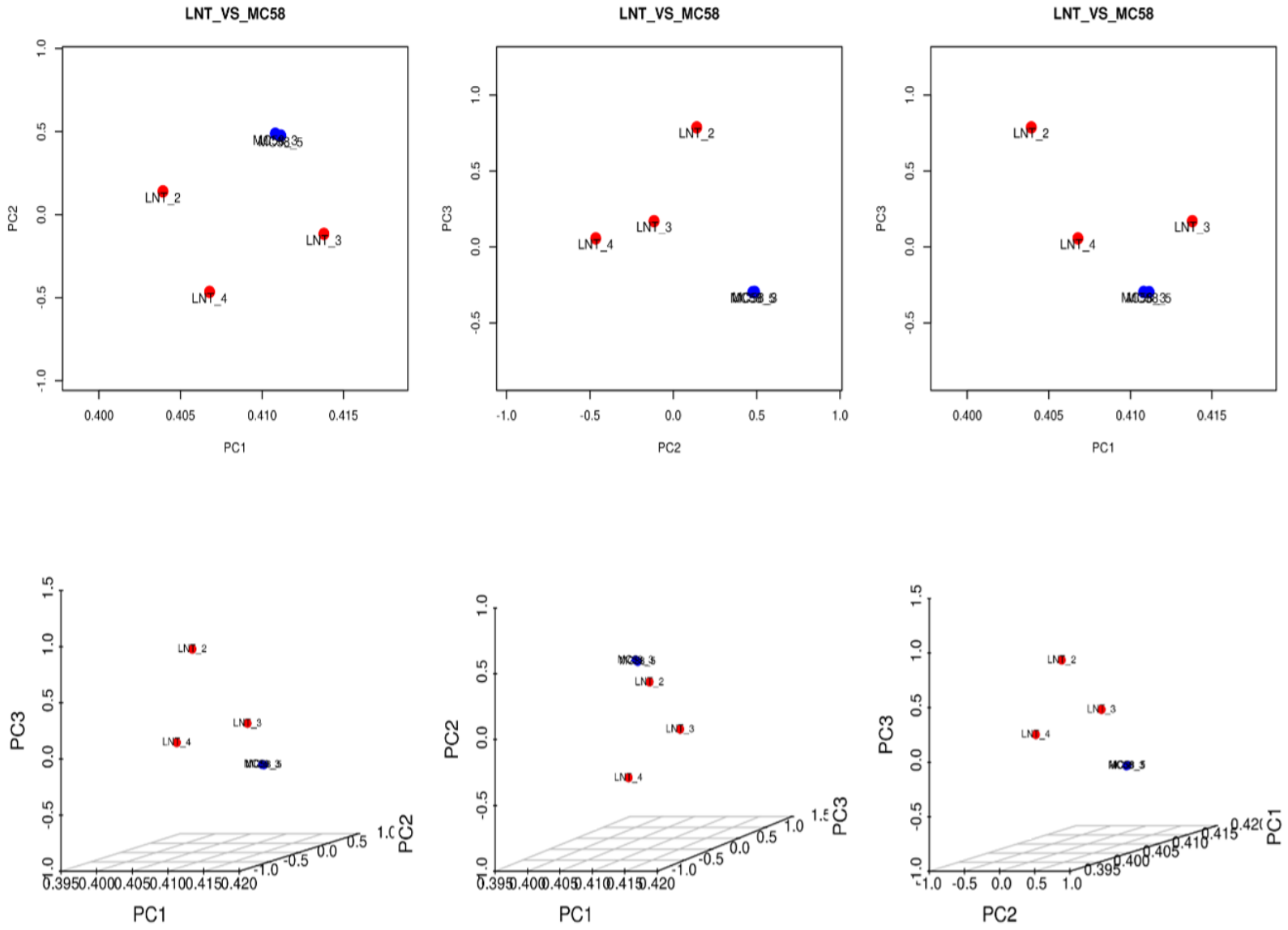

LNT_VS_MC58
PC2
PC1
LNT_2
LNT_3
LNT_4
LNT_VS_MC58
PC3
PC2
LNT_2
LNT_3
LNT_4
LNT_VS_MC58
PC3
PC1
LNT_2
LNT_3
LNT_4
PC3
PC2
PC1
PC2
PC3
PC1
PC3
PC2
PC1

Figure 4. 4- PCA plot of RNAseq performed for MC58 and MC58Lnt

PCA plot to illustrate homogeneity of the group of samples. The proximity of the dots reflects how biologically similar they are. MC58 samples (MC58_3 and MC58_5) are shown in blue whereas MC58Lnt (Lnt_2, Lnt_3 and Lnt_4) are shown in red.

After comparing the MC58 and MC58Lnt biological samples with PCA plots and analysing their homogeneity, DESeq2 was performed to determine the differentially expressed (DE) genes among individual samples. First, the negative binominal statistic method was used to display the DE genes as a heatmap (Fig 4.5), which correlates the submitted samples. The heatmap provides a global visual overview of how differentially expressed the genes are between the samples analysed. Clusters of downregulated and upregulated genes were apparent between MC58 and MC58Lnt.

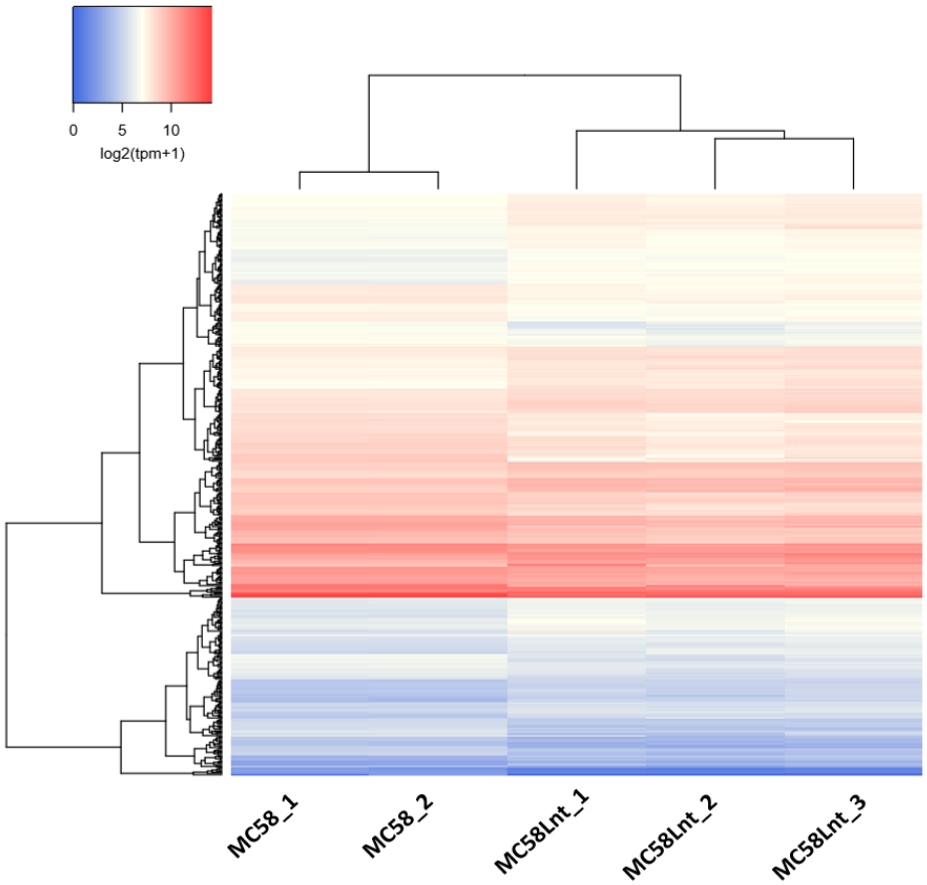


Figure 4. 5- Heatmap of RNAseq performed for MC58 and MC58Lnt

Heatmap generated to illustrate how individual RNA samples differ in relation to gene expression. Blue indicates downregulated genes and red indicates upregulated genes.

Next, a volcano plot was generated (Fig 4.6). Here, grouped analysed samples are shown for MC58Lnt vs MC58. Genes with a change in expression level greater than 1.6-fold were considered as being upregulated in MC58Lnt compared to the WT (padj <0.05, p-value adjusted) and similarly repressed genes were considered to be those with an expression lower than 1.6-fold compared to the WT level (padj <0.05). This fold difference was adopted as previously shown to be reliable for 99% of the genes in Nm in other studies (Dietrich *et al*., 2003; Guckenberger *et al*., 2002). The DE genes are shown in red in the volcano plot displayed below (Fig 4.6).

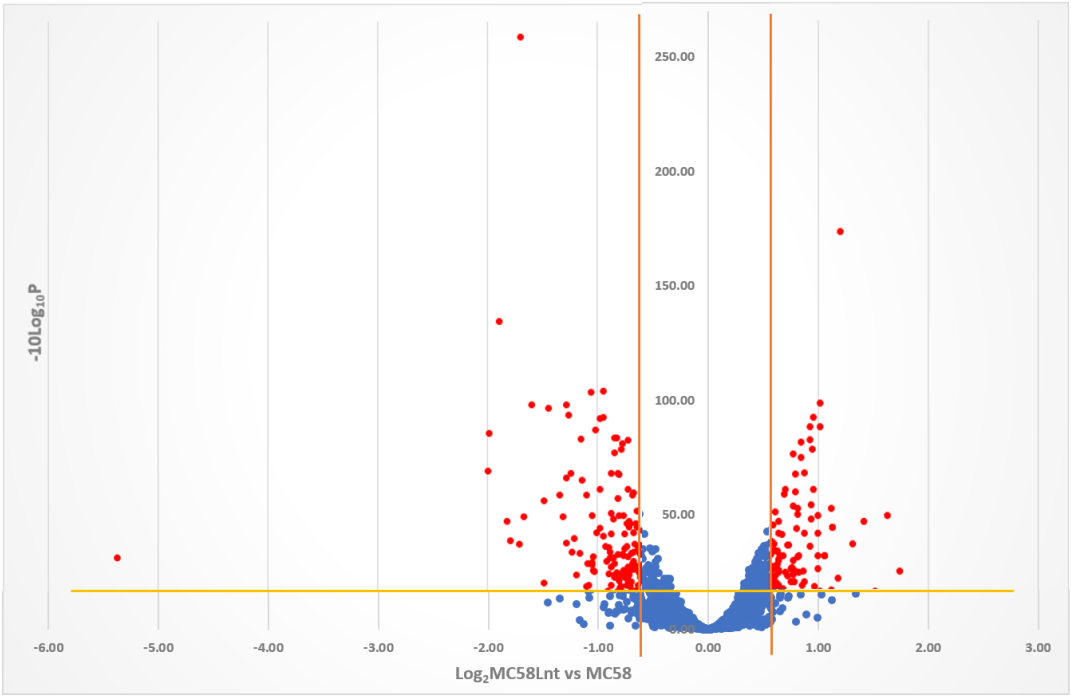


Figure 4. 6- RNAseq volcano plot of MC58Lnt vs MC58

DE genes are shown in red. Yellow line delimitates the established adjusted p-value (padj <0.05). Padj values are shown as $-10Log_{10}P$ values. Orange lines delimitate established fold difference adopted for greater and lower

expressed genes. 1.6-fold (or $\log_2$-fold greater or lower than 0.6) difference was accepted as significant.

From all the genes analysed by this methodology, 72 genes were shown to be upregulated and 111 were shown to be downregulated. Together, these genes are involved in several different cellular processes. The full list of genes analysed by RNAseq is provided in the appendix (Appendix XIII).

In order to verify the predicted cellular function of the DE genes, each DE gene annotation (NMB annotation for MC58 genes on NCBI) was checked for equivalent KO number (see appendix XII). KO numbers belong to the Kyoto Encyclopedia of Genes and Genomes (KEGG), a database resource that integrates genomic, chemical and systematic functional information (Kanehisa *et al*., 1999). After verifying each predicted gene function, it was clear that Lnt disruption affects several cellular processes (Figure 4.7).

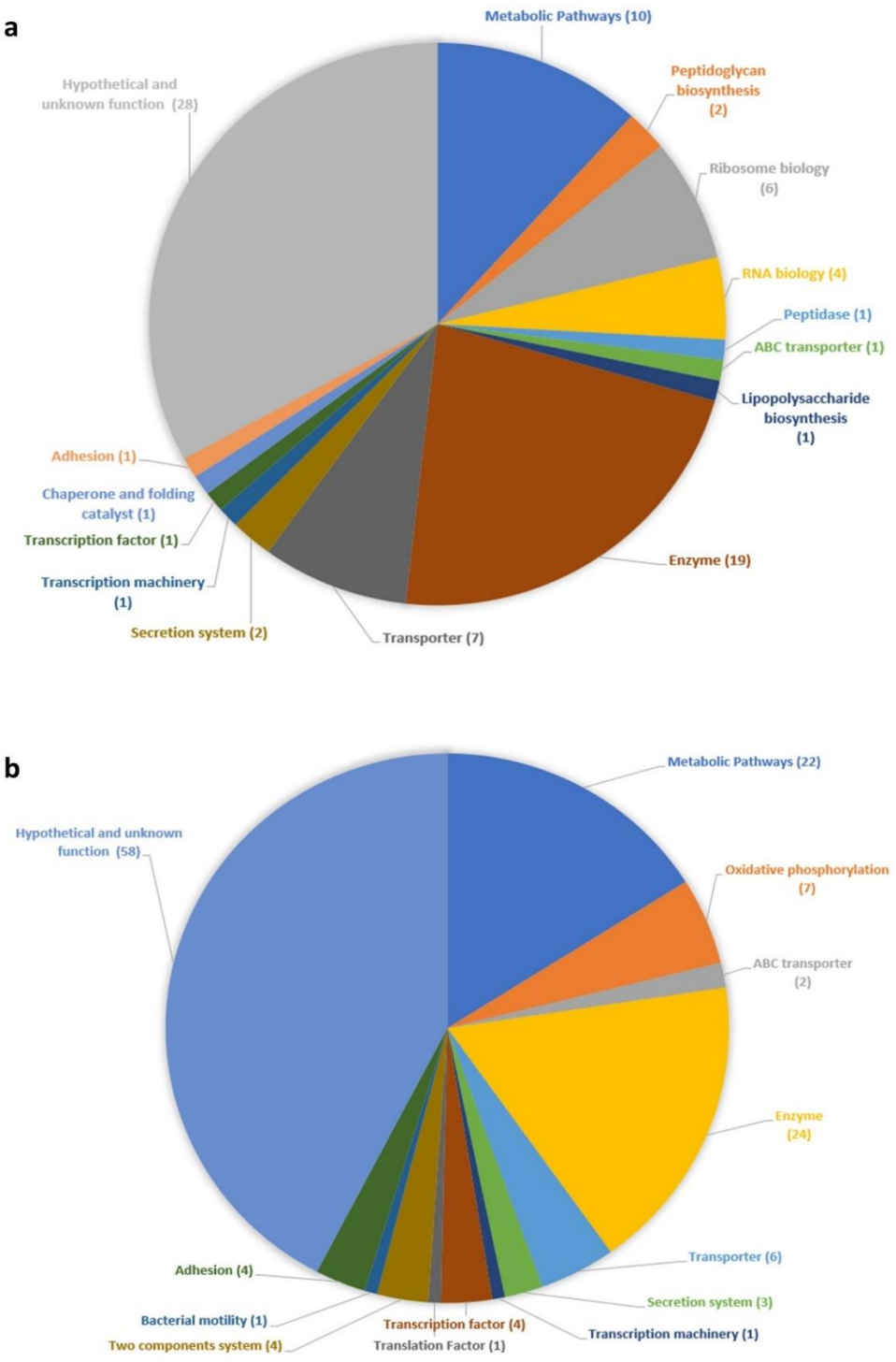


Figure 4. 7- Cellular function prediction of the DE genes

Database KEGG was used to retrieve predicted gene function for up and downregulated genes in MC58Lnt. **a.** Upregulated genes. **b.** Downregulated genes.

Further analysis of the genes being deregulated points to a global metabolic change caused by Lnt disruption. From the genes identified as playing a role in metabolism, 10 are upregulated while 22 are downregulated in comparison to MC58. This represents the majority (18%) of the DE genes with known function.

4.3.1- RNA biology genes

A closer look at upregulated genes gives insights into how the Neisserial cell is responding to the changes imposed by Lnt disruption at the growth phase tested. Four genes are affected that are related to RNA biology that may play a role in the maintenance of RNA levels in the cell. NMB1200, annotated as ribonuclease R, is 2-fold overexpressed. Ribonuclease R was previously implicated in broad mRNA recycling and maintenance (Hossaine *et al.*, 2016). Moreover, NMB0268 and NMB0868; RNA methyltransferase and Ribonuclease III, respectively, are linked to ribosomal RNA maintenance and influence translation rates (Court *et al*., 2013; Doi and Arakawa, 2007). Thus, it is plausible that Lnt loss may be resulting in conditions in the cell that are leading to adaptive changes that regulate the abundance of proteins.

4.3.2- Adhesion-encoding genes

In relation to downregulated genes, it is interesting to note that four genes previously linked to adhesion of the cell and, consequently, virulence are

less expressed when considering both cut-off criteria. For instance, the important pilus subunit, PilE (NMB0018) is 2.37-fold less expressed in MC58Lnt. This may represent major consequences for the biology of MC58Lnt. The presence of pilus was previously implicated in invasiveness of the neisserial cell, formation of microcolonies, DNA uptake and adhesion (Hung and Christodoulides, 2013). A more detailed analysis of adhesin expression is provided in session 4.2.7.

4.3.3- Genes of unknown function

Interestingly, a large proportion of DE genes are of unknown function. Out of 183 DE genes, 86 genes are hypothetical genes of unknown function. This highlights how little is known and this could include cellular processes the meningococcus employs to cope with membrane stresses.

4.3.4-Potential lipoproteins-encoding genes

We next analysed the predicted and known meningococcal lipoprotein-encoding genes (Table 4.1). The predicted lipoproteins analysed here came from the Master's thesis of Cansu Karyal (2016). Karyal used the bioinformatics tool, DOLOP, that predicts lipoproteins based on their N-terminus (Babu and Sankaran, 2002) and LipoP which predicts lipoproteins based on the presence of a signal peptidease II cleavage site (Juncker *et al*., 2003), to investigate all the annotated genes in Nm strain MC58. Some of the genes analysed by Karyal at the time of her thesis have been re-annotated so after eliminating these genes there is a list of 68 possible lipoproteins that we analysed for their gene expression in MC58Lnt. Out of the 68 possible lipoproteins 40 were downregulated and 17 of these were found to be statistically significant[a]. Interestingly, *fHbp* was found to be 1.29-fold less expressed in MC58Lnt but with a padj that was not significant. This finding indicates a lack of sensitivity with the RNAseq approach taken since we previously demonstrated by qRT-PCT that *fHbp* is significantly down-regulated in MC58Lnt (Chapter 3). Surprisingly, some of the predicted lipoproteins were found to be up-regulated; out of the 25 up-regulated, 10 were found with padj <0.05. It could be that these lipoproteins are those that normally localise to the IM and not sorted to the OM and thus there is less need to downregulate these lipoproteins as they are not mislocalised.

[a]Henceforth the reader is advised that DE genes were broadly accepted as padj <0.05 only. The second cut-off was not considered for the following analysis, unless otherwise stated.

Table 4. 1- List of predicted lipoproteins

Predicted lipoproteins by DOLOP and LipoP (Karyal, 2016). Statistically significant differences in gene expression are represented by *padj <0.05.

Gene ID	Name	Size (kDa)	Predicted Signal Peptide	Fold Difference	padj
NMB1162	Hypothetical protein	215	M K PLILGLAAVLA LSAC	-41.31	0.001*
NMB1541	Lactoferrin-binding protein	737	MC K PNYGGIVLLPLL LAS C	-3.23	0.000*
NMB1623	Pan1 major anaerobically induced outer membrane protein	390	MK R QALAAMIASLFA LAAC	-2.78	0.016*
NMB1211	Hypothetical protein	80	M K YIVSISLAMG LAAC	-2.42	0.000*
NMB1212	Hypothetical protein	112	M K YIVSISLAMG LAAC	-2.30	0.000*
NMB1213	Lipoprotein	120	M K YIVSISLAMG LAAC	-2.21	0.001*
NMB0462	PotD-1 spermidine/putrescine ABC transporter substrate-binding protein	459	MK K SVLAVLAALS LAAC	-2.21	0.000*
NMB1124	Hypothetical protein	215	M K PLILGLAAVLA LSAC	-1.95	0.000*
NMB1126	Hypothetical protein	223	M K TVSTAVVLAAAAVS LTGC	-1.83	0.001*
NMB2132	Transferrin-binding protein-like protein	488	MFK R SVIAMACIFA LSAC	-1.82	0.003*

NMB0653	MafB-like protein	422	M K PLRRLTNL LAAC	-1.65	0.019*
NMB1125	Hypothetical protein	123	MMNPKTLS R LSLCAAVLA LTAC	-1.62	0.144
NMB1785	Hypothetical protein	79	MRDSMKNW K QFTFFVIL VIAC	-1.57	0.119
NMB0787	Amino acid ABC transporter substrate-binding protein	275	MMLK K FVLGGIAALV LAAC	-1.57	0.144
NMB1035	Hypothetical protein	84	MN K LFITALSALA LSAC	-1.52	0.000*
NMB1433	Hypothetical protein	177	MFPPD K TLFLCLSALL LASC	-1.42	0.401
NMB1533	Outer membrane protein	183	M K AYLALISAAVIG LAAC	-1.40	0.025*
NMB1523	Hypothetical protein	98	MK K SLFAAALLSLV LAAC	-1.40	0.144
NMB0374	MafB-like protein	467	M K PLRRLTNL LAAC	-1.39	0.108
NMB898	Lipoprotein	171	MKIKQIV K PGLAVLAAGV LSAC	-1.38	0.072
NMB1578	Hypothetical protein	217	MFSVP R SFLPGVFVLAA LAAC	-1.38	0.041*
NMB1107	Hypothetical protein	200	MNMK K LISAICVSIV LSAC	-1.38	0.009*
NMB1811	Tfp pilus assembly protein PilP	181	M K HYALLISFLA LSAC	-1.35	0.036*
NMB1969	Serotype-1-specific antigen	1082	MRTTPTFPTKTF K PTAMALAVATT LSAC	-1.35	0.282
NMB1765	Hypothetical protein	99	MKKKLS K YSLFLSSVFC LTAC	-1.33	0.306
NMB1612	Amino acid ABC transporter substrate-binding protein	268	MNMK K WIAAALACSALA LSAC	-1.31	0.092*
NMB1592	Lipoprotein	162	MK K YLIPLSIAAV LSGC	-1.29	0.006*
NMB1870	Hypothetical protein	320	MTRSKPVN R TAFCCLSLTTALI LTAC	-1.29	0.261

NMB1017	Sbp sulfate ABC transporter substrate-binding protein	351	M K TYAPALYTAAL LTAC	-1.28	0.687
NMB0092	Hypothetical protein	75	MV R FFVLSFLTLINLCS LS AC	-1.27	0.132
NMB2147	Hypothetical protein	140	M R PIFLSFVLFPIL ITAC	-1.25	0.337
NMB1880	Hypothetical protein	321	MKP R FYWAACAVL LTAC	-1.19	0.695
NMB0923	Cytochrome c	152	M K TQISLAAAAITLL LSAC	-1.19	0.408
NMB0054	Hypothetical protein	135	MEIRAI K YTAMAALLAFT VAGC	-1.16	0.475
NMB2139	Hypothetical protein	297	MVTFSKI R PLLAIAAAAL L AAC	-1.12	0.346
NMB1946	Outer membrane protein	287	MKTFF K TLSAAALALI LAAC	-1.10	0.463
NMB0278	DsbA-1-Thiol:disulfide interchange protein DsbA	232	MKS R HLALGVAALFA LA AC	-1.10	0.695
NMB1672	Hypothetical protein	172	M R LFPIAAALS LAAC	-1.07	0.619
NMB0938	Hypothetical protein	278	MKN K TSSLLLWLTAIM LTAC	-1.06	0.784
NMB1470	Hypothetical protein	181	ML K TSFAVLGGCLL LAAC	-1.01	0.978
NMB0071	CtrA-Capsule polysaccharide export outer membrane protein	391	MFKVKFYI R HAVLLLCGSL IVGC	1.00	0.985
NMB1594	Spermidine/putrescine ABC transporter substrate-binding protein	376	MT K HLPLAVLTALL LAAC	1.00	0.999
NMB1010	Hypothetical protein	187	M K ILALLIAATCA LSAC	1.03	0.923
NMB1335	Hypothetical protein	186	MN R LLLLSAAVL LTAC	1.03	0.916
NMB1674	GDSL lipase	213	MPSEKPMNR R TFLLGAG ALLLTAC	1.06	0.705

NMB0033	Putative membrane-bound lytic mureintransglycosylase A	441	MKKYLF R AALYGIAAAI LA AC	1.10	0.439
NMB0580	Protein disulfide isomerase NosL	164	MK K TLLAIVAVSA LSAC	1.10	0.587
NMB0844	Hypothetical protein	107	M KKCILGI LTAC	1.13	0.531
NMB0532	HtrA-protease Do	499	MFK K YQYLALAALCAAS LAGC	1.13	0.230
NMB0032	Hypothetical protein	175	MEM K QMLLAVGVVAV LAGC	1.15	0.696
NMB2091	Hemolysin	202	MKPKPHTV R TLIAAIFSLA LSGC	1.15	0.394
NMB1468	Hypothetical protein	107	MK K LLIAAMMAAA LAAC	1.17	0.539
NMB1977	Hypothetical protein	56	M K YGVFFAAATALL LSAC	1.19	0.269
NMB0703	ComL-Competence lipoprotein	267	MK K ILLTVSLGLA LSAC	1.22	0.061
NMB0204	Lipoprotein	125	MN K TLILALSALLG LAAC	1.22	0.139
NMB0035	Hypothetical protein	388	MR K FNLTALSVMLALG LTAC	1.25	0.118
NMB0873	outer membrane lipoprotein LolB	193	M K HTVSASVILL LTAC	1.30	0.065
NMB1279	Membrane-bound lytic mureintransglycosylase B	369	MKKR K ILPLAICLAA LSAC	1.39	0.155
NMB0928	Hypothetical protein	398	MTHI K PVIAALALIG LAAC	1.46	0.000*
NMB1369	Hypothetical protein	184	MK K IIASALIATFA LAAC	1.48	0.041*
NMB1714	mtrE-Multidrup efflux pump channel protein	467	MDTTL K TTLTSVAAAFA L SAC	1.53	0.002*
NMB1567	Macrophage infectivity potentiator	272	MNTIF K ISALTLSAALA LSAC	1.55	0.002*

NMB0086	Hypothetical protein	338	MYR K LIALPFALL LAAC	1.61	0.004*
NMB0623	potD-2 Spermidine/putrescine ABC transporter substrate-binding protein	379	MK K TLVAAAILSLA LTAC	1.63	0.001*
NMB1949	Soluble lytic mureintransglycosylase	616	MYLPSM K HSLPLLAALV L AAC	1.73	0.002*
NMB1716	mtrC-Membrane fusion protein	412	MAFYAFKAM R AAALAAA VALV LSSC	1.76	0.000*
NMB2002	Hypothetical protein	72	MSMPEMP K WYDDDGQ IVSC	1.92	0.000*
NMB0294	DsbA-2-Thio:disulphide interchange protein DsbA	231	MKL K TLALTSLTLLA LAAC	2.18	0.005*

Next, we looked specifically at virulence and survival related genes such as the ones involved in LOS and capsule synthesis, and in adhesion of the cell in order to have a more in-depth idea of how Lnt mutation could have affected these aspects of meningococcal biology.

4.3.5- LOS synthesis genes

From the analysed genes involved in LOS production the only gene that met both cut-off criteria to determine DE genes for the KEGG analysis was the *lpxC* gene that encodes LptC, which was 1.83-fold more expressed (Table 4.2). Supposedly, LptC function in Nm is thought to be involved in the formation of a bridge connecting the IM and OM components of the LOS (Putker *et al*., 2014). Interestingly, this bridge is formed with the help of LptA; the gene encoding this protein (*lpxA*) is also statistically significantly overexpressed by 26% compared to the WT levels (padj = 0.010).

Table 4. 2- Genes involved in LOS synthesis

Some of the genes that are known to be involved in LOS synthesis as found in Kahler and Stephens, 1998. Statistically significant differences in gene expression are represented by *padj <0.05.

Gene ID	Name	Fold Difference	padj
NMB1801	-	-1.26	0.115
NMB2010	-	-1.12	0.313
NMB0828	***rfaD***	-1.10	0.72
NMB1418	-	-1.06	0.805
NMB0199	***lpxB***	1.00	1.000
NMB1704	***lgtF***	1.00	0.994
NMB1527	***rfaF***	1.02	0.847
NMB0544	-	1.05	0.778
NMB0014	***kdtA***	1.13	0.408
NMB0180	***lpxD***	1.26	0.139
NMB0178	***lpxA***	1.26	0.010*
NMB1919	-	1.26	0.034*
NMB0017	***lpxC***	1.83	0.001*

4.3.6- Capsule synthesis genes

Genes involved in capsule synthesis were then evaluated (Table 4.3). The genes *crgA* and *synX* involved in repression of capsule synthesis and export (Deghmane *et al.*, 2002) and its saccharide sialylation (Swartley *et al.*, 1996), respectively, were down-regulated. An additional five genes were also expressed at lower levels in MC58, but these differences were not significant.

Table 4. 3- Genes involved in capsule synthesis

Some of the genes that are known to be involved in capsule synthesis (Harrison *et al*., 2013a). Statistically significant differences in gene expression are represented by *padj <0.05

Gene ID	Name	Fold difference	padj
NMB1856	***crgA***	-1.82	0.000*
NMB0070	***synX***	-1.79	0.000*
NMB0083	***lipB***	-1.29	0.403
NMB0080	***rfbA-2***	-1.25	0.679
NMB0069	***siaB***	-1.13	0.551
NMB0079	***rfbB-2***	-1.05	0.928
NMB0078	**-**	-1.05	0.881
NMB0067	***siaD***	-1.02	0.946
NMB0071	***ctrA***	1.00	0.985
NMB0068	***siaC***	1.02	0.943
NMB0072	***ctrB***	1.10	0.375
NMB0073	***ctrC***	1.18	0.311
NMB0081	**-**	1.20	0.330
NMB0074	***ctrD***	1.23	0.072
NMB0082	***lipA***	1.47	0.000*

4.3.7- Adhesion and survival genes

Next, we investigated the expression of genes related to adhesion and survival of the meningococcus in the host (Table 4.4). From the analysed genes, this group of genes is the one with most statistically significant DE genes. For instance, out of the 16 genes linked to pilus synthesis, 5 are downregulated and 2 are upregulated. From the downregulated ones the gene responsible for expressing the major subunit PilE was 2.38-fold less expressed in MC58Lnt than in MC58. Out of the other adhesins analysed, a further 4 were downregulated including NadA and Opc, involved in intimate adhesion of Nm to cells (Hung and Christodoulides, 2013). Of the analysed genes linked to survival of the cell in the host, 7 genes were found DE and 6 of these were downregulated. Interestingly, genes linked to the two-partner secretion system were found down-regulated. This system has been shown to have more than one set of genes in MC58 (van Ulsen *et al*., 2008). Genes *tpsA3* (NMB1214) and *tpsB* (NMB1779) were the ones statistically significantly downregulated, with fold differences of -1.83 and -2.04 respectively. Other genes encoding for this secretion system, while not statistically significantly DE, were found to be less expressed in MC58Lnt; *tpsA2* (NMB1768), -1.20; *tpsB1* (NMB0496), -1.93; and *tpsA* (NMB1779), -1.09. Since these proteins have roles such as hemolysis/cytotoxicity, iron acquisition, and adhesion to host cells, disruption in their expression pattern could affect the ability of the meningococcus to survive in the host (Jacob-Dubuisson *et al.*, 2001; Jacob-Dubuisson *et al.*, 2004).

Table 4. 4- Genes involved in adhesion and survival

Some of the genes that are known to be involved in adhesion and survival of the meningococcus as found in Echenique-Rivera *et al.*, 2011; and Hung and Christodoulides, 2013. Statistically significant differences in gene expression are represented by *padj <0.05

Gene ID	Name	Fold difference	padj	
NMB0018	*pilE*	-2.38	0.000*	**Pili**
NMB1808	*pilM*	-1.44	0.010*	
NMB1810	*pilO*	-1.40	0.012*	
NMB1812	*pilQ*	-1.36	0.040*	
NMB1811	*pilP*	-1.35	0.036*	
NMB1809	*pilN*	-1.27	0.143	
NMB0052	*pilT*	-1.26	0.225	
NMB0886	*pilH*	-1.13	0.190	
NMB0887	*pilI*	-1.02	0.866	
NMB0890	*pilX*	1.00	0.991	
NMB0329	*pilT*	1.02	0.877	
NMB0888	*pilJ*	1.04	0.814	
NMB1309	*Tfp*	1.05	0.732	
NMB0889	*pilK*	1.07	0.704	
NMB0333	*pilG*	1.36	0.002*	
NMB0332	*pilD*	1.44	0.001*	
NMB0652	*mafA-2*	-1.48	0.036*	**Other Adhesins**
NMB1994	*nadA*	-2.03	0.019*	
NMB1053	*opc*	-1.85	0.000*	
NMB0653	*mafB*	-1.65	0.000*	
NMB1998	*ausI/mspA*	-1.37	0.150	
NMB1969	*nalP*	-1.35	0.282	
NMB0341	*tspA*	-1.20	0.555	
NMB2105	*mafB*	-1.08	0.005	
NMB1985	*app*	1.09	0.070	
NMB0992	*nhhA*	1.94	0.637	
NMB1869	*cbbA*	-1.27	0.006	

NMB0207	*gapA-1*	1.04	0.847	
NMB0442	*opaA*	-1.36	0.067	
NMB0926	*opaB*	1.03	0.917	
NMB1465	*opaC*	1.00	0.992	
NMB1636	*opaD*	-1.23	0.482	
NMB1623	*aniaA*	-2.78	0.016*	
NMB0663	*nspA*	-2.71	0.000*	
NMB1994	*nadA*	-2.03	0.019*	
NMB1398	*sodC*	-1.63	0.000*	
NMB0216	*kat*	-1.33	0.352	
NMB0278	-	-1.10	0.695	
NMB1220	-	1.19	0.454	
NMB1429	*porA*	1.20	0.334	
NMB0667	-	1.20	0.212	**Survival**
NMB2039	*porB*	1.28	0.189	
NMB1567	*miP*	1.55	0.002*	
NMB1768	*tpsA2*	-1.20	0.117	
NMB1214	*tpsA3*	-1.83	0.001*	
NMB0496	*tpsB1*	-1.93	0.104	
NMB0497	*tpsA1*	1.17	0.520	
NMB1779	*tpsA*	-1.09	0.640	
NMB1780	*tpsB*	-2.04	0.004*	

4.3.8- Other deregulated genes

We hypothesised that the accumulation of diacylated lipoproteins in the periplasm of MC58Lnt may result in envelope stress (Chapter 3). Alterations in the known Neisserial sigma factors RpoD, RpoH and RpoE (sigmaE) were investigated since they are linked to the expression of genes involved in house-keeping genes, translation of proteins, and replication and cell repair, respectively (Heidrich *et al.*, 2017). Other ones included the RNA chaperone Hfq which responds to envelope stress by transcriptionally controlling expression of envelope-localized proteins and protein complexes (Heidrich et al., 2017; Vogt and Raivio, 2014), protease DegQ, and chaperones DnaK, SurA, and Skp which are typically involved in general stress response (Arsène *et al.*, 2000; Volokhina *et al.*, 2011). Besides proteins DsbA, DsbD, PpiA, and FkpA that are known to correct protein-folding were also investigated (Baneyx and Mujacic, 2004; Sinha *et al.*, 2004).

The DE genes related to protein-folding include *dsbA.2, dsbD, ppiA and fkpA* and were all found to be upregulated in MC58Lnt. Gene *dsbA.2*, in particular, was 2.18-fold more expressed while *dsbD, ppiA* and *fkpA* were 1.46, 1.38 and 1.24-fold upregulated, respectively (Table 4.5). The gene *dsbA.2* encodes for the DsbA protein which is involved in the formation of disulphide bond and isomerization in exported proteins (Tinsley *et al*., 2004) (Table 4.5).

Table 4. 5- Genes involved stress response

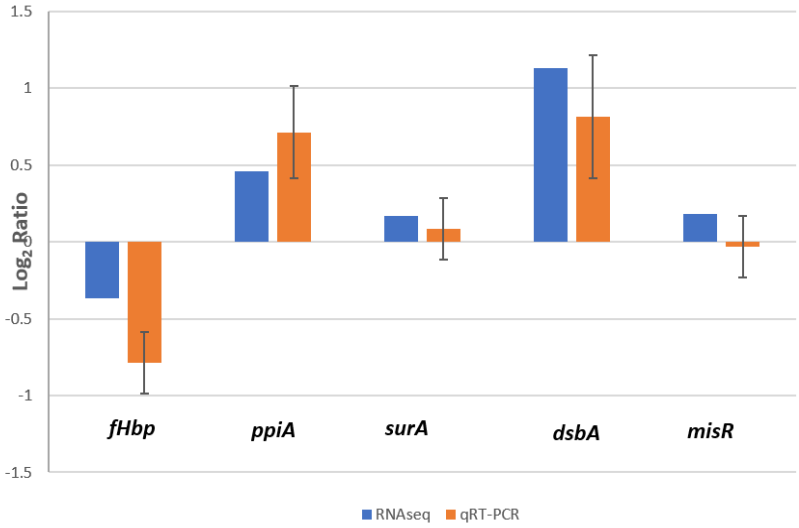
Some of the genes that are known to be involved in stress response in Nm. Statistically significant differences in gene expression are represented by *padj <0.05

Gene ID	Name	Fold difference	padj
NMB2144	*rpoE*	-0.82	0.381
NMB0278	*dsbA.1*	-0.91	0.695
NMB0407	*dsbA.3*	-0.92	0.540
NMB0748	*hfq*	1.04	0.798
NMB0281	*surA*	1.12	0.525
NMB0181	*skp*	1.12	0.312
NMB0712	*rpoH*	1.12	0.437
NMB0532	*degQ*	1.13	0.230
NMB1538	*rpoD*	1.19	0.402
NMB0027	*fkpA*	1.24	0.021*
NMB0554	*dnaK*	1.37	0.071
NMB1262	*ppiA*	1.38	0.013*
NMB1519	*dsbD*	1.46	0.005*
NMB0294	*dsbA.2*	2.18	0.005*

4.4- RNAseq Validation

In order to validate the RNAseq data, 5 genes were selected for evaluation by qRT-PCR, using the same methodology as that used in Chapter 3 (Fig 4.8a) (section 3.6.2). Validation of RNAseq data is recommended as best practice for RNAseq analysis although not strictly necessary (Fang and Cui, 2011). The genes *fHbp* (NMB1870), *ppiA* (NMB1262), *surA* (NMB0281), *dsbA* (NMB0294) and *misR* (NMB0595) were tested and their level of expression normalised against the house-keeping gene *recA*. Five RNA extractions were performed and analysed with Bioanalyser as previously described (Fig 4.3). These samples included the RNA samples sent for RNAseq plus an additional of 3 samples for MC58 and 2 for MC58Lnt, total of n=5 for each strain. The qPCR reaction efficiency and primer optimisations for each of these additional genes can be found in the appendix IV. The correlation, which means how well the RNAseq data is in accordance with the qRT-PCR results, showed a confidence of 90% for the RNAseq results (Fig 4.8b) meeting the high level of reliability required (Conesa *et al*., 2016).

a.



b.

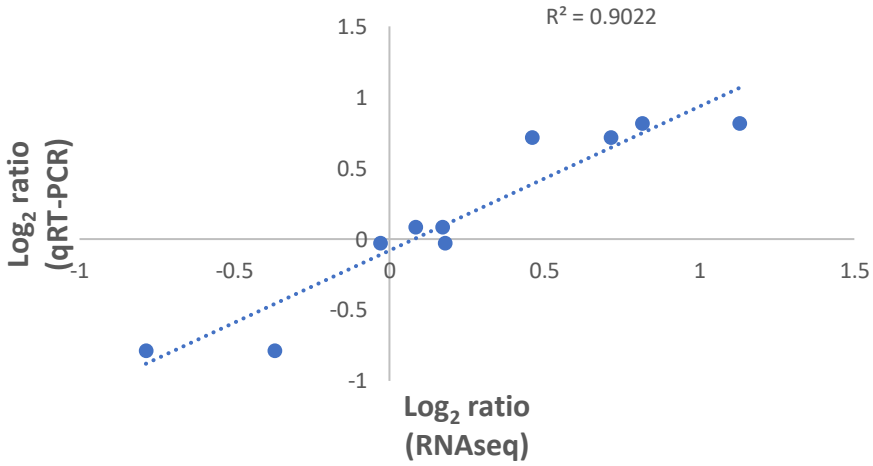


Figure 4. 8- Validation of RNAseq data by qRT-PCR

a. Comparison of RNAseq (blue bars) and qRT-PCR (orange bars) fold change results for 5 selected genes. Fold change qRT-PCR ratios represent the difference in transcript abundance/signal for these genes at OD_{600} 0.5. qRT-PCR normalization data was done using *recA* as a

reference gene. **b.** Correlation analysis of RNAseq and qRT-PCR transcript measurements for the 5 selected genes shown in panel a. The qRT-PCR and RNAseq log_2 values were plotted and the coefficient of correlation was calculated, $R^2 = 0.9022$.

4.5- Biological significance of MC58Lnt

Next we investigated whether the differences observed by RNAseq were reflected in the biology of the MC58Lnt mutant. Host-microbe interactions were analysed in a series of experiments including *in vitro* cellular assays, and biofilm formation assay.

First, growth curves of MC58 and MC58Lnt in both supplemented GC broth and in the media used for cell assays (RPMI supplemented with 10% FBS) were established. An aliquot of cells from each time point was plated to check if CFU counts were similar for both strains. No major difference in CFU counts was observed. The Lnt disruption appeared to reduce the growth rate slightly in GC broth for the period observed (7.6 h in total) but the difference did not remain when the mutant was grown for 24 h in the cell culture media used (Figs 4.9 and 4.10). The difference observed between the two media may well be due to how richer cell culture media is in comparison to GC broth.

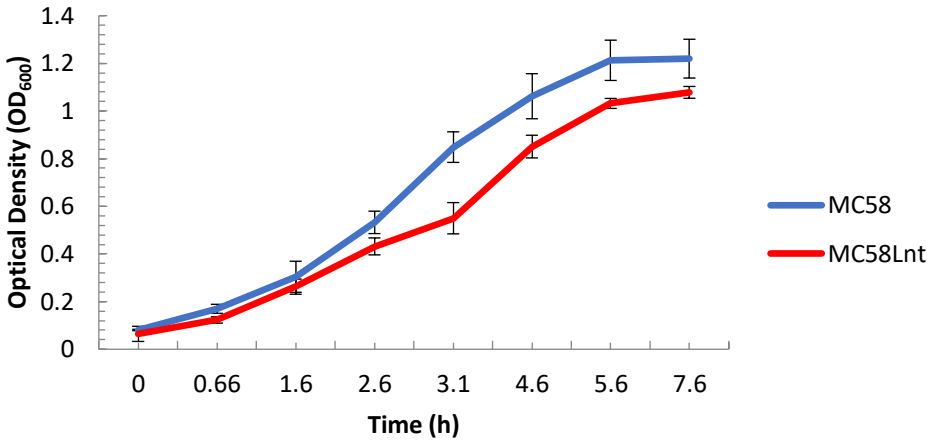


Figure 4. 9- Growth curve of MC58 and MC58Lnt in supplemented GC broth

Both strains had their growth monitored for a period of 7.6 h, when stationary phase is achieved. MC58 and MC58Lnt are shown in blue and red, respectively.

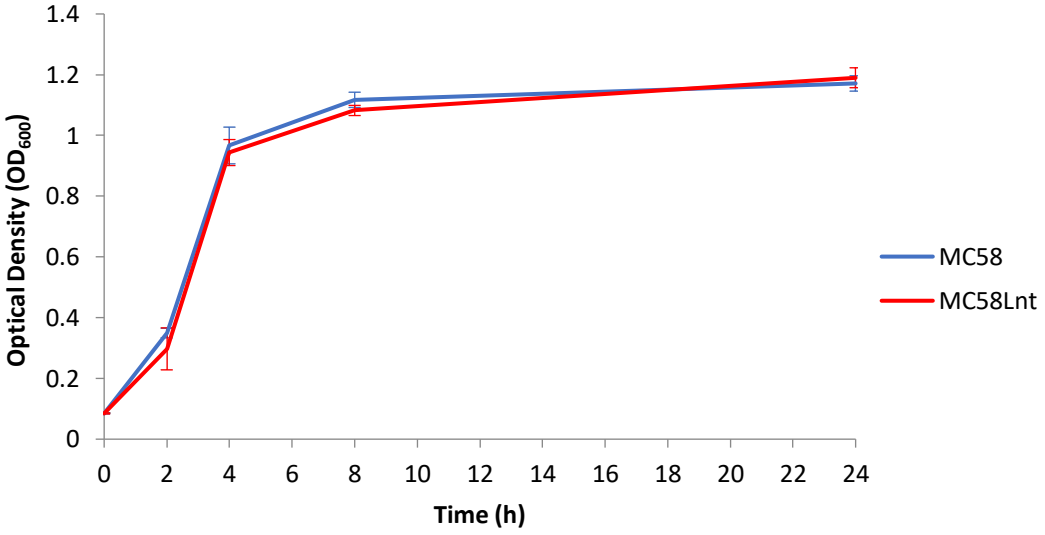


Figure 4. 10- Growth curve of MC58 and MC58Lnt in RPMI supplemented with 10% FBS

Both strains had their growth monitored for a period of 24 h, the maximum period of incubation for *in vitro* cell assays. MC58 and MC58Lnt are shown in blue and red, respectively.

4.5.1- Adhesion and invasion of MC58Lnt in HUVECs and HCEC cells are affected

Interactions with epithelial and endothelial cells are crucial steps for the development of meningococcal disease. Adhesion, followed by invasion of tissues characterise the primordial events that lead to meningitis as introduced in Chapter 1 (Carbonnelle *et al*., 2009; Corbett *et al*., 2004). In order to evaluate how Lnt disruption affects Nm interaction with these two different types of cells, adhesion and invasion rates were assessed with different *in vitro* assays using HUVEC (Human Umbilical Vein Endothelial Cells) and HCEC (Human Corneal Epithelial Cells). HUVECs have been used to represent endothelial cells in meningococcal infection studies for a long time (Virji *et al.*, 1991, 1992; Sa e Cunha *et al.*, 2009; Kuwae *et al.*, 2011). The model used for epithelial cells, HCEC, is also known as the Chang Conjunctival Epithelial Cell line (HCEC, CCL-20.2) (Hu *et al*., 2001).

4.5.1.1- MC58Lnt adheres to HCEC cells similarly to MC58 but invades less well

Adhesion and invasion were tested using an adapted version of the approaches used by Klee *et al.* (2000) and Sutherland *et al.* (2010). Invasion assays require the addition of gentamicin, a broad-spectrum antibiotic, to kill bacteria that did not invade or were not taken up by the cells. Treatment with gentamicin is given after incubation with Nm. The reason for specifically using gentamicin is its reduced penetration in

eukaryotic cells (Elsinghorst, 1994). All the data shown are the results of 3 biological replicates with technical replicates of n=5 for reliability.

Here it is important to emphasise that testing adherence and invasion of Neisserial cells to human cell lines are well-established *in vitro* surrogate approaches for investigating these steps of human infection deployed by Nm (Merz and So, 2002; Salit and Morton, 1981). Therefore, to investigate whether MC58Lnt had its adhesion and invasion abilities affected, first adhesion and invasion were tested in epithelial cells, HCEC.

MC58 and MC58Lnt were incubated with HCECs for a period of up to 6h after initial incubation of 1h to allow initial adherence. At each time point, the wells were washed with cell culture media to harvest non-adherent cells which were subsequently plated to determine CFU. After 6 hours, adherent CFU were quantified after the addition of 1 ml of PBS–1% saponin and scraping the bottom of the wells with a micropipette tip. Values of relative percentage of MC58Lnt to the WT were plotted (Fig 4.11). MC58Lnt adhered to HCECs at levels similar to those of the WT for up to 4h. However, by 6h adherence levels were only 44% of the WT levels, suggesting reduced capacity of the mutant to remain attached to these cells over longer periods.

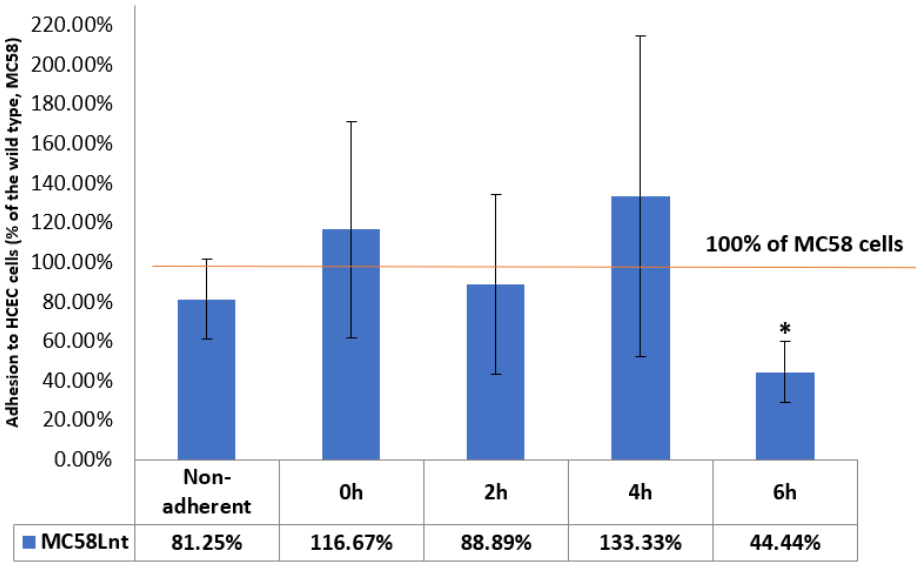
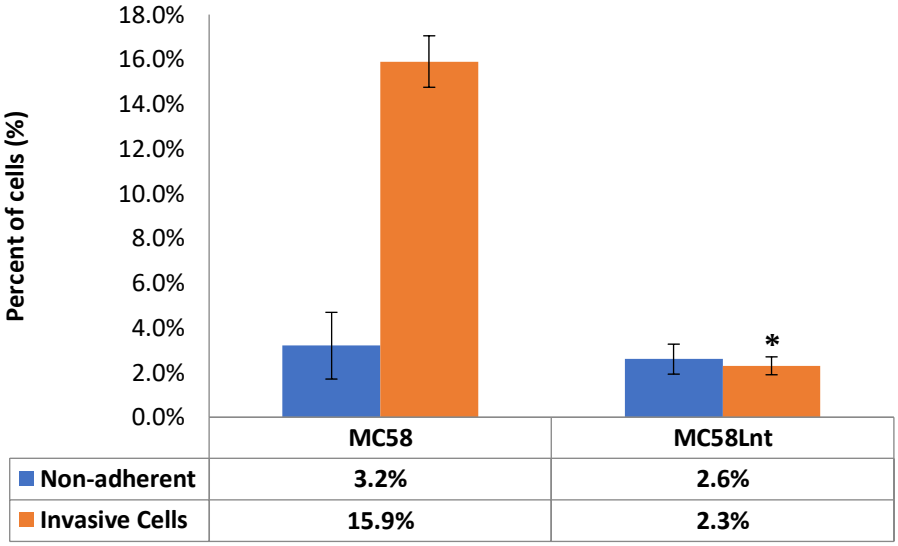


Figure 4. 11- Adhesion of MC58Lnt mutant to HCEC cells relative to MC58

Adhesion of MC58Lnt is represented as a percentage relative to the WT. Values for MC58Lnt are the averages of three experiments with technical replicates of n=5. Time points from 0h to 6h represent time points after infection and the removal of non-adherent bacteria. Unpaired T Tests with Welsch's correction were conducted in GraphPad v6 to compare WT and mutant. The values obtained with MC58Lnt were statistically significant: *, $p \leq 0.05$ vs WT.

We next investigated invasion of HCECs by both strains. After 6h of incubation with neisserial cells and washes after every hour with cell culture media, non-internalised but adherent cells were killed by the addition of gentamicin and incubation for a further for 4h. The MC58Lnt cells were less

invasive than cells of the WT (Fig 4.12). Only 2.3% of MC58Lnt cells invaded HCECs compared to 15.9% of MC58 cells.



	MC58	MC58Lnt
Non-adherent	3.2%	2.6%
Invasive Cells	15.9%	2.3%

Figure 4. 12- Invasion of MC58 and MC58Lnt strains to HCECs

Percentage of invasive cells is the number of internalized bacteria after 6 h of infection relative to the total initial number of cells added (10^5). Values for the MC58Lnt mutant are the averages of three experiments with n = 5. Unpaired T Tests with Welsch's correction were conducted in GraphPad v6 to compare WT and mutant. The values obtained with MC58Lnt were statistically significant: *, p≤0.05 vs WT.

4.5.1.2- MC58Lnt adheres less to HUVECs than MC58 but invades more

Following the analysis of adhesion and invasion in epithelial cells, HUVEC was chosen as the established *in vitro* model for endothelial cells studies (Virji *et al*., 1993c).

MC58Lnt was consistently less adherent to HUVECs compared to HCECs (Fig 4.13). Even at the highest adherence levels the mutant adhered to HUVECs at levels only a quarter of those seen for the WT. Adherence of only 8% cells was seen by 6h.

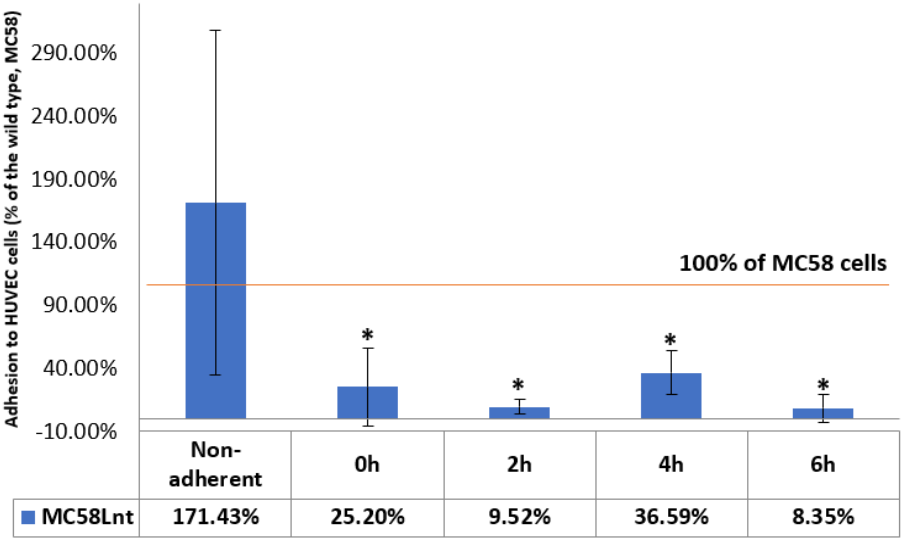


Figure 4. 13- Adhesion of MC58Lnt mutant to HUVECs relative to MC58

Adhesion is represented as a percentage relative to the WT. Values for MC58Lnt are the averages of three experiments with n=5. Time points from 0h to 6h represent time points after infection and the removal of non-adherent bacteria. Unpaired T Tests with Welsch's correction were conducted in GraphPad v6 to compare WT and mutant. The values obtained with MC58Lnt were statistically significant: *, p≤0.05 vs WT.

By contrast to the adhesion levels, the mutant invaded HUVECs at levels 3.1-fold higher than the WT (Fig 4.14).

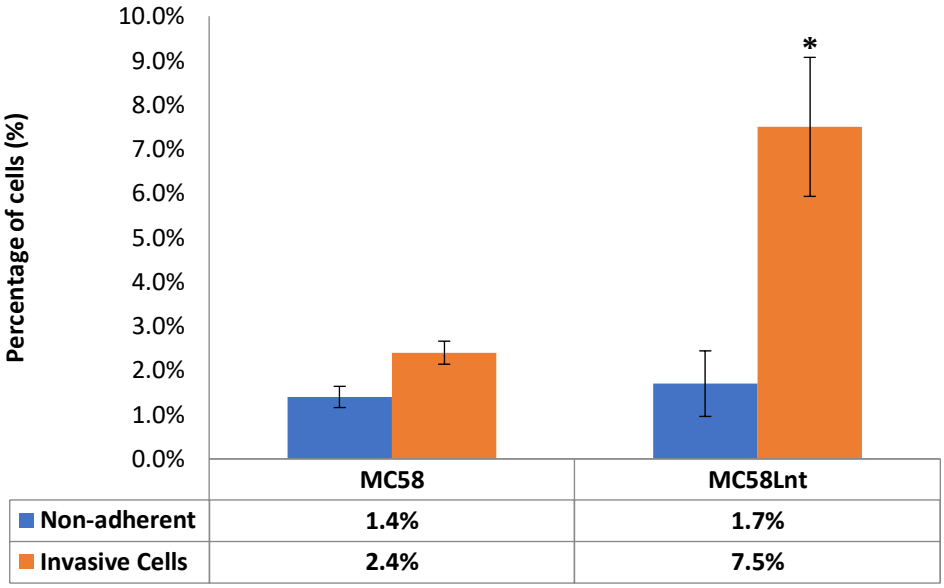


Figure 4. 14- Invasion of MC58 and MC58Lnt mutant strains to HUVECs

Percentage of invasive cells is the number of internalized bacteria after 6 h of infection relative to the initial number of cells added (10^5). Values for MC58Lnt are the averages of three experiments with n = 5. Unpaired T Tests with Welsch's correction were conducted in GraphPad v6 to compare WT and mutant. The values obtained with MC58Lnt were statistically significant: *, p≤0.05 vs WT.

In summary, MC58Lnt adheres to HCECs at similar levels to its parent, but invades less, while in HUVECs, MC58Lnt adheres less and invades more than its parent. Next, we investigated survival and replication of the MC58Lnt mutant in THP-1 cells.

4.5.2- Survival and Replication of MC58Lnt in THP-1 cells

Following the approach by LoVullo *et al.*, 2015, we investigated whether MC58Lnt can survive and replicate in THP-1 cells. These cells are the common used model for human monocytes, they are from a human monocytic cell line derived from an acute monocytic leukemia patient (Bosshart and Heinzelmann, 2016). The experiment was performed after initial induction with PMA to differentiate THP-1 cells into macrophage-like cells. Infection with both MC58 and MC58Lnt was then allowed up to 24h. CFU were counted after 6h and 24h as described in method section 2.2.1.

Monocytes have the ability to migrate from blood to tissue, where they differentiate into macrophages, and work as the sentinels mediating immune surveillance and inflammatory responses (Dick *et al*., 2017). The study of how THP-1 cells responds to MC58Lnt was performed in order to understand if disrupting Lnt could influence the survival and replication of this mutant intracellularly.

The data demonstrates that MC58Lnt survives and replicates better in THP-1 cells than the WT as shown in Table 4.6. There is a significant difference in uptake and replication between the two strains.

Table 4. 6- Survival and Replication of MC58Lnt compared to MC58 in THP-1 cells

Strain	THP-1 cells (CFU / ml) after[a]:	
	6h	24h
MC58	$2.38 \times 10^3 \pm 1.34 \times 10^3$	$1.64 \times 10^5 \pm 0.12 \times 10^5$
MC58Lnt	$1.28 \times 10^5 \pm 0.54 \times 10^5$*	$3.35 \times 10^5 \pm 0.46 \times 10^5$*

[a] Each experiment was performed in triplicate with n = 5, and the results are shown as averages. ±, standard errors of the mean (SEM). Unpaired T Tests with Welsch's correction were conducted in GraphPad v6 to compare WT and mutant. The values obtained with MC58Lnt were statistically significant: *, p≤0.05 vs WT.

4.5.3- HUVECs express more IL-8 and IL-6 than HCECs upon infection with MC58Lnt compared to MC58

A number of different cells secrete cytokines and chemokines in response to contact with bacterial cells or their products. While chemokines control the migration of monocytes, neutrophils and lymphocytes, cytokines act as a pro-inflammatory stimulus at the site of infection (Dick *et al*., 2017). Endothelial cells express IL-6 and IL-8 early after infection with Nm and both inflammatory cytokines have been shown to be up-regulated in HUVECs upon adherence by the meningococcus (Linhartova *et al*., 2006). Ocular epithelial cells were thought not to express cytokines and chemokines but, despite not being specialised cells for this task, studies have shown they express these immunomodulatory molecules when in contact with bacteria (Cole *et al*., 1999; Cubitt *et al*., 1993; Zhang *et al*., 2005). We aimed to investigate if the levels of expression of IL-6 and IL-8 in both cell models used in this study differ upon encounter with or MC58Lnt compared with MC58.

Both HCEC and HUVEC cultures were incubated with MC58 or MC58Lnt for up to 10h. A MOI of 100 bacterial cells was conducted, and after each time point, the cell culture supernatant was collected from a separate well and filtered with a 0.22μM filter to eliminate bacteria. ELISA was then performed to quantify IL-6 and IL-8.

MC58Lnt induced less expression of IL-8 and IL-6 at each time point compared to MC58 in HCECs (Fig 4.15a), whereas, it induced more expression of IL-8 and IL-6 in HUVECs (Fig 4.15b). Since MC58Lnt invades HCECs less and conversely invades HUVECs more, the degree

of invasion could be linked to the increase in expression of both inflammatory proteins. Perhaps the greater the invasion, the more the cells express IL-6 and IL-8 which signals to the immune system that an infection is happening allowing defence cells to be recruited to the infection site.

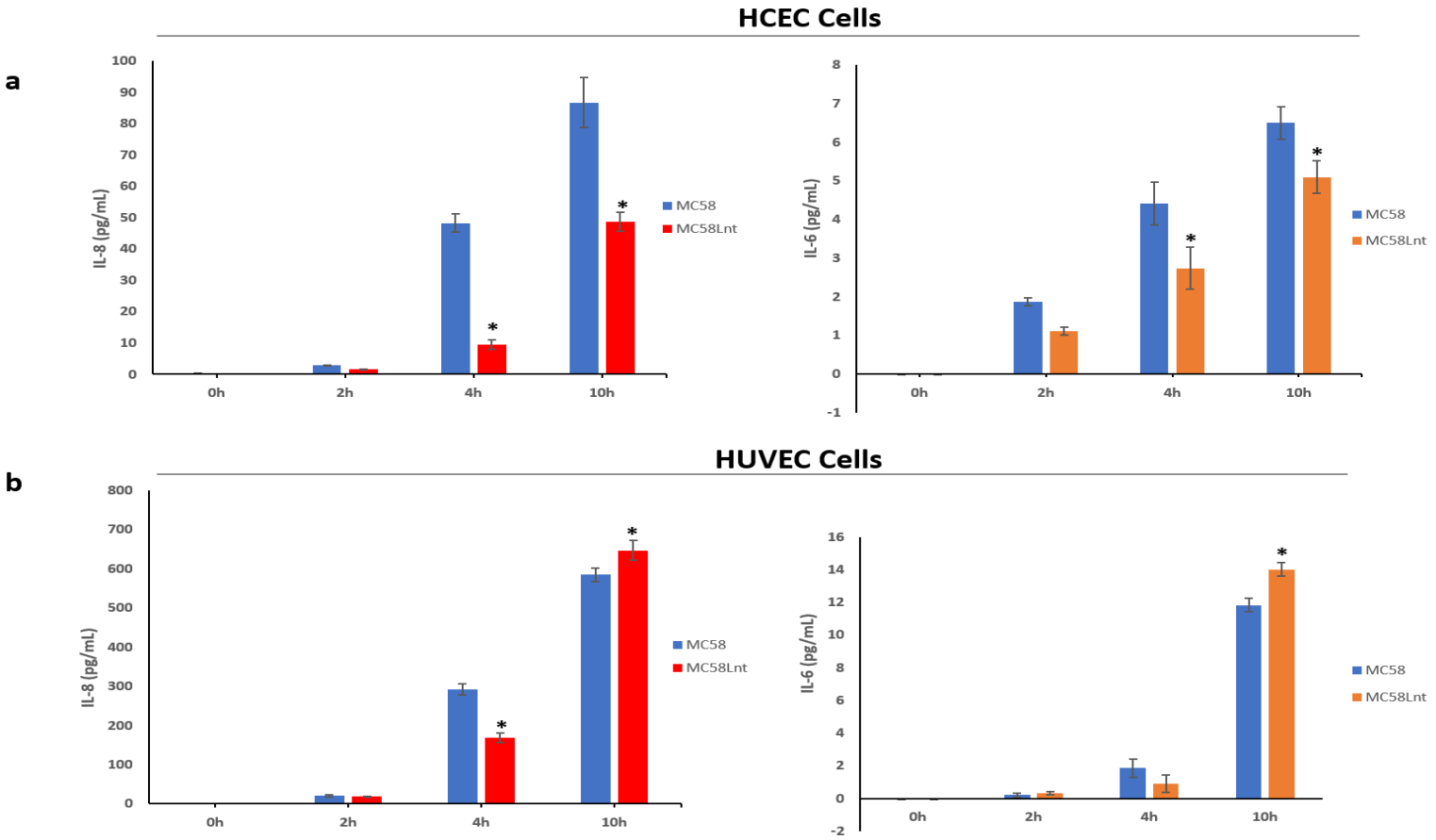


Figure 4. 15- Expression of IL-6 and IL-8 by HCECs and HUVECs when infected with MC58 and MC58Lnt strains
a. Amount of IL-8 and IL-6 expressed by HCECs when in contact with MC58 (blue) and MC58Lnt (red and orange). **b.** Amount of IL-8 and IL-6 expressed by HUVECs when in contact with MC58 (blue) and MC58Lnt (red and orange). The average level is shown from three independent experiments with n=3 for each time point. Unpaired T Tests with Welsch's correction were conducted in GraphPad v6 to compare the amount released by each cell type in contact with the strains MC58 and MC58Lnt. Error bars represent mean ± SEM. *p<0.05 vs wild type MC58 strain.

4.5.4- Expression of IL-6 and IL-8 by THP-1 cells when infected with MC58 and MC58Lnt

Since MC58Lnt was shown to survive and replicate better in THP-1 cells, compared with the WT, it was relevant to check the levels of expression of both IL-6 and IL-8 by this type of cell. IL-6 and IL-8 are important markers for meningococcal disease. High levels of both proteins are commonly found in patients with meningococcal septicaemia (Møller *et al*., 2005; Schultz *et al*., 2008; Waage, 1989). Since macrophages are important to tackle this disease, we evaluated both interleukin levels after infection with MC58 and MC58Lnt.

THP-1 cells were shown to express more IL-6 over time when infected with MC58Lnt than when infected with MC58 (Fig 4.16a), whereas they expressed similar levels of IL-8 when infected with both strains (Fig 4.16b). IL-6 expression could thus be linked to MC58Lnt higher capability of surviving in THP-1. Higher amounts of IL-6 allows increased permeability of blood vessels so more immune cells can tackle the bacterial infection (Narazaki and Kishimoto, 2018).

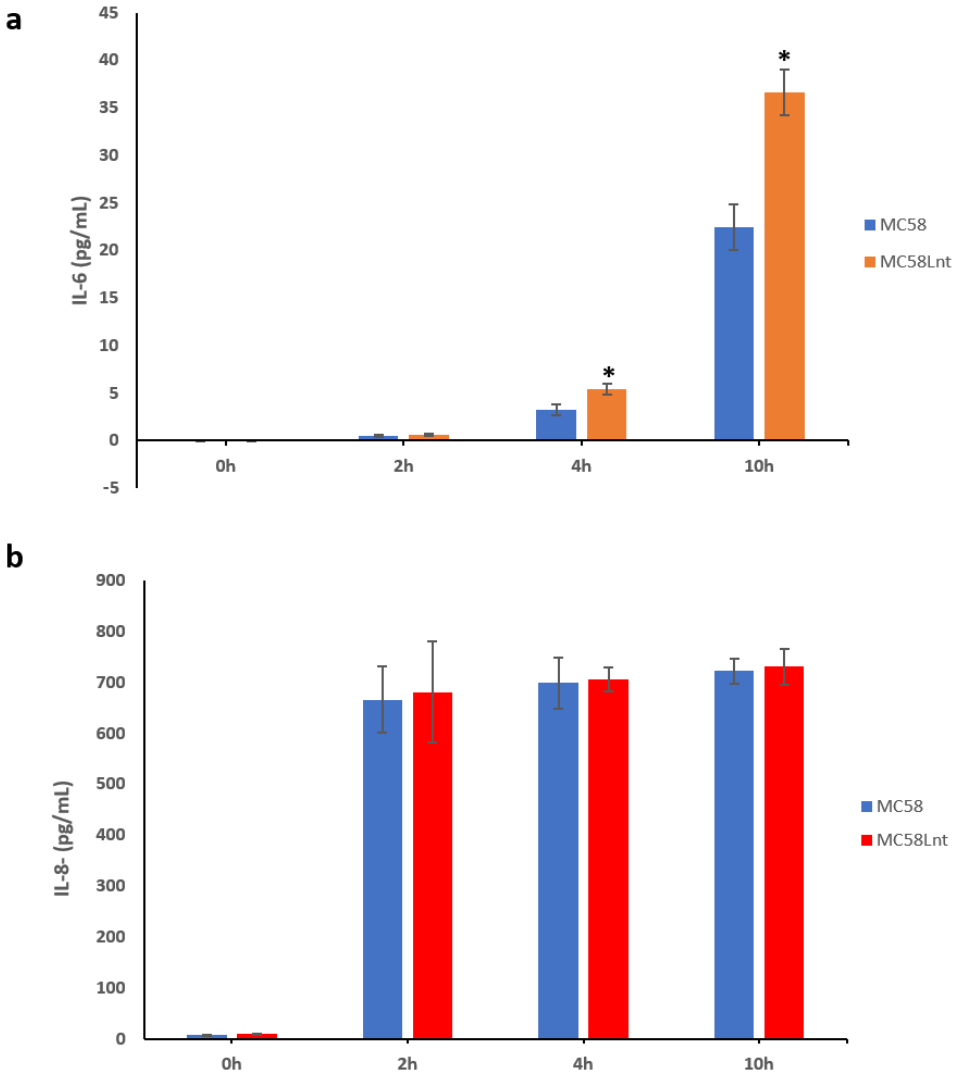


Figure 4. 16- Expression of IL-6 and IL-8 by THP-1 cells when infected with MC58 and MC58Lnt

a. Amount of IL-6 expressed by THP-1 cells when in contact with MC58 (blue) and MC58Lnt (orange). **b.** Amount of IL-8 expressed by THP-1 cells when in contact with MC58 (blue) and MC58Lnt (red). The average amount of three independent experiments with n=3 is shown for each time point. Unpaired T Tests with Welsch's correction were conducted in GraphPad v6 to compare the amount released by each cell type in contact with MC58

and MC58Lnt. Error bars represent mean ± SEM, *p<0.05 vs wild type MC58 strain.

4.5.5- MC58Lnt forms less biofilm than MC58

Using the approach by O'Dwyer *et al.* (2009), which is based on the ability of bacteria to form biofilms on abiotic surfaces such as polystyrene, we investigated the ability of MC58Lnt to form biofilm in comparison to MC58. The mutant strain formed 40% less biofilm when compared to the WT (Fig 4.17).

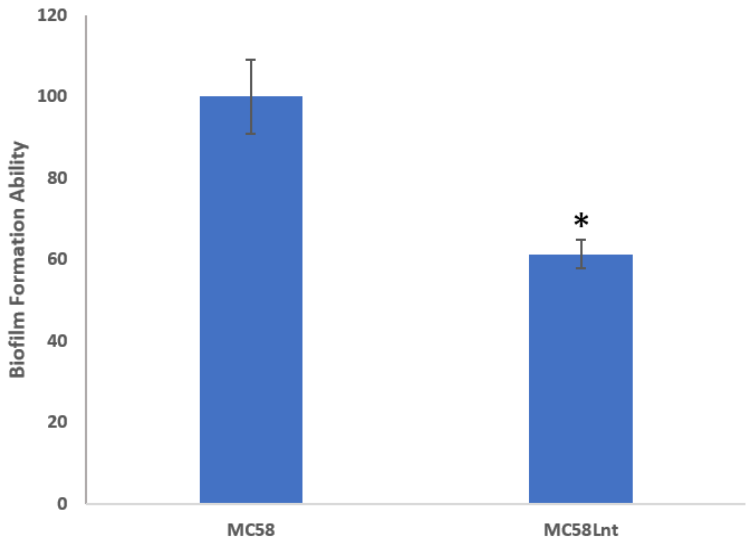


Figure 4. 17- MC58Lnt forms less biofilm than MC58

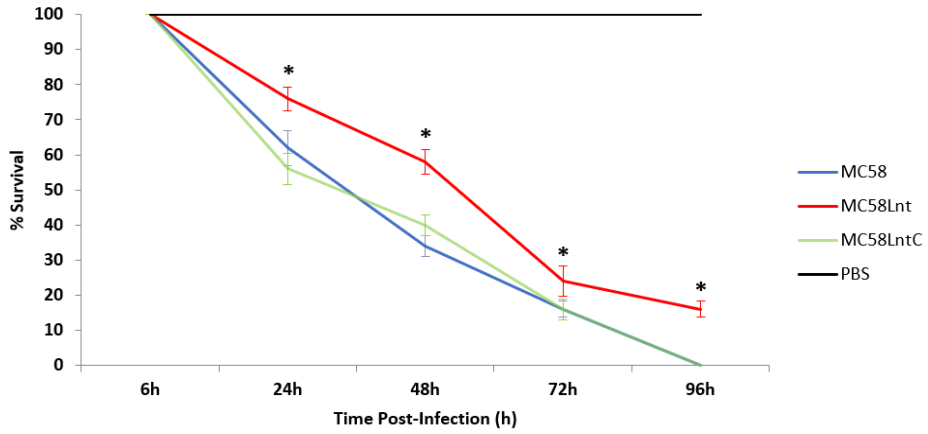
Microtitre plate assay based on the ability of bacteria to form biofilms on polystyrene. Biofilm formation was measured by crystal violet staining and the biofilms were quantified by measuring A_{600}. Absorbance of MC58 was considered 100% and values for MC58Lnt was calculated relative to that. Unpaired T Tests with Welsch's correction were conducted in GraphPad v6 to compare the ability of forming biofilm by MC58Lnt in comparison to MC58. Error bars represent mean ± SEM, *p<0.05 vs WT.

4.6- Comparison of pathogenicity of MC58, MC58Lnt and MC58LntC in *G. mellonella*

Lastly, to test whether MC58Lnt is less pathogenic or attenuated *in vivo* due to Lnt disruption, the waxworm *G. mellonella* was used as an *in vivo* model. To our knowledge, this is the first time this *in vivo* model has been used to test Nm virulence. However, it is an emerging model that has been used to test pathogenicity of several different microorganisms including *P. aeruginosa, Klebsiella pneumonia* and *E. coli* (Hernandez *et al*., 2019; Rossoni *et al*., 2019; Singkum *et al*., 2019). Larvae were injected with PBS or with 10^5 CFU of strains MC58, MC58Lnt and MC58LntC. Mortality was assessed based on absence of movement upon touching and change in colour of the larvae. After 96h, all larvae infected with strains MC58 and MC58LntC were dark-coloured and unresponsive to touch, whereas 16% of the larvae infected with MC58Lnt were still moving despite some of them being dark-coloured (Fig 4.18a and b).

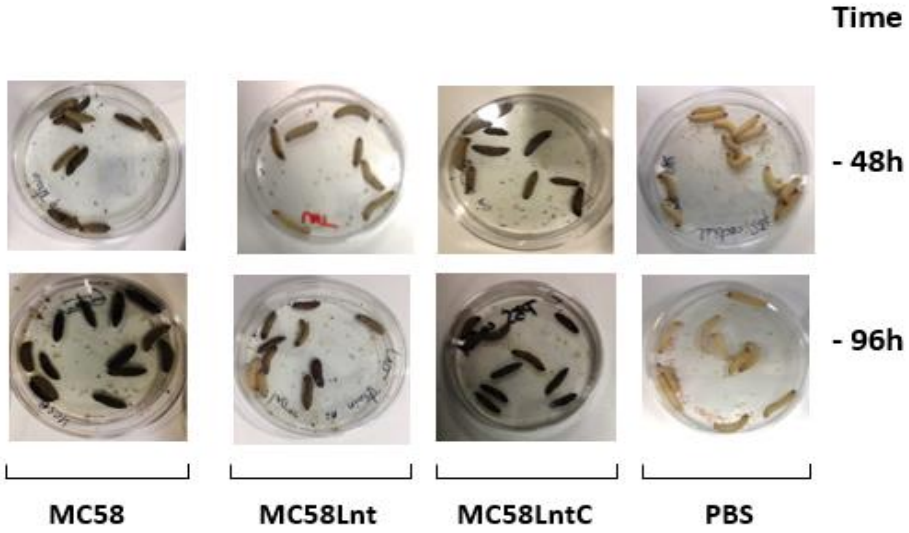
Based on CFU counts Nm was not only able to survive in *G*. *mellonella* but also multiply (Fig 4.18c), which is an indication that this model could be a suitable model to study the virulence of this microorganism. All three strains had similar CFU counts for all time points tested (Fig 4.18c).

a



b

c



STRAIN	6H	24H	48H	72H
MC58	$1.59 \times 10^2 \pm 0.12 \times 10^2$	$2.96 \times 10^4 \pm 0.24 \times 10^4$	$1.00 \times 10^5 \pm 1.63 \times 10^5$	$0.79 \times 10^5 \pm 0.57 \times 10^5$
MC58LNT	$0.66 \times 10^2 \pm 0.09 \times 10^2$	$2.40 \times 10^4 \pm 0.37 \times 10^4$	$0.91 \times 10^5 \pm 1.87 \times 10^5$	$1.03 \times 10^5 \pm 0.06 \times 10^5$
MC58LNTC	$1.44 \times 10^2 \pm 0.04 \times 10^2$	$3.16 \times 10^4 \pm 0.10 \times 10^4$	$0.89 \times 10^5 \pm 0.49 \times 10^5$	$0.78 \times 10^5 \pm 0.29 \times 10^5$

Figure 4. 18- MC58, MC58Lnt, and MC58LntC induced lethality of *G. mellonella* to different degrees

a. Larvae were injected with PBS or with 10^5 CFU of strains MC58, MC58Lnt and MC58LntC. Survival was monitored over 96h post infection. Mortality of larvae infected with Nm was strain dependent. The one-way

ANOVA followed by Dunnett's test was employed for statistical analysis in GraphPad v6. All values represent mean ± SEM, *p<0.05 vs strain MC58. **b.** Images of the strains after 48h and 96h of infection. **c.** CFU at different time points after infection.

4.7- The Lnt mutant is more susceptible to antibiotics

Lipoproteins are important for the integrity of the cell envelope and for maintaining homeostasis of the cell (Narita and Tokuda, 2017). An intact envelope is crucial for cells healthy functioning. Considering Lnt disruption could be affecting the lipoproteins in the cell and thus downregulating several lipoproteins that may play an important role in the OM biogenesis and integrity, it is possible that the integrity and permeability of the OM may be compromised in a Lnt mutant. We predicted that the envelope of this mutant would be more permeable to antibiotics. Several antibiotics were tested including those used to treat meningococcal infection (Table 4.7).

Table 4.7- Drug susceptibilities of MC58, MC58Lnt and MC58LntC

Drug	Dose(µg/ml)		
	MC58	MC58Lnt	MC58LntC
Tunicamycin[a]	50	50	50
Ceftriaxone[b]	0.00097	0.00049	0.0097
Penicillin G[b]	0.025	0.0125	0.025
Chloramphenicol[b]	2.0	1.0	2.0
Sulfalinamide[c]	100	25	100
Globomycin[c]	6.25	1.5625	6.25
Ciprofloxacin[d]	0.00391	0.00024	0.00391
Rifampicin[e]	0.125	0.00195	0.125

Minimum Inhibitory Concentrations (MICs) determined using Microbroth Dilution assays. [a] No significant fold change observed between strains. [b,c,d,e] MC58Lnt showed 2, 4, 16 and 64 fold increased susceptibility,

respectively, to the drugs tested. Experiments were performed in triplicates for at least 3 independent experiments. The one-way ANOVA followed by Dunnett's test was employed for statistical analysis in GraphPad v6. All values represent mean ± SEM, *p<0.05 vs strain MC58.

The MICs of 8 antibiotics were tested (Table 4.7). MC58Lnt showed significant increases in antibiotic susceptibility to seven of them as follows: 64-fold to rifampicin, 16-fold to ciprofloxacin, 4-fold to both globomycin and sulphanilamide and 2-fold to ceftriaxone, penicillin G and chloramphenicol. No change was seen in MIC for tunicamycin.

4.8- Discussion

Work described in this chapter explored the impact of disruption of Lnt in MC58 on the cellular and molecular level by TEM and RNAseq, respectively. *In vitro* cellular assays were used to evaluate whether the changes observed in gene expression reflected in the biology of this strain. MIC testing was performed to further explore the potential of Lnt as a novel drug target.

Our study found 111 genes DE in MC58Lnt that belong to several different functional classes in the cell (Fig 4.7). A recent study with an Lnt mutant of *Acinetobacter baylyi* found nearly 80 DE genes involved in different cellular processes (Gwin *et al*., 2018). In this study there was an emphasis on the possible use of their Lnt mutant for the study of membrane stress since they also observed increased susceptibility to antibiotics. We believe our strain could also be used in that way to elucidate how Nm responds to membrane

stress, however from the observations made at the chosen OD to perform RNAseq (OD_{600} 0.5), no strong difference in expression of possible known genes linked to membrane stress was observed (Fig 4.7 and Table 4.5) apart from higher expression (2.18-fold) of one of the copies of neisserial *dsbA*, known to help with protein folding (Kumar *et al*., 2011) and genes *dsbD* and *fkpA* also involved in protein-folding. It will be interesting to investigate if the many DE hypothetical genes found in this study are involved in membrane stress response. Important to also mention Lnt disruption in Nm impacted on the growth of MC58Lnt considerably less than mutation of the same gene in *A. baylyi* when grown in LB broth. Gwin *et al.* (2018) attributed the growth defect of their mutant to OM disturbances (shown by slight ultrastructural changes). Like MC58Lnt, this mutant showed increased susceptibility to antibiotics (Gwin *et al*., 2018).

The *in vitro* cellular assays in this chapter used the cells lineages HCEC, HUVEC, and THP-1 to investigate adherence, invasion and survival. MC58Lnt interacted differently with each of these cells when compared to the WT. Whilst MC58Lnt adheres similarly to HCECs and invades them less (Figs 4.11 and 4.12), this strain adheres less to HUVECs and invades them more (Figs 4.13 and 4.14). Higher invasion in HUVECs could imply MC58Lnt is successfully subverting these cells for its own benefit or, alternatively, HUVECs could be more active in engulfing the cells with such disruption. Whilst endothelial cells are considered to be inert and non-phagocytic, several recent studies have now recognised that, on the contrary, they play an important role in managing infection (Dick *et al*., 2017). As reviewed by Carbonnelle *et al.*, 2009, some groups observed that

adhesion of Nm to endothelial cells promotes the local formation of membrane protrusions originating from cellular microvilli that surround bacteria which could benefit bacterial invasion but also be a mechanism of defense by the endothelial cells (Eugène *et al*., 2002; Merz and So, 1997; Merz *et al*., 1999). From their observations, it was seen that specific molecular complexes, named cortical plaques, are formed from the contact of cells with the meningococcus, precisely beneath the bacterial colonies.

Regarding THP-1 cells survival assay, MC58Lnt did not have lower ability to survive and replicate in THP-1 cells (Table 4.6). This result is similar to the one observed by LoVullo *et al.* (2015). LoVullo and co-workers did not observe lower intracellular growth for their *F. tularensis* Lnt mutant.

Bacterial infection by MC58Lnt in cell lineages induced more expression of IL-6 and IL-8 when testing HUVECs than when testing HCECs (Fig 4.15). It is difficult to explain such data; however, it seems that the greater ability of invading HUVECs by the mutant could be linked to the higher expression of these proinflammatory cytokines. The more invasion, the more important it might be to signal to the immune system an invader is present. Cytochalasin D which prevents bacterial invasion (Finlay *et al.*, 1991) could be used in future work to test this.

An interesting question is why the Lnt mutation could have caused an enhanced ability of MC58Lnt to invade HUVECs. The literature about meningococcal adhesion is vast and sometimes conflicting, however, it is known that in the absence of pili, other major adhesins including the opacity proteins (Opa and Opc) as well as minor adhesins govern how well Nm will adhere to and invade cells. The RNAseq data showed downregulation of

genes encoding pili and Opc (Table 4.4) while Opa-encoding genes were not significantly affected by the Lnt mutation. As reviewed by Hung and Christodoulides (2013), low expression of Opc allow more Opa-dependent invasion. And it could well be that in the absence of other major adhesins, Opa could be playing a bigger role in the invasion of HUVEC cells. The absence of the major adhesins may allow intimate adhesion to happen quicker providing a type of interaction that favours Opa-mediated invasion. The availability of specific cell receptors for these adhesins is also a key influencing factor. Epithelial and endothelial cells display differences in their abundance of different receptors. In fact, more inflammation induces more expression of CEACAM receptors which will in turn favour cellular invasion in an Opa-CEACAM-depend manner (Griffiths *et al*., 2007; Hill *et al*., 2010a; Rowe *et al*., 2007). It would be interesting to repeat the above studies with a double mutant of Lnt and Opa and also to evaluate the cell receptor expression on HCEC and HUVECs.

MC58Lnt showed reduced ability (60%) to form biofilm on abiotic surfaces (Fig 4.17) which indicates potential reduced virulence since biofilm formation is crucial for meningococcal disease development (Hung and Christodoulides, 2013). The reduced expression of genes related to pili formation shown earlier in this chapter, may be an influencing factor (Yi *et al*., 2004).

The *G. mellonella* model of virulence used here demonstrated that *N. meningitidis* is able to both survive in this invertebrate model and causes disease (as evidenced by CFU and the change in colour and response by the worm) (Fig 4.18). MC58Lnt proved to be less pathogenic compared to

MC58 and MC58LntC. It will be important to expand this study to a greater panel of strains including mutants of well-characterised virulence phenotypes. The three strains used in this study should also be compared in more established *in vivo* models such as mice, including human transgenic mouse models that have been used extensively to study meningococcal virulence (Yi *et al.*, 2003).

The integrity of the OM is crucial as a physical barrier and for active protection against antibiotics (Graef *et al*., 2016). LoVullo *et al.* (2015) observed less profound effects of antibiotics on their Ng Lnt mutant than found for MC58Lnt in this thesis. To test our prediction of the fragility of the Lnt mutant's envelope and our model of inefficient sorting of diacylated lipoproteins by MC58Lnt in Chapter 3, we tested antibiotics that are commonly used to treat infection with Nm including Penicillin G, Ceftriaxone, Ciprofloxacin and Rifampicin (Nadel, 2016) as well as less common ones like Chloramphenicol, Globomycin and Tunicamycin. MC58Lnt showed significant increases to antibiotic susceptibility to 7 of them. 64-fold to Rifampicin, 16-fold to Ciprofloxacin, 4-fold to both Globomycin and Sulphanilamide and 2-fold to Ceftriaxone, Penicillin G and Chloramphenicol (Table 4.7). No change was seen in MIC for Tunicamycin.

Rifampicin inhibits bacterial DNA-dependent RNA polymerase which impairs protein synthesis (Calvori *et al.*, 1965). The higher susceptibility to Rifampicin may be indicating a heavier dependence of MC58Lnt on functional RNA machinery to control RNA synthesis for translation. Indeed, the RNAseq data showed significant changes in the expression of certain genes involved in RNA biology. The disruption of Lnt is likely to be causing

membrane stress such as that the mutant requires greater activity from RNA machinery to produce more periplasmic proteases to degrade mislocalised lipoproteins. The added deleterious effect of Rifampicin on RNA machinery, specifically on RNA polymerase to a mutant already disturbed in its RNA functionality would explain the dramatically increased bactericidal impact of Rifampicin on MC58Lnt.

The other antibiotics tested affect important cellular mechanisms. Since MC58Lnt is already affected metabolically, the bactericidal impact of these antibiotics is unsurprisingly greater for the mutant than the WT. Ciprofloxacin, for instance, is a broad-spectrum antibiotic of the fluoroquinolone class. It functions by inhibiting DNA gyrase, and types II and IV topoisomerase, thereby inhibiting cell division (Drlica and Zhao, 1997; Pommier *et al.*, 2010). Globomycin and Sulfalinamide affect the function of LspA (jamming the IM with unfully processed lipoproteins) and para-aminobenzoic acid (PABA) enzyme (affecting folic acid production and thereby affecting bacterial replication), respectively (Castelli *et al.*, 2001; Kiho *et al.*, 2004). Chloramphenicol inhibits protein synthesis (Kehrenberg *et al.*, 2005) and Ceftriaxone and Penicillin G affect bacterial cell wall formation (LeFrock *et al.*, 1982; Rasmussen *et al.*, 1980). Tunicamycin affects the formation of peptidoglycan. Therefore it is a potent antibiotic against gram-positive and not so much against gram-negative bacteria (Zhu *et al*., 2018). Here, Lnt disruption does not affect the cell to the point to make it more susceptible to killing by Tunicamycin.

Our observations from Chapter 3 regarding the poor efficiency of LolFD for sorting diacylated lipoproteins to the OM which likely weakens its tertiary

structure, rendering it more permeable to antibiotics offers an explanation to the susceptibility of MC58Lnt to several antibiotics observed in this chapter. The weakened OM with increased susceptibility to antibiotics in combination with the reduced virulence point to Lnt as a candidate drug target. The dual action of an Lnt inhibitor and an antibiotic of lower dose than normally administered could be a potent therapy and this could have applications for other AMR pathogens.

Importantly this chapter shows that the mutation of Lnt could be exploited to help in the identification of lipoproteins. We investigated the expression of 68 possible lipoproteins in this chapter (Table 4.1). As many as 25 putative lipoproteins were DE in MC58Lnt. We observed that 10 potential lipoproteins had their expression upregulated in this strain. It will be interesting to experimentally test if these proteins are indeed lipoproteins lipidated by Lnt. In conclusion, this chapter provided an evaluation of the global effects the Lnt mutation had on the meningococcus.

Chapter 5

Importance of the signal peptide in FHbp's translocation, processing and localisation

5.1- Introduction

Having elucidated the role of Lnt in adding the third fatty acid to FHbp and having found all the meningococcal orthologues[7] of the conserved lipoprotein machinery pathway established for *E. coli;* the question remained as to why some strains display FHbp poorly at the surface. Whilst efforts to characterise upstream regulatory regions of *fHbp* have provided strong evidence for transcriptional efficiency differences between isolates (Cayrou *et al.*, 2018). These studies do not fully explain poor surface display. Indeed, it is the surface abundance of FHbp that is critical for target recognition by FHbp-based vaccines. In this chapter, we describe the critical importance of the SP of FHbp in dictating its processing fate as well as surface abundance.

SPs are cleavable amino acid sequences that direct protein modification, translocation and secretion (Austen, 1979; von Heijne and Blomberg, 1979; Nielsen *et al.*, 2019). Located at the N-terminus of proteins, SPs in prokaryotes are recognised by one of the secretory systems, Sec or Tat, for translocation across the inner membrane. Lipoproteins have their SP removed by LspA and lipid added to the conserved cysteine residue of the lipobox (+1 position) (Zückert, 2014). A typical lipoprotein SP will have the presence of positively charged amino acids in the initial residues, the presence of at least 7 to 22 residues between the predicted lipobox and the charged residues and the lipobox located within the first 40 residues from the N-terminus with the consensus; [LVI][ASTVI][ASG][C] (figure 5.1). These regions are called the n-region, h-region and c-region, respectively (Inouye *et al.,* 1977; Sankaran and Wu, 1993; Braun *et al.,* 1993).

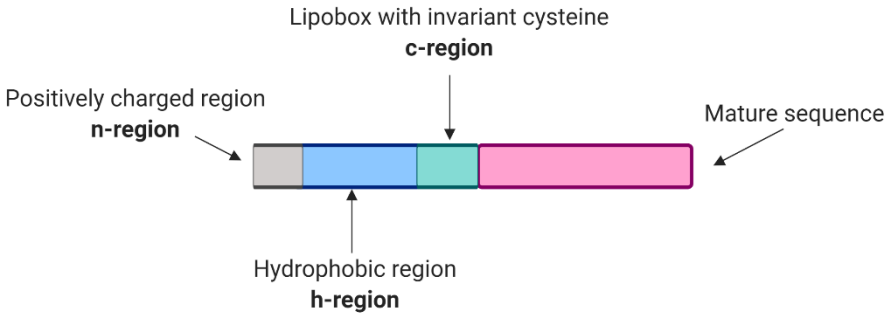


Figure 5. 1- Tripartite nature of the signal peptide

A SP will normally have the n-region, a variable h-region and a c-region containing the lipobox.

Lipidation and SP cleavage follow before sorting to the OM and, in some cases, subsequent flipping to face the external environment (Hooda *et al.*, 2016a). Until now, it had assumed that all meningococcal isolates that express FHbp synthesise the mature lipoprotein. Lipidation of FHbp was assumed to occur for all isolates and its surface localisation conducted by surface lipoprotein assembly modulator, Slam. This chapter aims to expand the current knowledge on SPs and their role in lipoproteins processing in prokaryotes, with a focus on FHbp.

5.2- FHbp sequence alone is responsible for failure to localise FHbp at the surface in strain L91543

In Chapter 3, we show that strain L91543 shows extremely low levels of FHbp on the surface compared to strain MC58 supporting observations by Newcombe *et al.* (2014) (Fig 3.14). In Newcombe *et al.* (2014) study, L91543 strain was shown to be resistant to killing by serum anti-FHbp antibodies, unlike strain MC58, we predicted L91543 to be a vaccine-escape strain.

From WB observations using whole cell lysates, a clear difference was observed in mobility of FHbp of MC58 and L91543 (Fig 5.2). Strain MC58 has a 27 kDa FHbp, known to be tri-palmitoylated, whereas our WB suggests the FHbp of L91543 is about 3 kDa larger (Fig 5.2).

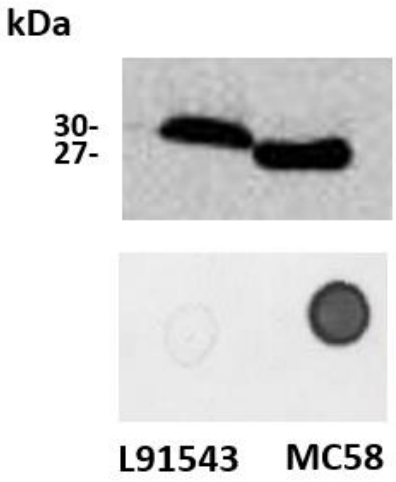


Figure 5. 2- FHbp expression phenotypes of L91543 and MC58

WCL Western immunoblot (upper panel) and WC suspensions immuno-dot blot (lower panel) of strains L91543 and MC58 from fresh plate cultures performed using mAb JAR4.

In order to investigate the reasons for a different FHbp size and poor surface localisation in L91543, genomic DNA of this strain was sequenced (GenBank accession number CPO16685) and all genes known to be involved in translocation, processing and surface localisation of FHbp (da Silva *et al.*, 2017) were compared to MC58 genes. No deletions, insertions or frame shifts were identified that could explain the lack of surface exposure of FHbp in L91543 (Table 5.1). The enzyme LspA, which is responsible for SP cleavage, for instance, was 100% similar at the AA level between both strains. All other canonical proteins share >97% identity across their protein sequences. The biggest difference observed was for FHbp itself with 93% AA similarity, and the most divergent region is the SP. Specifically, there are two non-synonymous SNPs in the SP of L91543; leucine (L) substituted by phenylalanine (F) at position 15 (SNP1) and threonine (T) substituted by alanine (A) at position 19 (SNP2) (Fig 5.3).

Table 5. 1- Amino acid identity of proteins involved in translocation, processing and surface localisation of FHbp in L91543 compared to MC58

MC58 (ACCESSION NUMBER: NC_003112)			L91543 (ACCESSION NUMBER: CP016684)	
PROTEIN	**ID**	**Gene**	**ID**	**AA Identity (%)**
FHBP	NC_003112.2/ NMB1870	*fhbp*	ANW71006.1	93
		PROCESSING MACHINERY		
LNT	NP_273755.1/ NMB0713	*lnt*	ANW71313.1	99
LSPA	NP_274829.1/ NMB1832	*lspA*	ANW71045.1	100
LGT	NP_274105.1/ NMB1072	*lgt*	ANW71667.1	98
		SORTING MACHINERY		
LOLA	NP_273666.1/ NMB0622	*lolA*	ANW71222.1	99
LOLB	NP_273914.1/ NMB0873	*lolB*	ANW71457.1	99
LOLF	NP_274259.1/ NMB1235	*lolF*	ANW71760.1	99
LOLD	NP_274258.1/ NMB1234	*lolD*	ANW71759.1	97
SLAM	NP_273362.1/ NMB0313	*Slam*	ANW70408.1	99
		SEC TRANSLOCON		
SECA	NP_274543.1/ NMB1536	*secA*	ANW70027.1	99
SECB	NP_274788.1/ NMB1789	*secB*	ANW71093.1	100
SECD	NP_273651.1/ NMB0607	*secD*	ANW71209.1	99
SECE	NP_273183.1/ NMB0125	*secE*	ANW70781.1	100
SECG	NP_274884.1/ NMB1888	*secG*	ANW70989.1	99
SECF	NP_273652.1/ NMB0608	*secF*	ANW71210.1	99
SECY	NP_273220.1/ NMB0162	*secY*	ANW70814.1	100

Figure 5. 3- Protein sequence alignment of FHbp from MC58 against L91543

Alignment generated by SnapGene v4.3.11. SNPs 1 and 2 of L91543 FHbp SP are shown. Consensus sequences are shown at the top. Highlighted in yellow amino acids that don't match.

To investigate phenotypic differences in FHbp further, the FHbp expression levels of both strains were evaluated by WB and immuno-dot blotting with the monoclonal antibody JAR4 at different ODs. To investigate FHbp expression at different stages of growth, cells were harvested at OD_{600} 0.1, 0.5 and 1. Despite expressing FHbp at high levels at late OD_{600} as observed after WB with WCL, strain L91543 failed to express FHbp on the surface of the cell (Fig 5.4) at this OD, as well as at the earlier ODs. This result shows FHbp is present in L91543, but this strain fails to localise it to the surface, retaining it within the cell and thus failing to either sort FHbp to the OM and/or surface localise it.

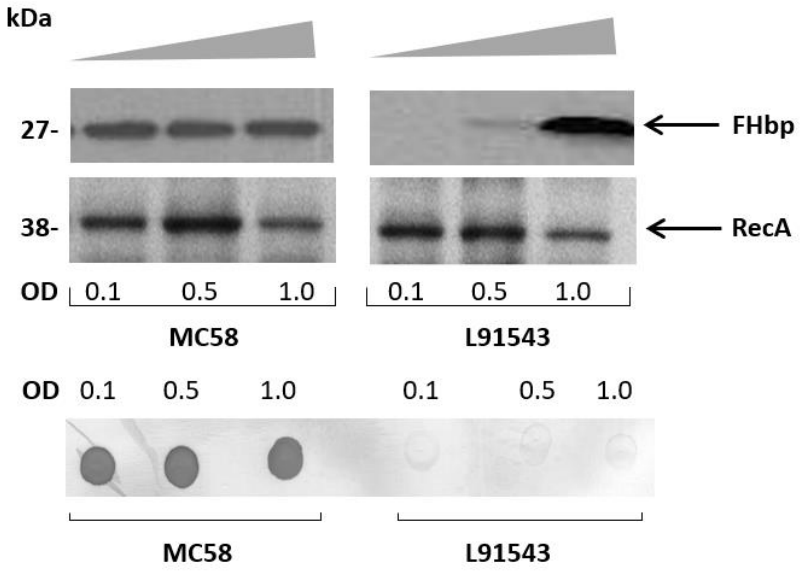


Figure 5. 4- FHbp expression phenotypes of MC58 and L91543 in different growth phases

Different growth phases of broth cultures of MC58 and L91543 analysed by WCL WB (upper panel), including anti-RecA antibody to verify loading

control, and WC suspensions immuno-dot blot (lower panel), representative of 3 independent experiments.

To crudely test if strain L91543 was able to translocate and surface expose FHbp, MC58 *fHbp* was amplified using primers PacI-fHbp-for and PmeI-fHbp-rev (table 2.5) and cloned into the Neisseria complementation vector pGCC4 at the *PacI* and *PmeI* restriction sites. *E. coli* transformants were selected on kanamycin and successful cloning of MC58 *fHbp* into pGCC4 verified by PCR using primers PacI-fHbp-for and PmeI-fHbp-rev, and DNA sequencing with pGCC4-fwd and pGCC4-rev (Fig 5.5a and appendix III). This vector was named pGCC4*fHbp*MC58 and subsequently used to transform strain L91543. Successful Nm transformants were selected on erythromycin and designated L91543*fHbp*MC58. Transformation of MC58*fHbp* into L91543 was verified by PCR with primers pGCC4-fwd and pGCC4-rev (Table 2.5 and Fig 5.5b).

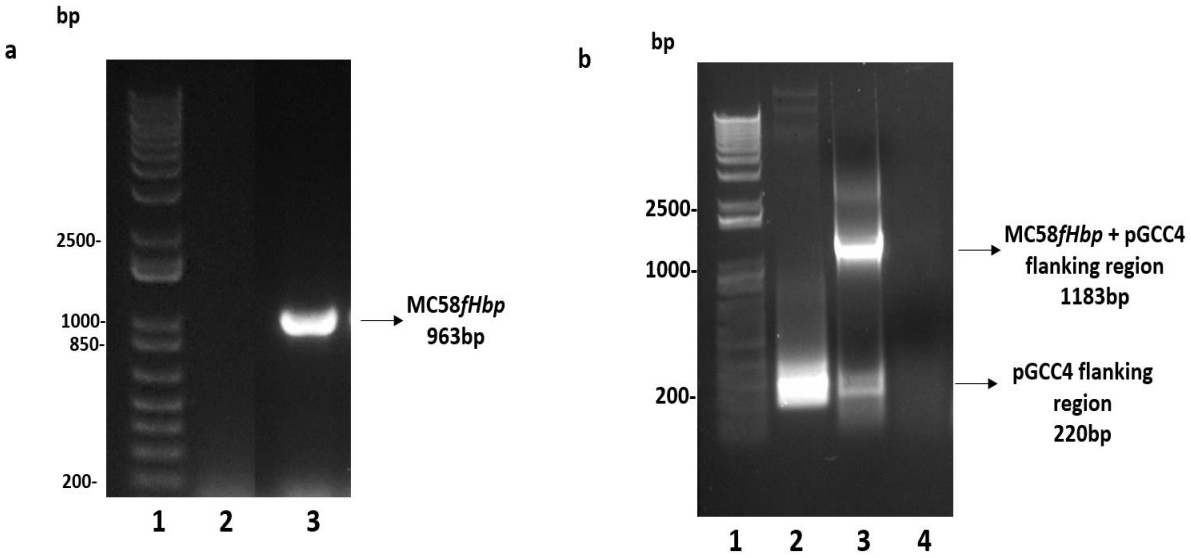


Figure 5. 5- PCR verification of pGCC4*fHbp*MC58

a. PCR amplification of MC58 *fHbp* using primers PacI-fHbp-for and PmeI-fHbp-rev to verify successful cloning of MC58 *fHbp* into pGCC4. Sequencing was performed with flanking primers pGCC4-fwd and pGCC4-rev. Lanes; 1, ladder; 2, no DNA negative control; 3, MC58 *fHbp* in pGCC4 (963bp). **b.** PCR amplification of L91543*fHbp*MC58 with the flanking primers pGCC4-fwd and pGCC4-rev to verify integration of MC58 *fHbp* in the genome of L91543. Lanes; 1, ladder; 2, pGCC4 empty vector; 3; L91543*fHbp*MC58 displaying a band of 1183bp corresponding to MC58*fHbp* gene (963bp) plus flanking region of 220bp; 4, no DNA negative control.

Restoration of FHbp to the cell surface was observed (Fig 5.6) suggesting that the pathway for translocation and sorting in L91543*fHbp*MC58 is functional in L91543 and the inability to process FHbp is due to the sequence of the L91543 FHbp protein itself.

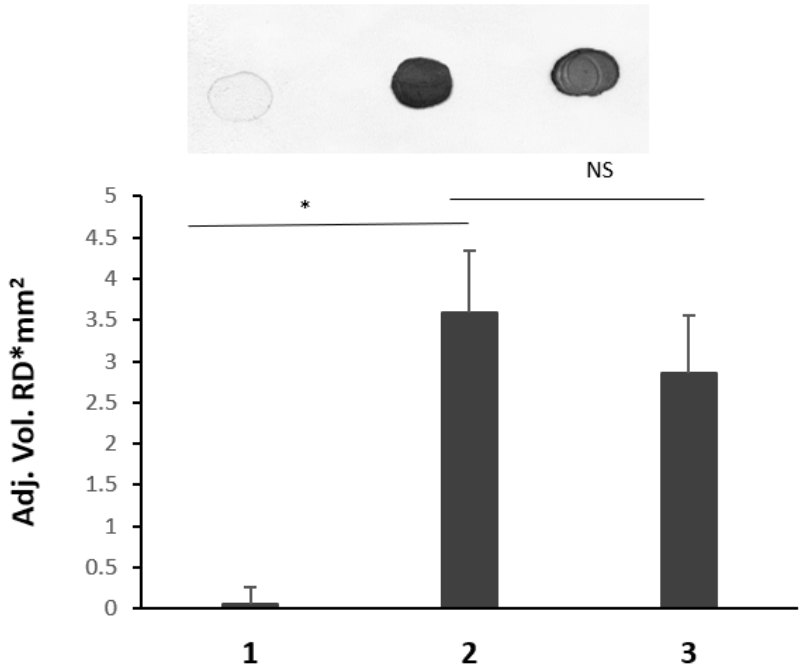


Figure 5. 6- Restoration of FHbp surface localisation in L91543*fHbp*MC58

WC suspensions tested by immuno-dot blot with JAR4. Lanes; 1, L91543; 2, MC58; 3, L91543*fHbp*MC58. Data are representative of 5 independent experiments. The reflective density was measured by a GS-800™ calibrated densitometer. The one-way ANOVA followed by Dunnett's test was employed for statistical analysis in GraphPad v6. All columns represent mean ± SEM, *p<0.05 vs strain MC58.

Upon immunoblotting of WCLs, both species of FHbp were shown to be present in L91543*fHbp*MC58 (Fig 5.7) providing further evidence that in L91543 the FHbp sequence alone was responsible for the failure to cleave and export FHbp.

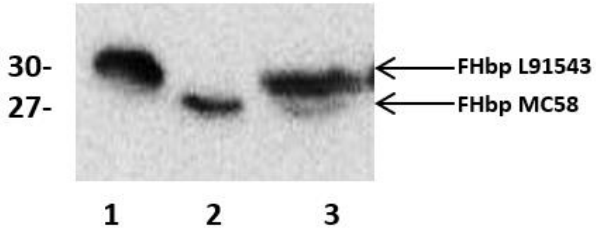


Figure 5. 7- Both MC58 and L91543 FHbp can be observed in L91543*fHbp*MC58

WB. Lanes; 1, L91543; 2, MC58; 3, L91543*fHbp*MC58. Data are representative of 5 independent experiments.

5.3- SNPs in the SP of FHbp affect its processing and translocation

To investigate the combined and individual contributions of each L91543 SP SNP on FHbp processing and translocation, first the *fHbp* gene was deleted in L91543, and designated L91543Δ*fHbp*. This strain was then complemented with *fHbp* of L91543 or derivatives with corrected SNP and their respective FHbp expression profiles were compared.

5.3.1- Deletion of *fHbp* in L91543

Gene Splicing by Overlap Extension (gene SOEing) was used to create a fusion PCR product to replace *fHbp* in L91543 with the kanamycin resistance gene (*kanR*) from EZ::Tn5<KAN-2> insertion kit (Epicentre) following the approach described by Horton (1995) and also described in section 2.5.10.

Initially, in the first round of PCRs, homology arms (HA1 and HA2) of approximately 600 bp flanking *fHbp* were amplified from genomic DNA of strain L91543 using HA1_FHbp_Fwd and HA1_FHbp_Rev, and HA2_FHbp_fwd and HA2_FHbp_Rev, and the kan gene amplified using primers Kan_fwd and Kan_Rev (Fig 5.8; table 2.5). The HA1 and kan products obtained were gene-cleaned then used as template for the second round of PCR with primers HA1_FHbp_Fwd and Kan_Rev and the annealed product generated cleaned and used as template along with the HA2 PCR product for a third round of PCR with primers HA1_FHbp_Fwd and HA2_FHbp_Rev. The final PCR product generated containing HA1-

kan-HA2 was gel extracted, gene cleaned and sequenced for confirmation (Fig 5.8, see appendix III for sequences).

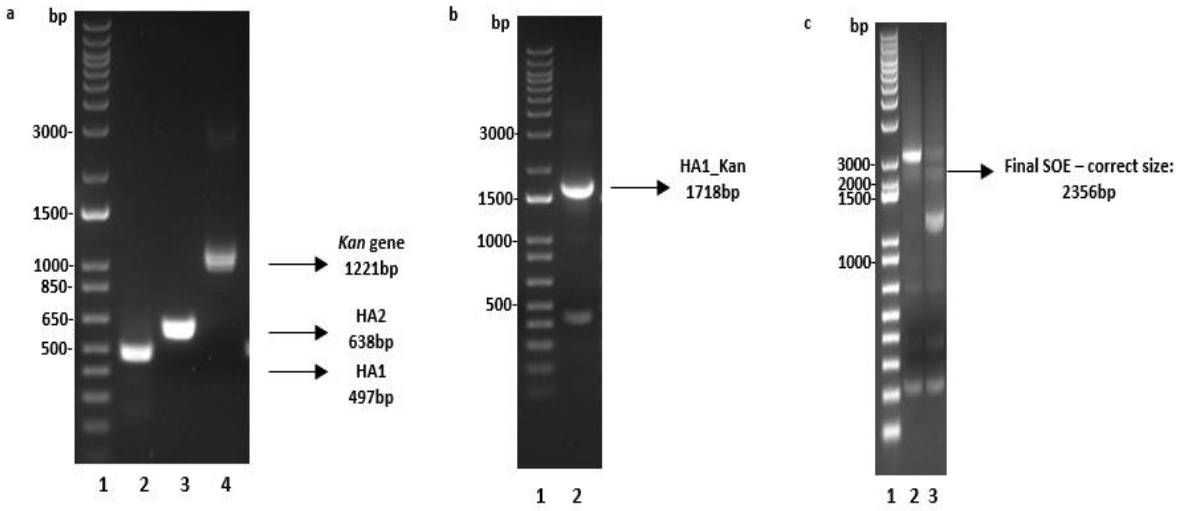


Figure 5. 8- PCR amplification steps involved in the deletion of *fHbp* in L91543

a. First round of PCR amplification is shown. Lanes; 1, ladder; 2, HA1 upstream of *fHbp* (497bp); 3, HA2 downstream of *fHbp* (638bp); 4, *kan* gene (1221bp). **b.** Second round of PCR amplification using HA1 and *kan* products as template for this round. Lanes; 1, ladder; 2, HA1_kan product (1718bp). **c.** Final round of PCR amplification. HA1_kan and HA2 were used as template to generate the last PCR product to be used for transformation. Lanes; 1, ladder; 2 and 3, attempts of incorporating HA2 to HA1_kan, respectively. Right size bands of the product HA1-kan-HA2 (2356bp) were excised, gene cleaned and sent for sequencing for verification before transformation into L91543.

The verified SOE construct was used to transform L91543 strain with selection on kan. Deletion mutants were confirmed by PCR, WGS and WB (Fig 5.9).

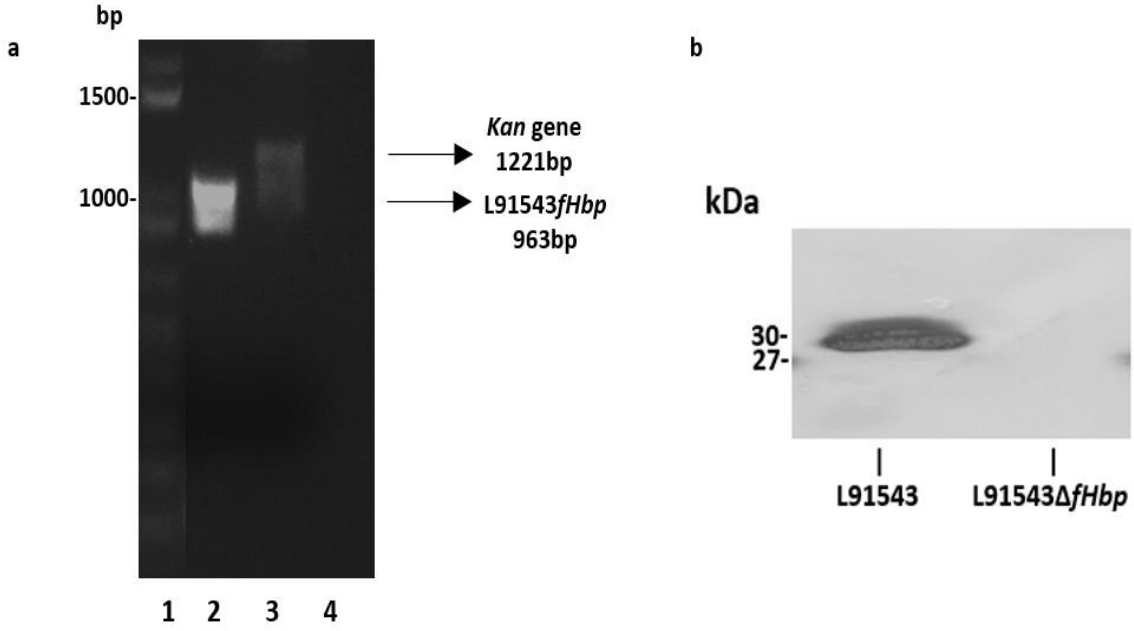


Figure 5. 9- Deletion of *fHbp* in L91543 by SOE

a. A representative agarose gel of the PCR amplification to verify L91543Δ*fHbp*. Lanes; 1, ladder; 2, *fHbp* of L91543 (963bp) as positive control; 3, *kan* gene (~1200bp) of L91543Δ*fHbp* amplified with kan_fwd and kan_rev primers; 4, *fHbp* deletion checked with *fHbp* primers, PacI-fHbp-for and PmeI-fHbp-rev. **b.** Western immunoblotting of WCL of L91543 and L91543Δ*fHbp* with JAR4.

5.3.2- SDM of L91543 *fHbp* in pGCC4*fHbp*L91543 vector

After cloning *fHbp* of L91543 into the vector pGCC4 similarly to the way used to clone MC58*fHbp* (Fig 5.10), this plasmid, designated pGCC4*fHbp*L91543, was used as template for Site directed mutagenesis (SDM). SDM was used to mutate the SNPs in the SP of L91543 FHbp to the corresponding MC58 amino acids.

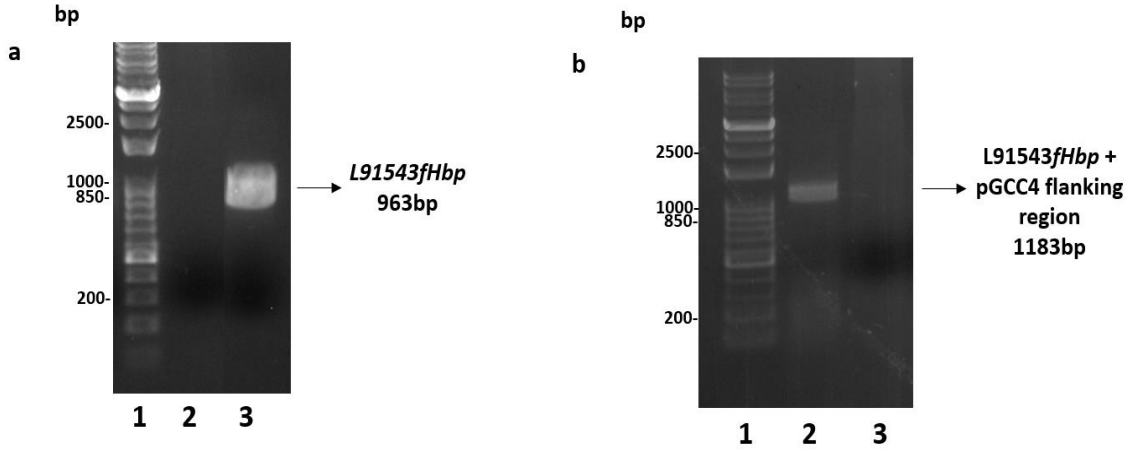


Figure 5. 10- PCR amplification of L91543*fHbp* to verify correct construction of pGCC4*fHbp*L91543

a. Initial PCR amplification of L91543*fHbp* using primers PacI-fHbp-for and PmeI-fHbp-rev. Lanes; 1, ladder; 2, no DNA negative control; 3, L91543*fHbp* (963bp). **b.** PCR verification of pGCC4*fHbp*L91543 with the flanking primers pGCC4-fwd and pGCC4-rev to verify correct cloning of L91543 into pGCC4. Lanes; 1, ladder; 2, pGCC4*fHbp*L91543 vector. The band shown corresponds to 1183bp of L91543*fHbp* gene (963bp) plus flanking region of pGCC4 backbone (220bp); 3, no DNA negative control.

SNP1 and SNP2 were corrected to resemble the amino acids found in the SP of FHbp of MC58 using SDM. Primers SP1_fwd and SP2_rev (table) were used to substitute phenylalanine (F) with leucine (L) at position 15 (SNP1) and primers SP2_fwd and SP2_rev (table) to substitute alanine (A) with threonine (T) at position 19 (SNP2) of L91543's SP (Fig 5.11). After SDM and transformation with the SDM product, several *E. coli* transformants were selected for verification. The verified constructs were designated pGCC4L*fHbp*SNP1 and pGCC4L*fHbp*SNP2 (appendix III). It was difficult to repair both SNPs by SDM. Instead, the PacI-PmeI fragment of pGCC4*fHbp*L91543 was commercially synthesized (Life Technologies) with the 2 SNPs repaired and the DNA cloned into the PacI-PmeI sites of pGCC4 to create pGCC4L*fHbp*SNP1+2 (appendix V). The construct was verified by sequencing with pGCC4-specific primers (Table 2.5 and appendix V).

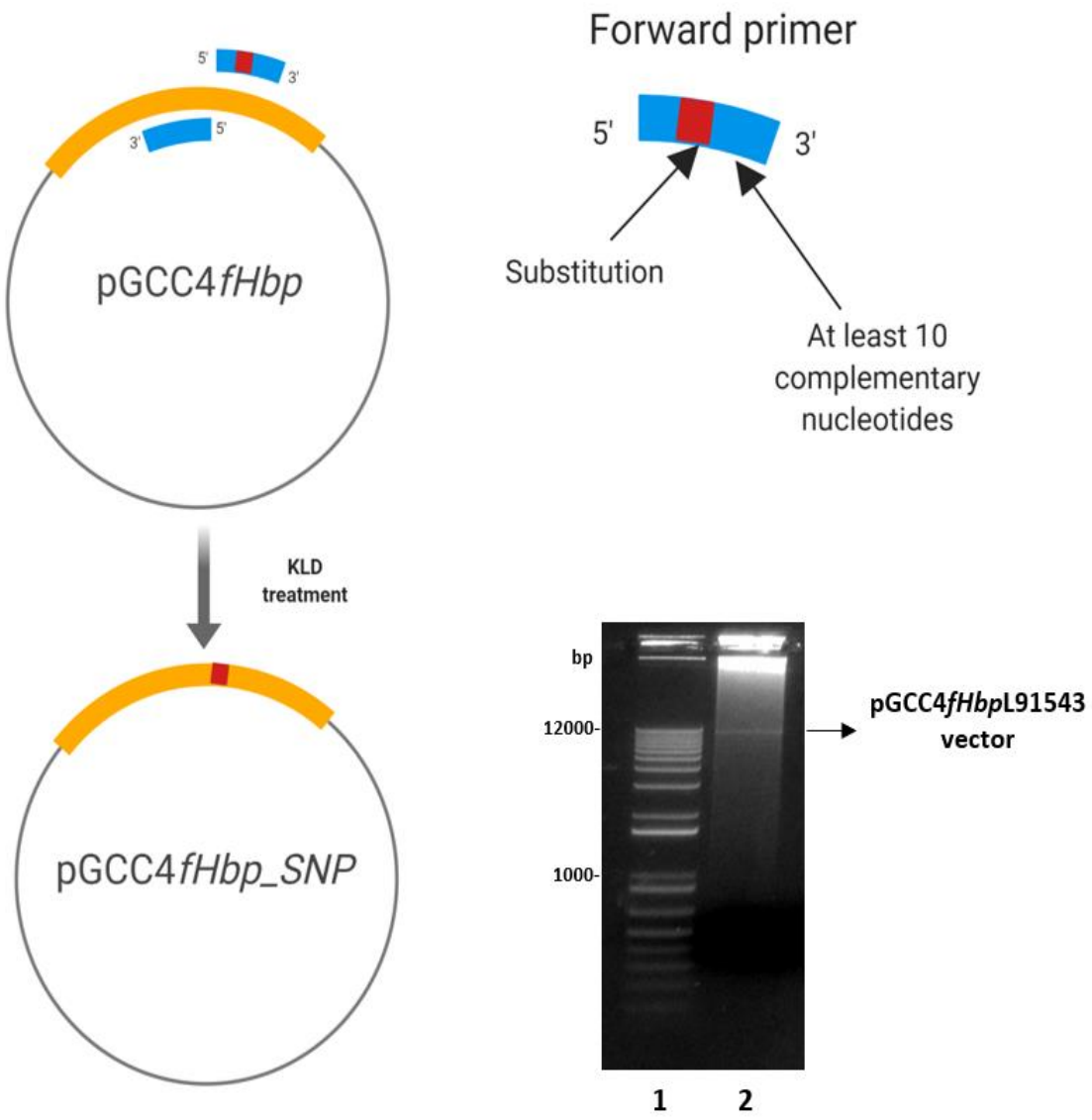


Figure 5. 11- SDM of L91543 *fHbp*

Schematic figure of SDM. Forward primer contains the nucleotide sequence that will substitute the sequence of interest. PCR is performed and full plasmid is amplified, KLD treated and used to transform *E. coli* (see Methods section 2.5.9 for full procedure). Several colonies were screened for correct mutation by DNA sequencing. Also shown is a representative agarose gel of amplified vector after SDM reaction. Lanes; 1, ladder; 2, mutated vector.

Strain L91543Δ*fHbp* was transformed with pGCC4*fHbp*L91543 to create L91543Δ*fHbp*L*fHbp* with no SNP corrections as a negative control. Strain L91543Δ*fHbp* was transformed with pGCC4L*fHbp*SNP1 to generate recombinant strain L91543Δ*fHbp*+L*fHbp*SNP1, with pGCC4L*fHbp*SNP2 to generate strain L91543Δ*fHbp*+L*fHbp*SNP2 or with pGCC4L*fHbp*SNP1+2 to create strain L91543Δ*fHbp*+L*fHbp*SNP1+2 (Fig 5.12). Strains were checked with pGCC4-specific primers (table 2.5).

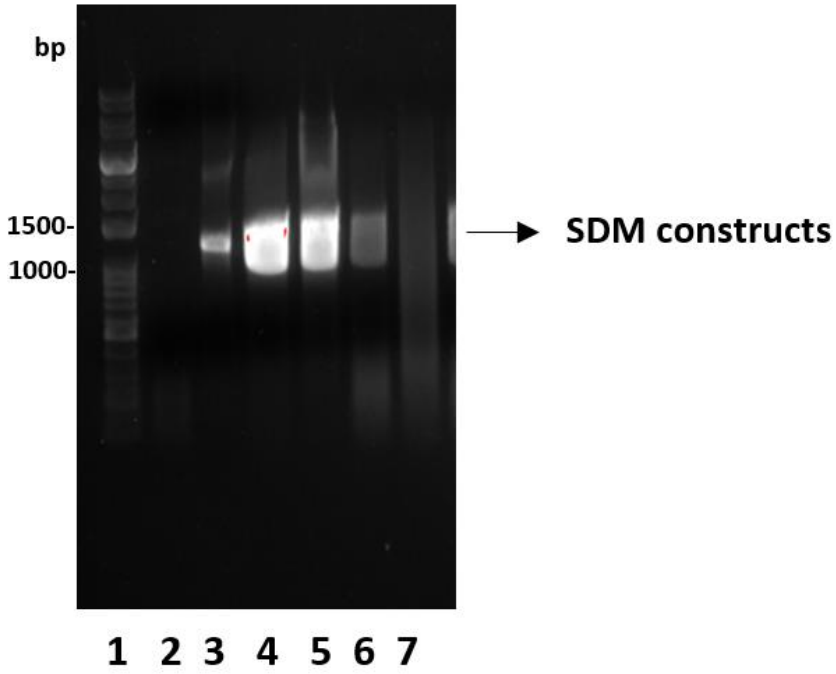


Figure 5. 12- Verification of L91543Δ*fHbp*L*fHbp*, L91543Δ*fHbp*+L*fHbp*SNP1, L91543Δ*fHbp*+L*fHbp*SNP2 and L91543Δ*fHbp*+L*fHbp*SNP1+2 by PCR

Lanes; 1, ladder; 2, no DNA negative control; 3, L91543Δ*fHbp*+L*fHbp*; 4, L91543Δ*fHbp*+L*fHbp*SNP1; 5, L91543Δ*fHbp*+L*fHbp*SNP2; 6,

L91543Δ*fHbp*+L*fHbp*SNP1+2; 7, negative control L91543 genomic DNA. Expected band size for constructs is the same 1163bp.

5.3.3- SP SNPs in FHbp prevent cleavage, but not surface localisation

To investigate the individual and combined contribution of the 2 SNPs in SP cleavage and surface localisation, WB and immuno-dot blotting were performed for the recombinant strains of L91543.

L91543Δ*fHbp*+L*fHbp*SNP1 and L91543Δ*fHbp*+L*fHbp*SNP2 showed moderate levels of surface expression of FHbp whereas L91543Δ*fHbp*+L*fHbp*SNP1+2 displayed similar surface localisation to MC58 (Fig 5.13). WB suggested no cleavage of the SP in L91543Δ*fHbp*+L*fHbp*SNP1 and L91543Δ*fHbp*+L*fHbp*SNP2 since they displayed the lower mobility like that of L91543 compared with MC58 FHbp and strain L91543Δ*fHbp*+L*fHbp*SNP1+2 (Fig 5.13). The higher mobility for the FHbp expressed by L91543Δ*fHbp*+L*fHbp*SNP1+2 suggested the SP had been cleaved. Surface expression of FHbp for this strain was similar to MC58.

This result suggests both AA substitutions impact on the FHbp translocation-processing pathway at some point up to, or at, the SP cleavage step and surprisingly that repair of either SNP individually can permit partial localisation of FHbp to the cell surface without the need for prior SP cleavage. Furthermore, this result corroborates the initial hypothesis that the SP is influencing FHbp surface exposure.

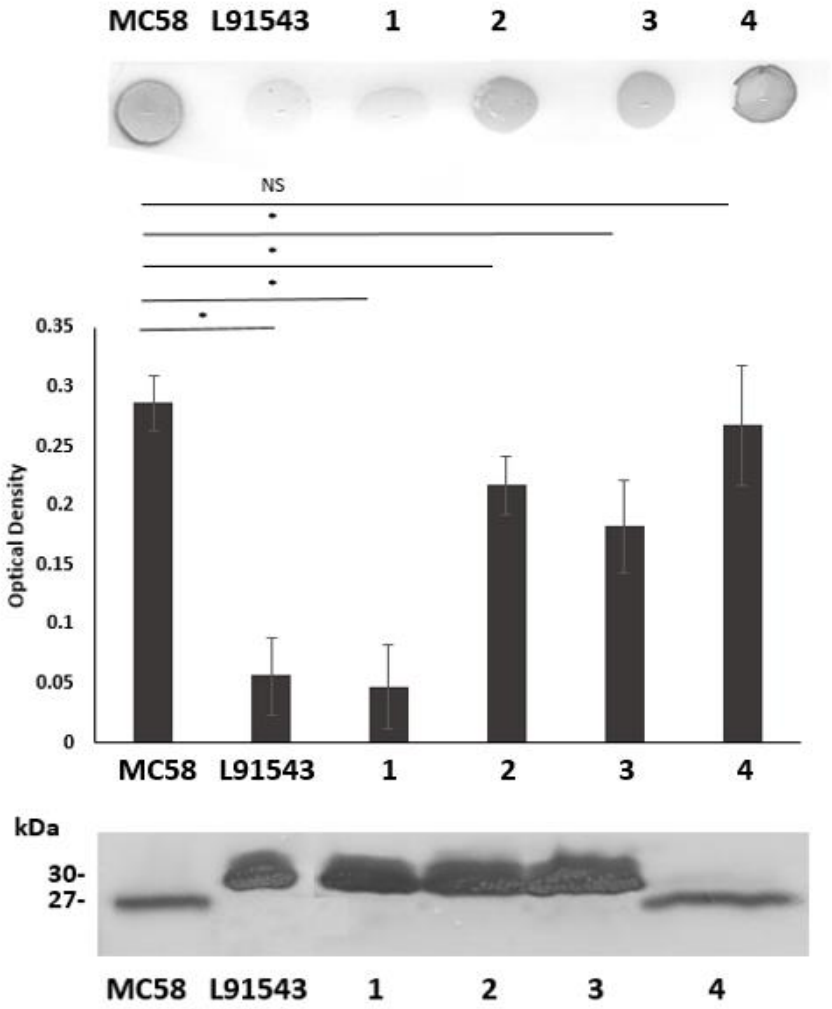


Figure 5. 13- SP SNPs in FHbp prevent cleavage but not surface localisation

Whole cell suspensions and WCL from fresh plate cultures analysed by immuno-dot blot (upper panel) and Western immunoblot (lower panel) respectively with JAR4. Lanes; 1, L91543Δ*fHbp*+L*fHbp*; *2,* L91543Δ*fHbp*+L*fHbp*SNP1; 3, L91543Δ*fHbp*+L*fHbp*SNP2; 4, L91543Δ*fHbp*+L*fHbp*SNP1+2, representative of 3 independent experiments. The density of spots was measured by ImageJ and one way ANOVA followed by Dunnett's test was employed for statistical analysis in GraphPad v6. All columns represent mean ± SEM, *p<0.05 vs strain MC58.

5.4- FHbp with SP SNPs show reduced binding to SecA

Sec-dependent proteins are translocated to the periplasm via the translocase SecYEG-SecA machinery (Tsirigotaki *et al.*, 2017). Most of the proteins destined for the OM employ this pathway to reach their final destination. The essential ATPase motor SecA binds to the SP of preproproteins on the ribosome or binds to the whole unfolded protein in the cytoplasm in order to move them to SecYEG (Bauer *et al.*, 2014). Allosterically, SecA interacts with the SP at the N-terminus of preproproteins. Whilst the hydrophobic region of SPs gives the affinity SecA requires for intermolecular interactions with the nascent protein, the positively charged acidic residues of the SP n-region permit the electrostatic trapping of the preprolipoprotein (Gelis *et al.*, 2007, Gouridis *et al.*, 2009).

Due to its importance in the translocation of OM proteins, we decided to investigate SecA binding to FHbp SPs of both strains MC58 and L91543 using a bacterial 2-hybrid approach. We hypothesised that the SNPs in L91543 SP would affect SecA binding. The approach employed here relied on induced expression of FHbp and SecA using *E. coli* as the host organism.

5.4.1- Cloning of *fHbp* and *secA* for bacterial 2-hybrid

As described in methods section 2.5.14, the protein–protein interaction of MC58 FHbp and L91543 FHbp with SecA was investigated using the Bacterial Adenylate Cyclase Two-Hybrid System (BACTH) Kit (Euromedex). According to the BACTH Euromedex manufacturer's manual, the system exploits the fact that the catalytic domain of adenylate cyclase (CyaA) from *Bordetella pertussis* made of T25 and T18 subunits are not active when physically separated (Ladant and Ullmann, 1999). When these two fragments are fused to interacting polypeptides, X and Y, heterodimerization of these hybrid proteins results in functional complementation between T25 and T18 fragments resulting in cAMP synthesis. Proteins Y and X in this study represent FHbp of MC58 and L91543, each separately cloned into the T18-encoding plasmid, and SecA from MC58 cloned into the T25-encoding plasmid. SecA of L91543 is 99% identical at the amino acid level to SecA of MC58 (Table 5.1). The reconstituted chimeric enzyme produces cyclic AMP. The newly produced AMP then binds to the catabolite activator protein, CAP (Karimova *et al.*, 1998). The cAMP/CAP complex is a pleiotropic regulator of gene transcription in *E. coli* which will switch on the expression of genes in the *lac* and *mal* operons involved in lactose and maltose catabolism (Fig 5.14). Thus, bacteria containing interacting T18-T25 hybrid proteins become able to use the carbohydrates lactose or maltose as carbon source and can be easily distinguished on indicator or selective media, such as IPTG-MacConkey used in this study.

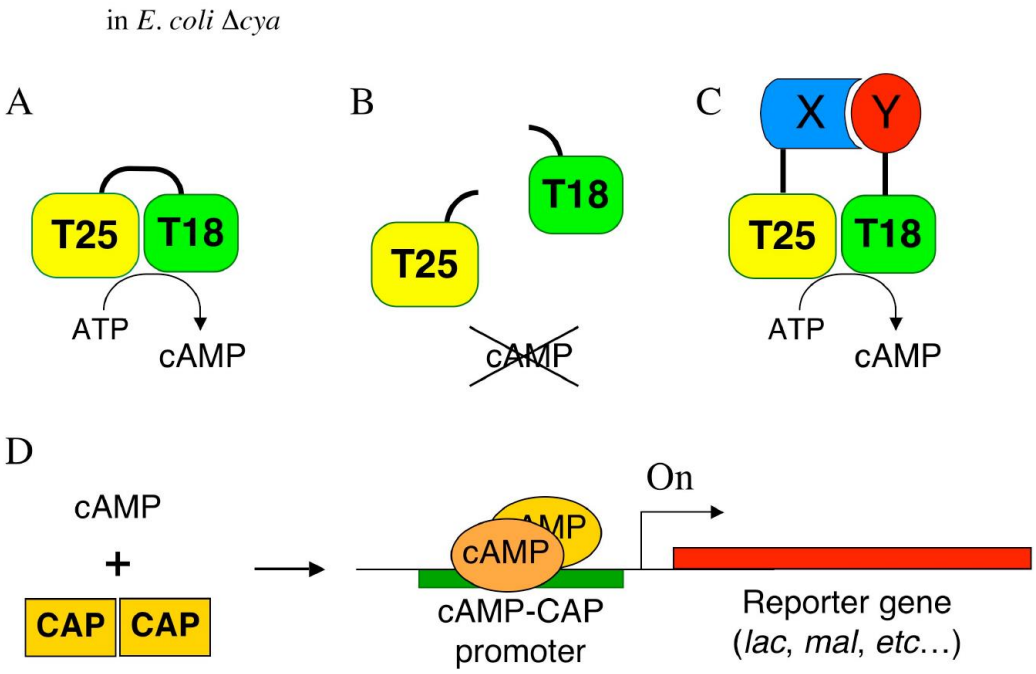


Figure 5. 14- Schematic figure of the bacterial two-hybrid system

The catalytic domain of adenylate cyclase (CyaA) from *B. pertussis* consists of two fragments, T25 and T18 (Fig 5.14A), that complement each other and are not active when spatially separated (Fig 1B). When fragments T25 and T18 are fused to interacting polypeptides, X and Y, heterodimerization of these hybrid proteins results in complementary functional activity between these fragments and, in consequence, cAMP synthesis (Fig 1C). CAP binds to the produced Cyclic AMP. The cAMP/CAP complex is a regulator of gene transcription in *E. coli.* It turns on the expression of several genes, including genes of the *lac* and *mal* operons involved in lactose and maltose catabolism (Fig 1D), which means *E. coli* can now utilise lactose and maltose as carbon source. Interaction between hybrid proteins means higher β-galactosidase activity (image and adapted legend from Euromedex BACTH manufacturer manual).

Firstly, *fHbp* from MC58 and L91543 were PCR-amplified with primers BamHIfwd_MC58FHbp and EcoRIrev_MC58FHbp, and BamHIfwd_L91543FHbp and EcoRIrev_L91543FHbp, respectively (Table 2.5), and the PCR products cloned separately into vector pUT18 (Fig 5.15a), these plasmids were named pUT18_*fHbp*MC58 and pUT18_*fHbp*L91543. The gene encoding SecA from MC58 was PCR-amplified using primers PstIfwd_SecA and SmaIrev_SecA (Table 2.5), and then cloned into vector pKT25 (Fig 5.15a), this plasmid was named pKT25_*secA*. The appropriate prey (pKT25-based construct) and the equivalent concentration of appropriate bait (pUT18-based construct) were co-transformed into *E. coli* BTH101 cells and plated on IPTG-MacConkey and appropriate antibiotics. Bacteria expressing interacting hybrid proteins formed pink/purple colonies, whilst cells expressing non-interacting proteins remained white/light pink. As a positive interaction control, a co-transformant containing commercial pKT25-zip and pUT18-zip constructs was used. Co-transformants containing empty vector pKT25 and/or pUT18 were used as negative controls. Pink colonies were isolated and grown in LB broth and plasmid DNA extracted for verification by PCR and sequencing (Fig 5.15b, see appendix III for sequences).

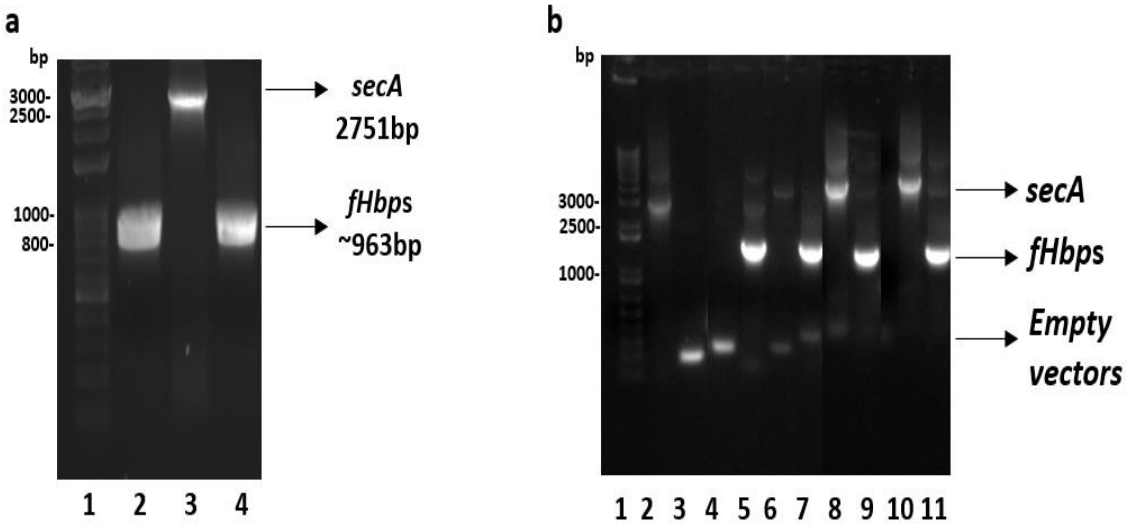


Figure 5. 15- Cloning of *fHbp* of MC58 and L91543 and *secA* of MC58 for bacterial 2-hybrid

a. Initial PCR amplification of *fHbp* of MC58 and L91543, (~963bp) and *secA* of MC58 (2751bp). Lanes; 1, ladder; 2, MC58 *fHbp*; 3, MC58 *secA*; 4, L91543 *fHbp*. **b.** PCR amplification of pKT25_*secA* and pUT18_*fHbp* construct combinations to verify *E. coli* transformed with both plasmids. Sequencing primers specific for pKT25 (T25F and T25R) and pUT18 (T18F and T18R) (Table 2.5) were used. Lanes; 1, ladder; 2 and 3, pair pKT25_*secA* and empty pUT18; 4 and 5, pair empty pKT25 and pUT18_MC58*fHbp*; 6 and 7, pair empty pKT25 and pUT18_L91543*fHbp*; 8 and 9, pair pKT25_*secA* and pUT18_MC58*fHbp*; 10 and 11, pair pKT25_*secA* and pUT18_L91543*fHbp*.

5.4.2- ß-galactosidase assay

After successful cloning and transformation with the appropriate construct combinations, a ß-galactosidase assay was performed to evaluate the level of interaction between FHbps and SecA. This assay showed a significant 1.9-fold lower binding of SecA to L91543 FHbp when compared to MC58 FHbp (Fig 5.16). This result suggested that the SP SNPs in the FHbp of L91543 may influence recognition by SecA for subsequent translocation.

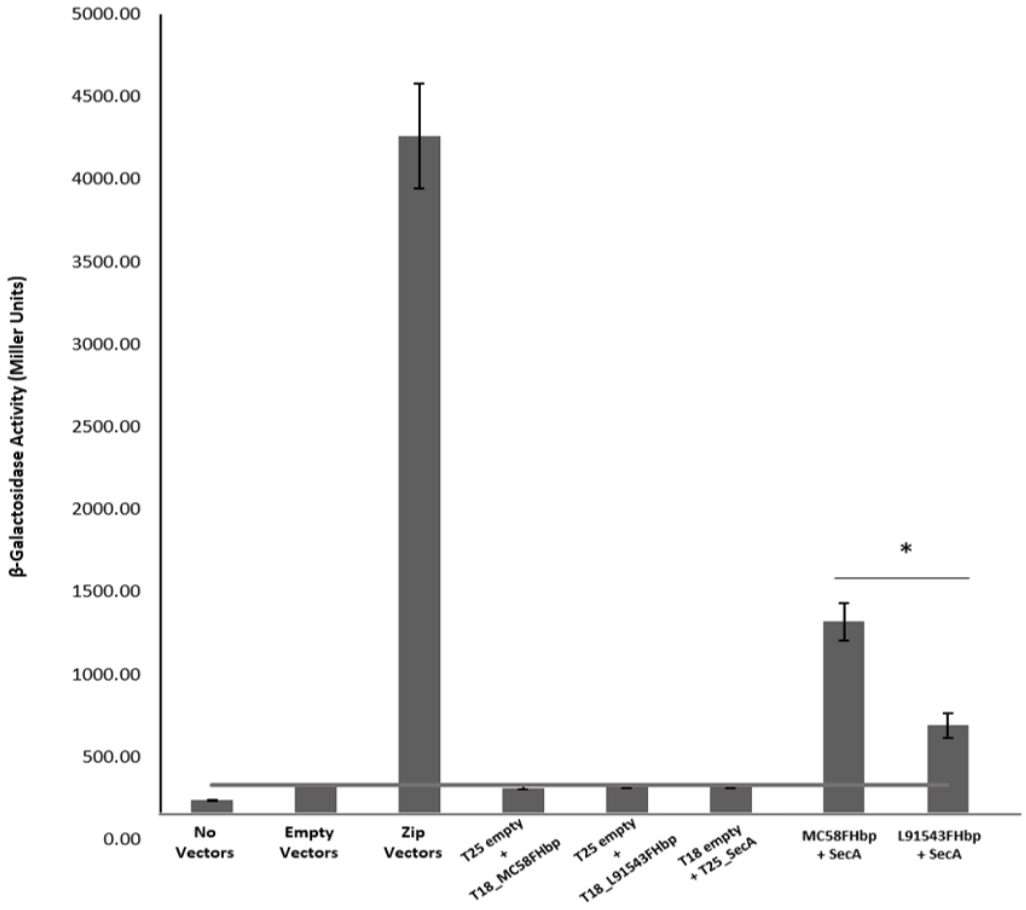


Figure 5. 16- FHbp from L91543 shows reduced binding to SecA compared to MC58 FHbp

Bacterial 2 hybrid experiments to compare MC58 and L91543 FHbp binding to SecA. The *fHbp* gene of strains MC58 and L91543 and *secA* gene of MC58 were cloned into the two-hybrid pUT18 (T18-prey) or pKT25 (T25-bait) vectors, respectively and the appropriate prey and bait pair of vectors were co-transformed in BTH101. In parallel, zip prey and zip bait vectors provided in the BACTH kit were co-transformed in BTH101 as a positive control for protein-protein interaction and likewise, different pray-bait combinations but with one vector empty were used as negative controls. The interactions were quantified by measuring the corresponding ß-

galactosidase levels expressed in Miller units (values of 4 independent clones). Unpaired T Tests with Welsch's correction were conducted to compare the interactions. All columns represent mean ± SEM, *p<0.05 vs strain MC58.

5.5- Clinical isolates with FHbp SP SNPs show SP retention yet surface localization

Before further investigating the impact of FHbp SP SNPs on the canonical pathway for lipoprotein processing and surface localisation; it was important to ascertain if the L91543 FHbp SP SNPs occurred rarely in meningococci, or whether these SNPs were highly prevalent in invasive isolates. The Meningitis Research Foundation (MRF) Meningococcus Genome Library database of invasive isolates collected in the UK between 2003-2017 was therefore interrogated by Dr Neil Oldfield. Surprisingly, only 9% of the 1,895 *fHbp*-containing capsule group B isolates encoded a FHbp SP with an identical sequence to MC58. 48% of isolates had only SNP2, 23% had both SNPs 1 and 2, and 17% had SNP2 plus an additional SNP, alanine substituted to threonine, called SNP3 in their SP. A small percentage (3%) had other SNPs (Table 5.2). On the basis of their FHbp SP SNPs, the strains were classified into four classes (Table 5.2).

Table 5. 2- Frequency of different FHbp SP classes in serogroup B isolates in the MRF Meningococcus Genome Library database. Exception made to include strain L91543 which is part of serogroup C.

The n-region of the SP is shown in burgundy, h-region in green and lipobox in red.

SP Class	SP sequence	No. of isolates (%) n = 1895	Isolates used in this study
1 (NO SNPs)	MTRSKPVN R TAFCCLSLTTALI LTAC	179 (9.4)	MC58 and 1 - 4
2 (SNP1 and SNP2)	MTRSKPVN R TAFCCFSLTAALI LTAC SNP1 SNP2	435 (22.9)	L91543 and 10 - 13
3 (SNP2)	MTRSKPVN R TAFCCLSLTAALI LTAC SNP2	904 (47.7)	5 – 9
4 (SNP2 and SNP3)	MTRSKPVN R TTFCCLSLTAALI LTAC SNP3 SNP2	317 (16.7)	14 – 18
Other SNPs		60 (3.2)	

To test whether there was a correlation between SNP class and phenotype, 5 strains from each class were analysed by WB and WC immunodot blotting to determine the size and surface localisation of FHbp. Except for strains L91543, MC58 and H44/76 (which has identical AA FHbp protein sequence to MC58), the isolates were randomly selected from a sub-panel of 79 serogroup B Nm isolates from the MRF genome library database which were used in a previous study (Cayrou *et al*., 2018). Strain MC58 and isolates 1 to 4 represented Class 1; strain L91543 and isolates 10 to 13 represented Class 2; isolates 5 to 9 represented Class 3; and finally isolates 14 to 18 represented Class 4 (Table 5.2).

WB of WCLs with the mAb JAR4 revealed lower mobility of FHbp for all classes except for Class I (Fig 5.17a). This result pointed to the retention of SP when SNPs are present. All isolates tested express a FHbp variant 1, subfamily B and are predicted to bind to JAR4 since they present JAR4 epitope, as shown in appendix VII and VIII. However, isolate 4 exhibited no binding to JAR4 and was tested with a polyclonal FHbp antibody kindly provided by Dr Christoph Tang.

Immuno-dot blotting of these strains surprisingly showed that most isolates display FHbp on the surface despite having at least one SNP in their SP (Fig 5.17b). An overall 2.2-fold reduction in FHbp surface localisation was observed when comparing all pooled non-class 1 isolates with pooled class 1 (excluding isolate 4). Exceptions to surface localisation of FHbp include isolates 12 and 13. Both isolates belong to Class 2 (like strain L91543). The reasons for this are explored later on in this thesis.

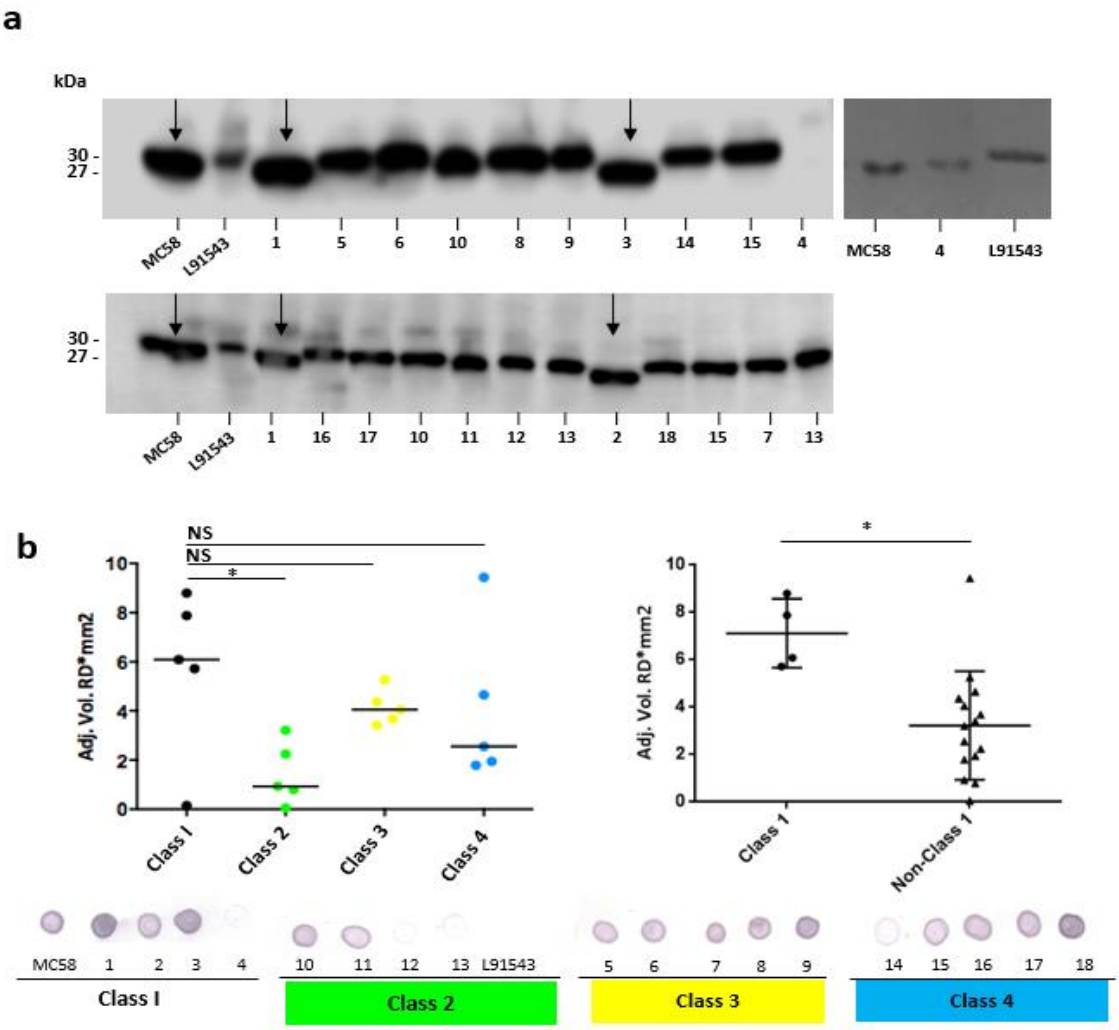


Figure 5. 17- Comparison of FHbp size and surface localisation in clinical isolates

For all non-class 1 isolates, the SP is uncleaved, yet for most isolates, FHbp is surface localised. WCL or whole cell suspensions were prepared from fresh plate cultures and JAR4 used except where indicated**. a.** Western immunoblot. For isolate 4, polyclonal anti-FHbp antibody was also used; **b**, Whole cell immuno-dot blot. The density of spots was measured by ImageJ and the one-way ANOVA followed by Dunnett's test employed for statistical analysis in GraphPad v6. All columns represent mean ± SEM, *p<0.05 vs class 1. Pooled data of class 1 (excluding isolate 4) and non-class 1 were

analysed using un-paired T Tests with Welsch's correction. Both columns represent mean ± SEM, *p<0.05 vs class 1.

5.6- FHbp with SP SNPs localise to the surface via Lnt and Slam with escape from processing by Lgt and LspA

Interestingly all strains shown to express uncleaved FHbp have SNP2, T substituted by A, a polar amino acid substituted by a hydrophobic one. Back in 1984, it was postulated that a similar substitution in *E. coli* could be affecting Lpp as early as translocation to the IM or affecting direct recognition of the SP by Lgt or LspA (Vlasuk *et al.*, 1984). This study led us to question whether the meningococcal Lgt and LspA enzymes fail to recognise FHbp with class 2 (SNP1 and SNP2), class 3 (SNP2) and class 4 (SNP2 and SNP3) SPs (Table 5.2) and how these preprolipoproteins localise to the surface.

To test whether Lgt fails to recognise the FHbp SP of non-class 1 isolates, click chemistry was used. One isolate of each class was grown in the presence of palmitic acid alkyne. FHbp was then immunoprecipitated and clicked with biotin azide, then detected using Streptavidin-HRP after SDS-PAGE separation. Only MC58 FHbp was shown to be lipidated using this approach. Strain L91543 (Class 2) and isolates 6 (Class 3) and 18 (Class 4) did not display a band suggesting their FHbp is not lipidated (Fig 5.18). The same samples were probed with JAR4 confirming the presence of FHbp for each strain.

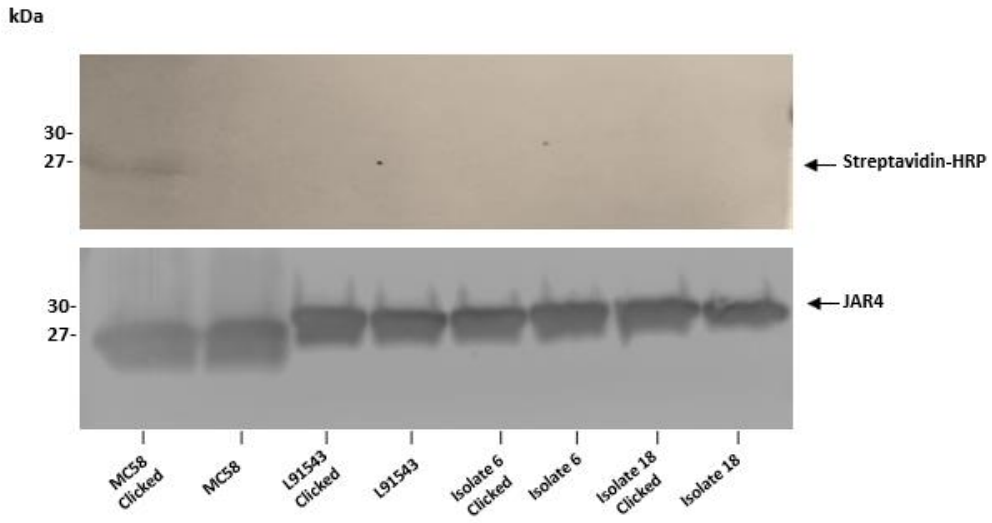


Figure 5. 18- Amino acid changes resulted from SNPs in the FHbp sequence of the SP affect recognition by Lgt

WB after growing strains in palmitic acid alkyne and clicking immuno-precipitated FHbp with biotin-azide; biotin incorporation into FHbp was detected with Streptavidin-HRP (upper panel) and FHbp by JAR4 (lower panel). One representative figure of 5 experiments is shown.

The same 4 isolates were treated with Globomycin, a known inhibitor of LspA function (Vogeley *et al*., 2016). After treatment, surface localisation and FHbp mobilities were investigated by immuno-dot blotting and WB, respectively (Fig 5.19). Immuno-dot blotting showed reduced surface localisation of FHbp for all strains. This was expected since Globomycin is known to have a global effect on the lipoproteome including LolB, directly involved in OM translocation of lipoproteins (Tokuda, 2009; Buddelmeijer, 2017). More interestingly, treatment with Globomycin partially affected LspA recognition of MC58 FHbp (class 1) as inferred by the detection of a second band on the WB, corresponding to the size of uncleaved FHbp. This WB showed that the FHbp of the non-class I isolates was unaffected by Globomycin treatment demonstrating that inhibition of LspA had no effect on FHbp in these isolates (Fig 5.19).

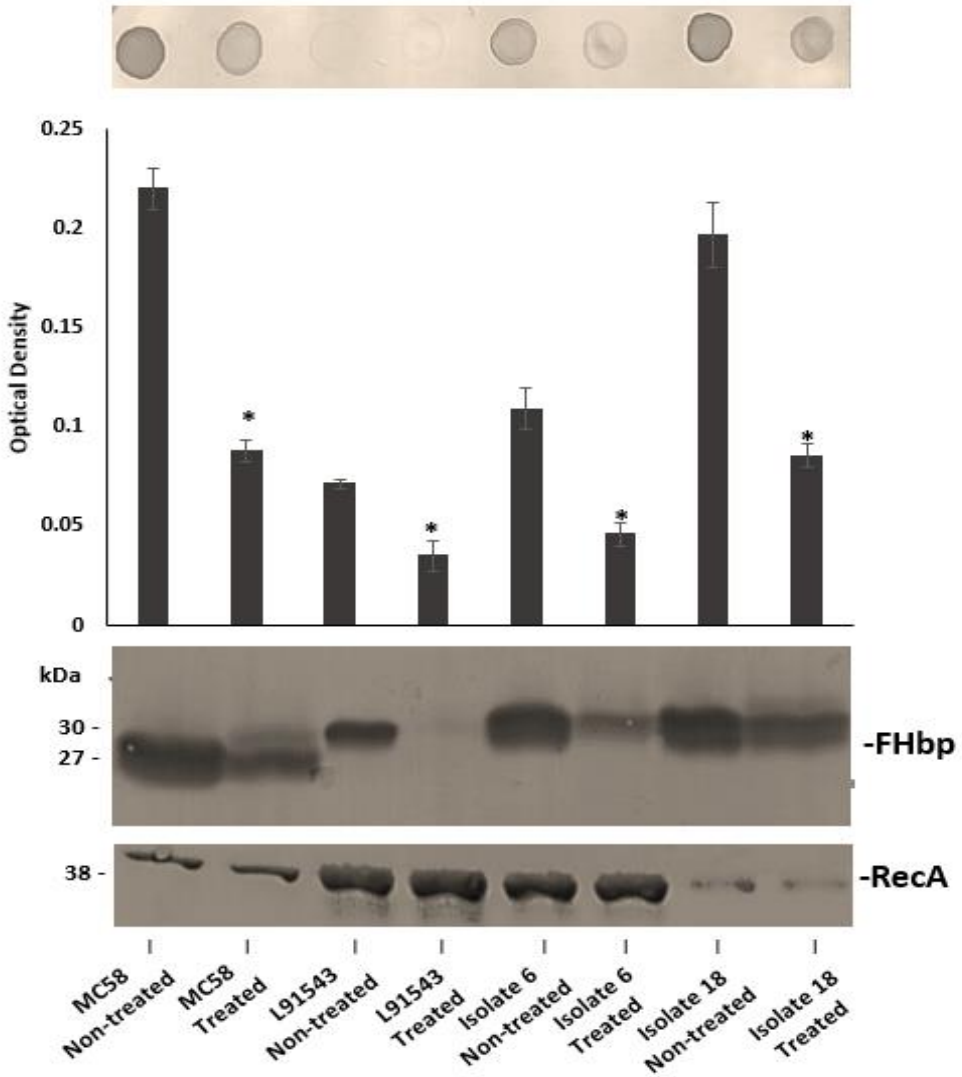


Figure 5. 19- Amino acid changes resulted from SNPs in the FHbp sequence of the SP affect recognition of FHbp by LspA

Immuno-dot blot of broth cultures (A_{600} 0.4) and WB of WCL with JAR4 after Globomycin treatment. Unpaired T Tests with Welsch's correction were conducted to compare the surface localisation of FHbp between Globomycin-treated and untreated strains. All columns represent mean ± SEM, *p<0.05 vs corresponding untreated strain. One representative figure of 3 experiments is shown. Equal protein loading was confirmed for isogenic pairs of strains by the determination of RecA protein in each sample.

Together these results suggest a failure of Lgt and LspA to process non-class 1 FHbps. Next, it was investigated whether the canonical pathway is resumed by Lnt. The *lnt* gene of these four isolates was disrupted by transformation with the PCR product obtained following amplification of the disrupted *lnt* gene in MC58Lnt, with primers lnt_for and lnt_rev (Chapter 3) with selection on kanamycin (Fig 5.20) and sequence verified (appendix III). True transformants were designated MC58Lnt, L91543Lnt, Isolate6Lnt and Isolate18Lnt.

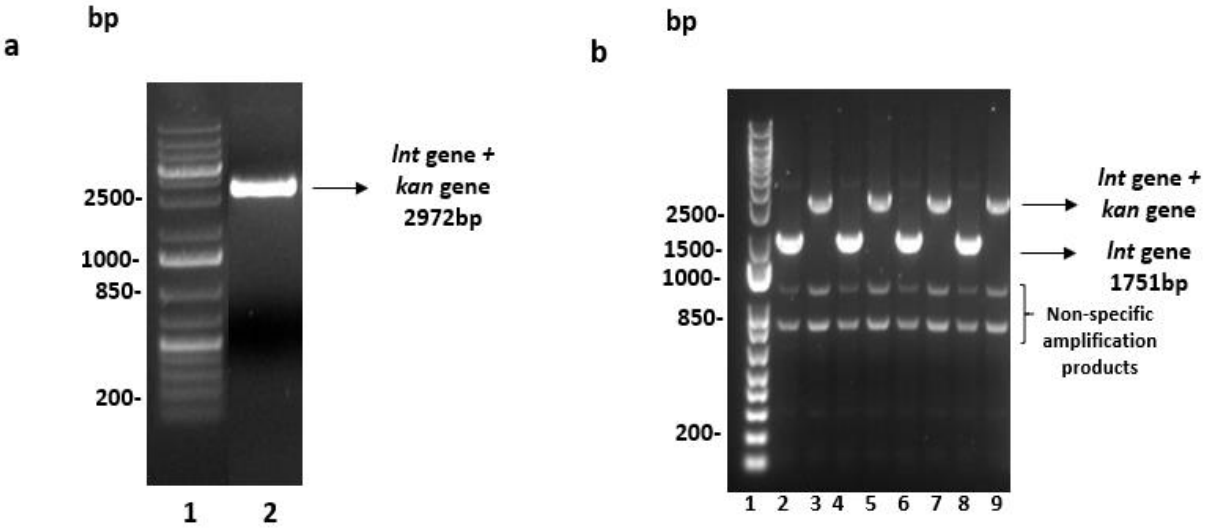


Figure 5. 20- Disruption of *lnt* in strains MC58, L91543, 6 and 18

a. PCR amplification with primers lntFseIfor and lntPacIrev of *lnt* disrupted by *kan* gene from MC58Lnt. Lanes; 1, ladder; 2, *lnt* gene disrupted by *kan* gene PCR product (2972bp). **b.** PCR amplification of the *lnt* gene region for verification of *lnt* mutants with the primers lntFseIfor and lntPacIrev. Lanes; 1, ladder; 2 and 3, MC58 and MC58Lnt; 4 and 5, L91543 and L91543Lnt; 6 and 7, isolate 6 and Isolate6Lnt; 8 and 9, isolate 18 and Isolate18Lnt.

Lnt disruption not only affected surface localisation of FHbp but also reduced overall levels of FHbp (Fig 5.21). This supports previous findings in chapter 3 where it was shown that MC58Lnt employs mechanisms to reduce FHbp by lowering its expression and possibly by employing periplasmic proteases as well as shunting some of diacylated FHbp to the surface (da Silva *et al*., 2017). From these results, it is inferred that Lnt plays a role in the pathway leading to surface localisation in non-class 1 isolates, as well as triacylating FHbp in class I strains. It is possible that Lnt acts as a chaperone delivering the FHbp precursor to Lol in non-class 1 strains since it seems Lgt and LspA are not involved in the process but Lnt is.

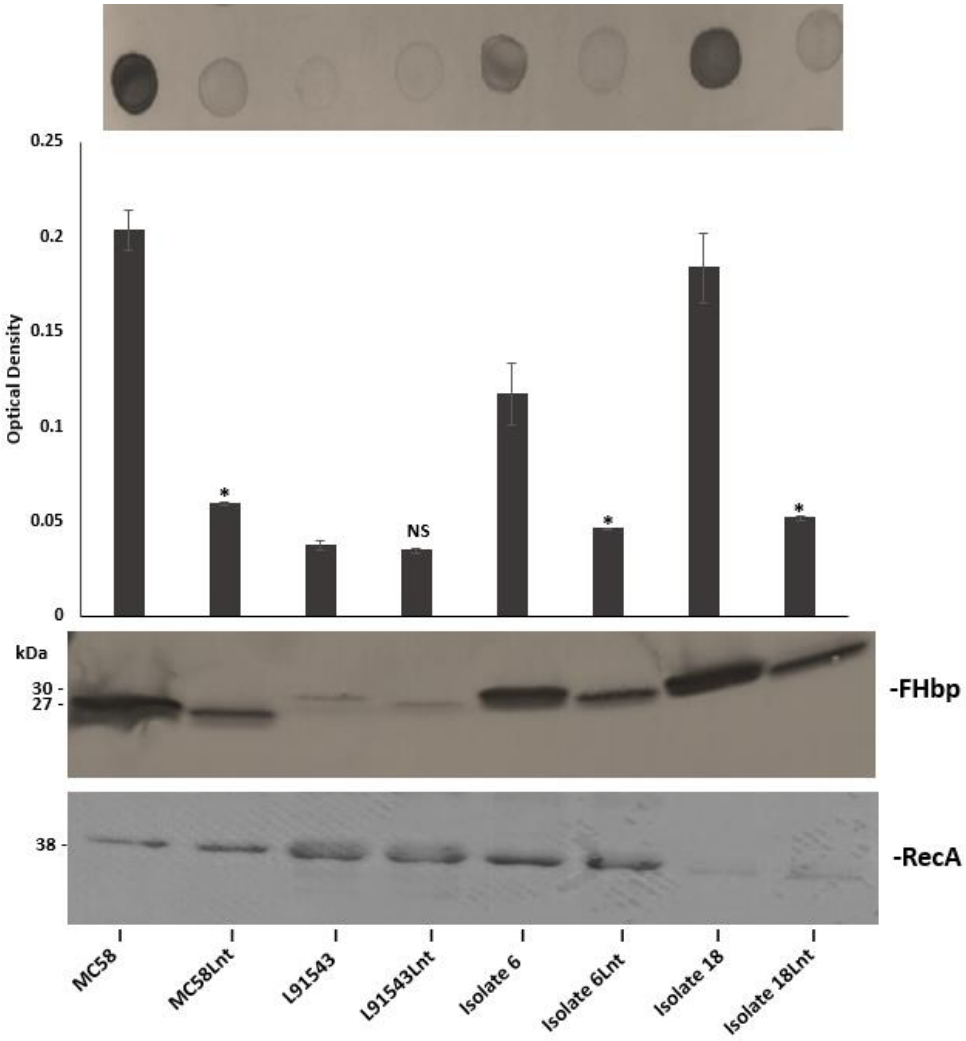


Figure 5. 21- Lnt disruption affects FHbp surface display and expression

Immuno-dot blot of broth cultures (A_{600} 0.5) and Western immunoblot of WCL with JAR4. Unpaired T Tests with Welsch's correction were conducted to compare WT and isogenic mutant strains. All columns represent mean ± SEM, *p<0.05 wild type vs isogenic strain. One representative figure of 3 experiments is shown. Equal protein loading was confirmed for isogenic pairs of strains by the determination of RecA protein in each sample.

Attempts were made to knock out LolA and LolB but without success (see appendix XI for SOE construction of these KOs). Since Slam was previously shown to be important for FHbp surface localisation (Hooda et al., 2016a), Slam was deleted next in all 4 isolates. The SOE technique was used to generate the deletion of Slam (Fig 5.22). First, it was verified that all four strains have the same genomic organisation that would permit homologous recombination with the SOE product generated with *Slam* flanking regions of MC58 genomic DNA. Alignment of *Slam* and flanking regions showed MC58, L91543, and isolate 18 to be highly similar (99% identical) (see appendix VI).

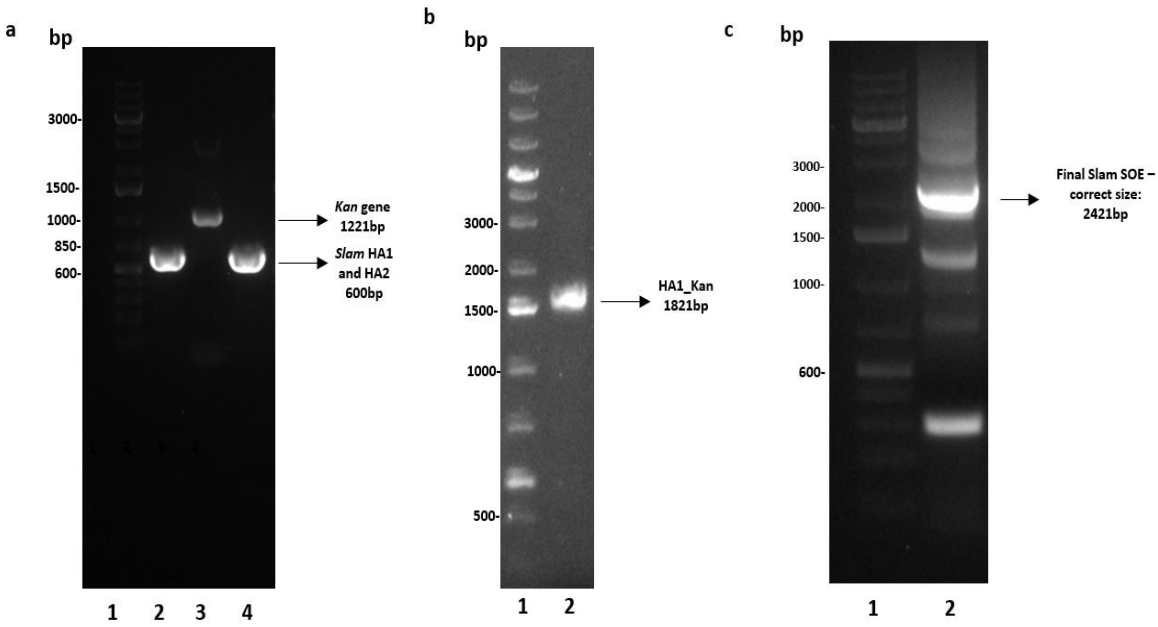


Figure 5. 22- PCR amplification steps involved in deletion of MC58 *Slam* by SOE

a. First round of PCR amplification is shown. Lanes; 1, ladder; 2 HA1 upstream of *Slam* (600bp), 3; *kan* gene (1221bp), 4; HA2 downstream of *Slam* (600bp). **b.** Second round of PCR amplification using gene cleaned

HA1 and *kan* products as template. Lanes; 1, ladder; 2, HA1_kan product (1821bp). **c.** Final round of PCR amplification using HA1_kan and HA2 as template to generate the last PCR product to be used for transformation. Lanes; 1, ladder; 2 Attempt to incorporate HA2 to HA1_kan. Right size band of the product HA1-kan-HA2 (2421bp) was excised, gene cleaned and sent for sequencing for verification before transformation into strains MC58, L91543, isolate 6 and isolate 18.

Successful transformants with deleted Slam were confirmed by PCR (Fig 5.23). Transformants were designated MC58Slam, L91543Slam, Isolate6Slam, Isolate18Slam.

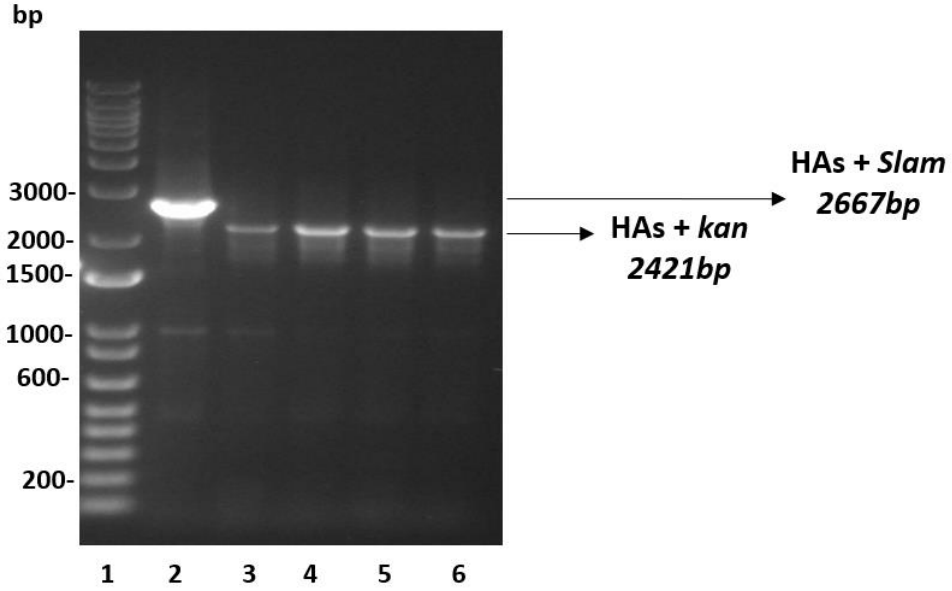


Figure 5. 23- PCR confirmation of MC58Slam, L91543Slam, Isolate6Slam, Isolate18Slam

PCR amplification with primers HA1_0313_Fwd and HA2_0313_Rev of *Slam* region. Lanes; 1, ladder; 2, *Slam* gene (1467bp) and HAs (1200bp)

from MC58 as positive control (2667bp); 3, MC58Slam; 4, L91543Slam; 5, Isolate6Slam; 6, Isolate18Slam.

As expected for MC58, Slam deletion prevents surface display of FHbp. Surprisingly the deletion of Slam also affected surface localisation in the non-class 1 isolates (Fig 5.24). This finding suggests that Slam is involved in the correct display of non-lipidated proteins, as well as lipidated proteins, since non-class 1 FHbps are not lipidated. This result establishes a broader role for Slam then originally reported (Hooda *et al.*, 2016b).

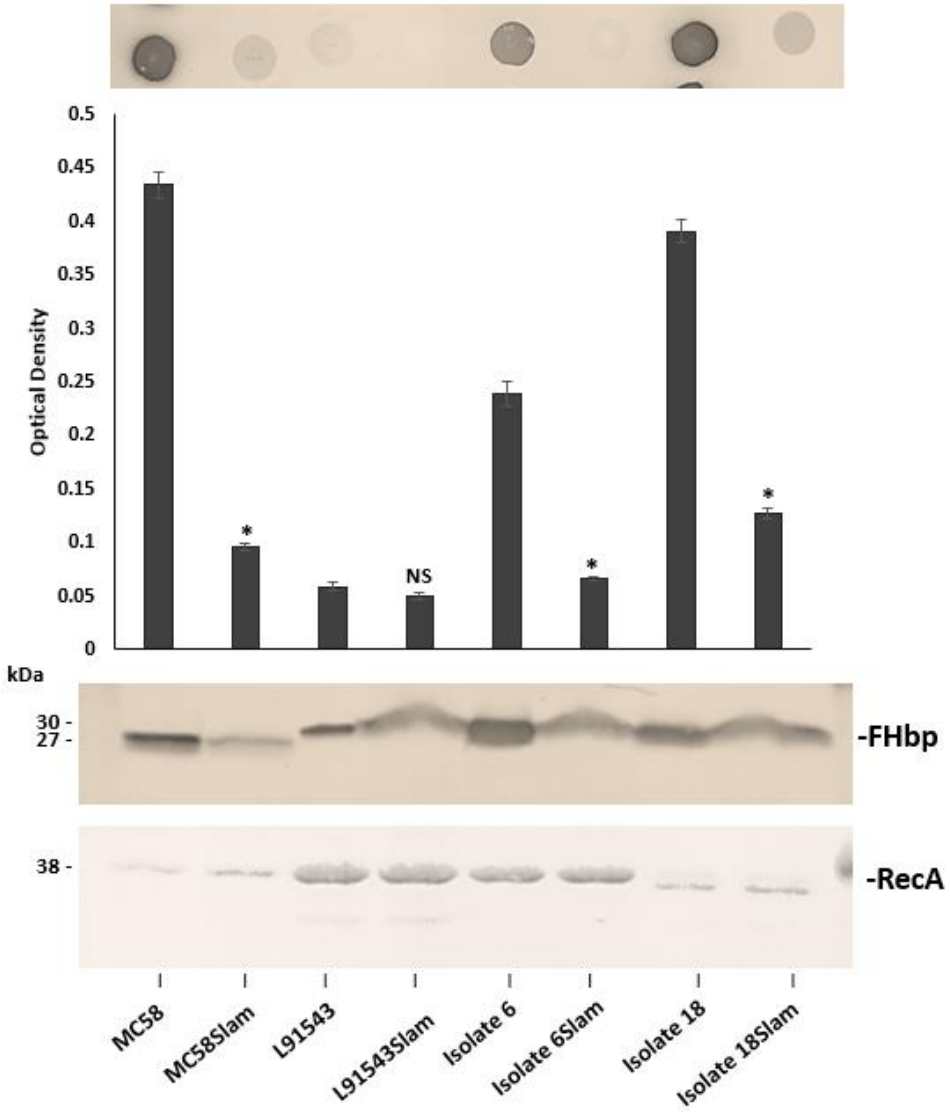


Figure 5. 24- Slam is involved in FHbp surface localisation

Immuno-dot blot of broth cultures (A_{600} 0.5) and Western immunoblot of WCL with JAR4. Unpaired T Tests with Welsch's correction were conducted to compare the WT and respective isogenic strains. All columns represent mean ± SEM, *p<0.05 wild type vs isogenic strain. One representative figure of 3 experiments is shown. Equal protein loading was confirmed for isogenic pairs of strains by the determination of RecA protein in each sample.

We next investigated the localisation of FHbp in the different cell compartments of the 4 WT isolates. Given that our BACTH results showed reduced recognition of SecA for L91543 FHbp with SP SNPs which could affect IM translocation, and the pathway for surface localisation was resumed by Lnt and Slam, we expected to see differences in the sub-cellular distribution of FHbp between MC58 (class 1) and the three non-class 1 isolates.

In MC58, FHbp of around 27 kDa size is seen mainly in the IM and is also detected in the other cell compartments except cytoplasm. The extremely low abundance of FHbp in the cytoplasm indicates efficient translocation to the IM as expected by the efficient binding of SecA to the SP of MC58 FHbp, (Fig 5.25). In contrast, for isolates 6 and 18, unprocessed precursor was detected in all 4 cellular compartments; cytoplasm, IM, periplasm and OM, and at high levels (Fig 5.25). These findings demonstrate that the efficiency of translocation from cytoplasm to IM is reduced in these isolates (supporting our BACTH data) but surprisingly their OM translocation efficiency is comparable with that of MC58.

For strain L91543, unprocessed FHbp was noticeably less abundant and all of this localised to the IM and periplasm suggesting complete translocation across the IM (Fig 5.25). Less overall FHbp in L91543 seems to provide more opportunity for SecA to bind this FHbp. Conversely, for isolates 6 and 18, the greater overall abundance of preprolipoprotein appears to have exceeded the capacity of SecA which has reduced binding affinity to fully translocate it. This results in cytoplasmic retention of FHbp. To conclude, we propose that in non-class 1 strains, when the FHbp

preprolipoprotein is abundant, SecA, which is hampered in its binding affinity for non-class 1 SPs, translocates only a portion of this precursor to the IM.

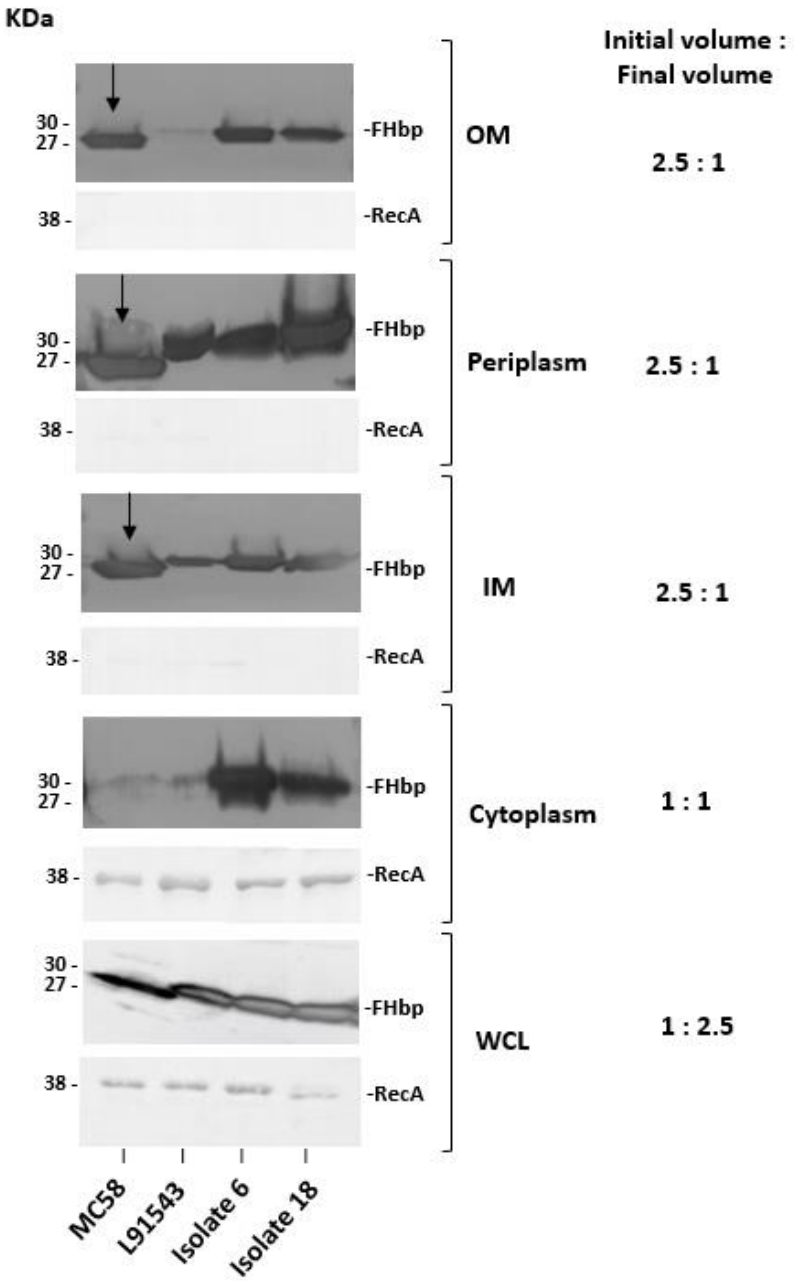


Figure 5. 25- FHbp sub-cellular localisation in 4 isolates collectively representing of each SP class

Western immunoblot of different cellular fractions with JAR4. (Lane 1, MC58; 2, L91543; 3, isolate 6; 4, isolate 18.). One representative figure of 3 experiments is shown. Equal protein loading was confirmed by the determination of RecA protein in each sample.

5.7- Transcript levels of *fHbp* influence surface localisation of FHbp

To investigate if the transcript levels of *fHbp* also influence FHbp localisation, RNA was extracted from the 20 representative isolates from FHbp SP classes 1-4 (Fig 5.26a) and RT-PCR performed from cDNA of the RNA extracted (Fig 5.26b). Primers that bind to a conserved region in all *fHbp* genes were designed (Table 2.5, appendix IX). Strain L91543 and isolates 12 and 13 which do not expose FHbp at the surface generated lower transcript levels when compared to all other isolates that display FHbp at the cell surface. L91543 exhibited 5.8-fold reduction in *fHbp* transcript level, and a reduction of 4.6-fold was observed for isolates 12 and 13, compared to MC58 (Fig 5.26b). These 3 isolates were the same isolates previously found to display FHbp poorly at the cell surface (Fig 5.17) hence transcription efficiency influences surface abundance as expected

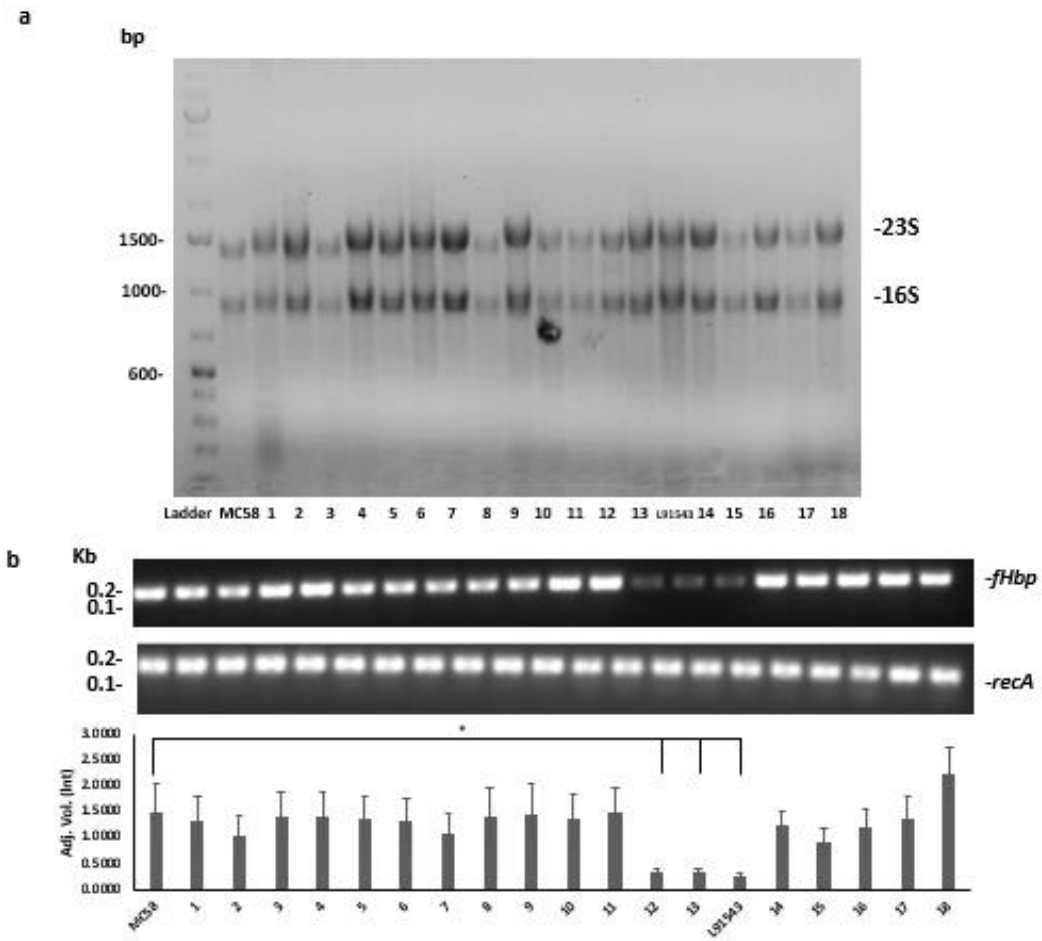


Figure 5. 26- Comparison of *fHbp* transcript levels between isolates

a. Verification of RNA integrity by agarose gel electrophoresis. **b.** RT-PCR of *fHbp* and *recA* (standard house-keeping gene) using primer RTfHbpFwd and RTfHbpRev and recA_fwd and recA_rev, respectively. One representative experiment of 3 is shown. Band intensity was measured using Image lab v4.0.1 and the data acquired using Linear non-threshold model (lnt). The one-way ANOVA followed by Dunnett's test was employed for statistical analysis in GraphPad v6. All columns represent mean ± SEM, *p<0.05 vs strain MC58.

To test if increasing the level of expression of *fHbp in* L91543 results in cytoplasmic retention of FHbp as observed for the other two non-class 1 isolates, recombinant strain L91543Δ*fHbp*+L*fHbp* was tested. Here the expressed copy of *fHbp* is under the control of the pGCC4 *lacZ* promoter. Thus, L91543 transcription could be induced by IPTG.

When IPTG-induced, localisation of FHbp on the surface was demonstrated for L91543Δ*fHbp*+L*fHbp* (Fig 5.27a). Similar to isolates 6 and 18 which naturally have a high transcript level of *fHbp*, induction of *fHbp* expression in L91543Δ*fHbp*+L*fHbp* resulted in cytoplasmic retention and detection of FHbp in all sub-cellular compartments as observed for isolates 6 and 18 (Fig 5.27b). The data suggest that a threshold level of accumulation of FHbp is tolerated in the periplasm as shown for strain L91543 (Fig 5.16) but an over-accumulation as shown for L91543Δ*fHbp*+L*fHbp* seems to force FHbp translocation to the surface as also seen for isolates 6 and 18 (Fig 5.16). This data corroborates our earlier speculation that reduced binding of the non-class 1 SP of FHbp to SecA lowers the capacity for SecA to mediate translocation when this FHbp is abundant. In other words, SecA fails to translocate it effectively when there is an accumulation of non-class 1 FHbps.

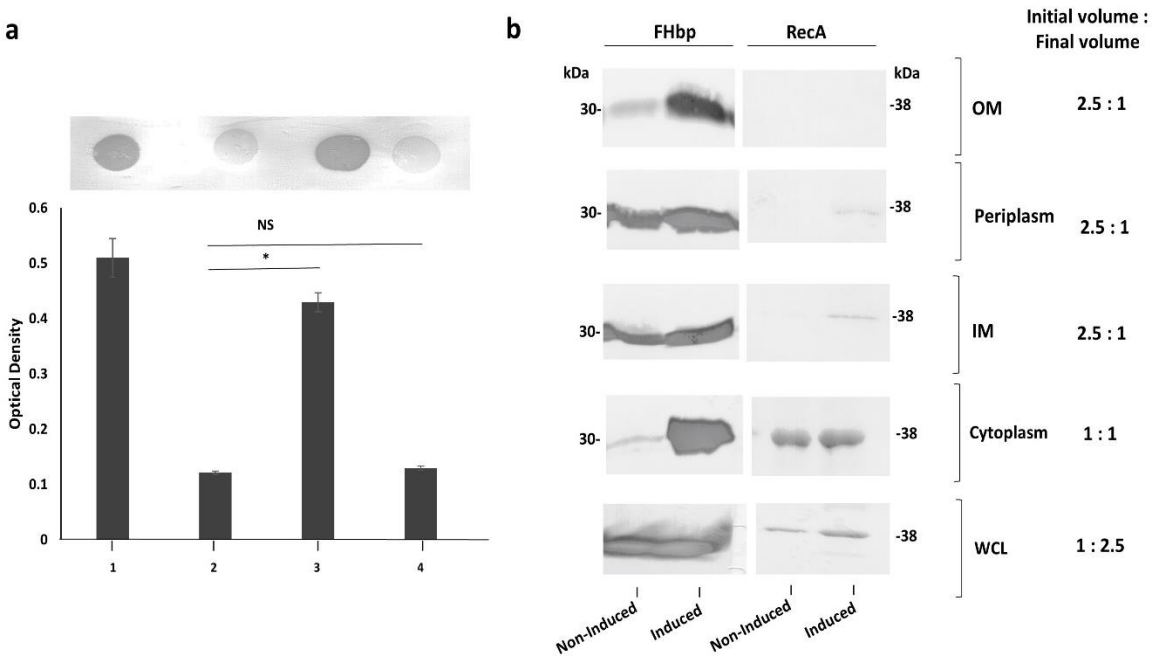


Figure 5. 27- The effect of increasing transcription of *fHbp* on FHbp subcellular distribution and surface localization in L91543Δ*fHbp*+L*fHbp*

a. Immuno-dot blot of WC with JAR4. Lanes; 1, MC58, 2, L91543, 3, L91543Δ*fHbp*+L*fHbp* induced with IPTG, 4, L91543Δ*fHbp*+L*fHbp* non-induced. One representative experiment of 5 is shown. The optical density was measured by ImageJ. The ANOVA followed by Dunnett's test was employed for statistical analysis in GraphPad v6. All columns represent mean ± SEM, *p<0.05 vs strain L91543; **b.** WB with JAR4 of different subcellular compartments of strain L91543ΔfHbp+LfHbp non-induced versus induced. Equal protein loading was confirmed by the determination of RecA protein in each sample.

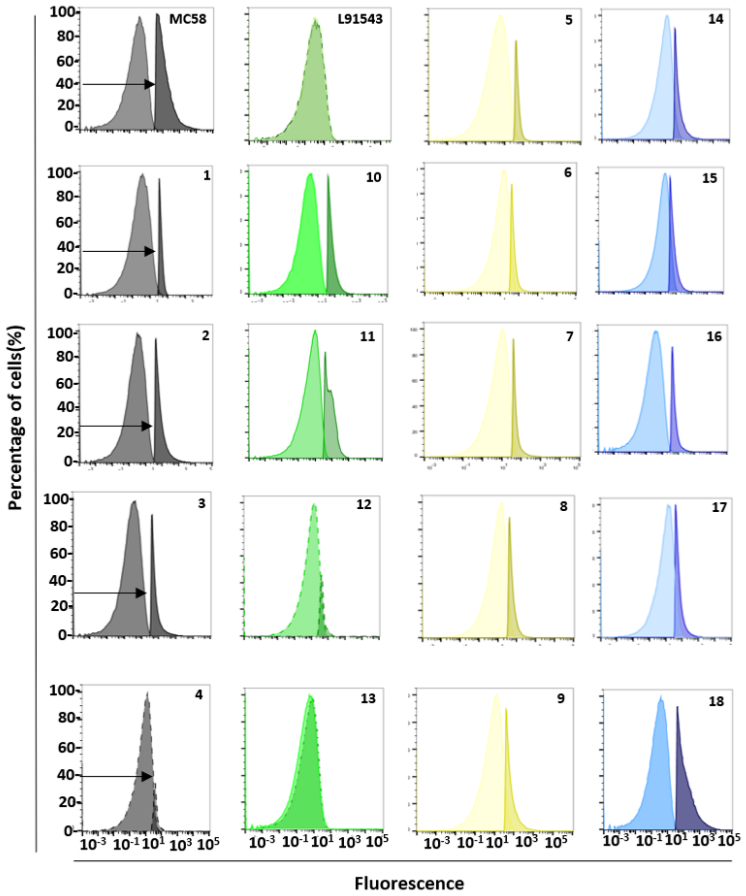
5.8- Comparison of biological activities of unprocessed and processed surface localised FHbp

Having identified the presence of non-lipidated unprocessed FHbp in all non-class 1 meningococcal isolates tested, we set out to investigate the biological relevance of this. First FACs was performed to rigorously evaluate the levels of FHbp on the cell surface of the 20 representative isolates (see appendix X for the adopted gating strategy). Secondly, the ability of anti-FHbp to mediate antibody mediated-killing of the isolates was tested. It is known that for effective killing by FHbp directed antibodies, the number of FHbp molecules on the Neisserial surface must be above a certain threshold (Jiang *et al*., 2010; McNeil *et al*., 2018), specifically above 757 FHbp molecules per cell (Biagini *et al*., 2016). JAR4 binds to one epitope on FHbp (appendix VII) and saturation of Neisserial cells with this mAb should mean a maximum of one antibody binds per FHbp molecule. This enabled us to infer the number of antibodies bound per cell (ABC) using commercially available microspheres (Bangs Lab). Such microspheres contain a known number of antibodies linked to their surfaces, which means that when incubated with the secondary fluorescent antibody used in this study, they will have a maximum fluorescence corresponding to their known initial number of antibodies that attached to them. Microspheres with increasing number of antibodies will allow inference of number of antibodies based on fluorescence after a standard curve (number of antibody vs fluorescence) is plotted (see appendix X for generated fluorescence vs number of antibodies curve by Quickcal v2.3). Strains MC58 and L91543 were used to benchmark all other isolates as

these have previously been shown to have extreme opposite phenotypes, with MC58 demonstrating strong surface display and susceptibility to killing by SBA and, conversely, L91543 exhibited very poor surface localisation of FHbp and resistance to killing by SBA (Newcombe *et al.*, 2014).

As expected, strain L91543 and isolates 12 and 13 that have low transcription of *fHbp* did not surface localise FHbp to the level found to be required for effective killing. All the other isolates presented FHbp molecules above the minimum threshold, indicating these would be susceptible to killing by antibodies. We next investigated if this was the case using SBA (Fig 5.28).

a



b

	Strain	(ABC)	FHbp expression inferred by ABC
Class 1	MC58	**8427** ± 3003	+
	1	**8992** ± 3045	+
	2	**7147** ± 3318	+
	3	**6911** ± 4600	+
	4	**410** ± 96	-
Class 3	5	**1994** ± 1042	+
	6	**1977** ± 553	+
	7	**2465** ± 1021	+
	8	**2785** ± 938	+
	9	**2279** ± 564	+
Class 2	L91543	**376** ± 35	-
	10	**5354** ± 3043	+
	11	**3961** ± 2802	+
	12	**406** ± 91	-
	13	**481** ± 324	-
Class 4	14	**4925** ± 3316	+
	15	**4544** ± 2227	+
	16	**4371** ± 1164	+
	17	**9041** ± 2634	+
	18	**10868** ± 2105	+

c

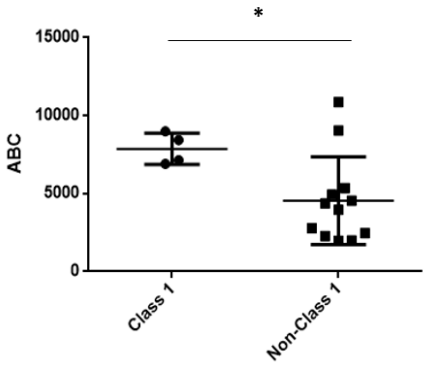


Figure 5. 28- FACS analysis with Mab JAR4 of the 20 isolates

a. A representative flow cytometry plot for each isolate is shown. The read-outs from negative control samples, cells incubated with secondary antibody only, (left peak) were gated (as shown by the arrows). The read-outs from samples incubated with both primary and secondary antibody were overlaid. **b.** The number of JAR4 antibody molecules bound per cell (ABC) and corresponding prediction for successful killing in SBA assays is denoted by +. The mean ABC for each isolate was derived from 3 independent FACS experiments. **c.** Pooled data for class 1 and non-class 1 isolates, excluding the outliers (isolates that do not express FHbp on the cell surface or failed to be recognised by JAR4). Values were analysed by unpaired T Tests with Welsch's correction. Columns represent mean ± SEM, *$p<0.05$ vs class 1.

Following FACS analysis, SBA was performed with the antibodies JAR4 and JAR5. Like JAR4, JAR5 is also a mAb. JAR5 binds the AA residues 84-91 and 115-123 of FHbp (appendix VII) and acts synergistically with JAR4 to exert a bactericidal activity on meningococcal cells in the presence of complement (Beernink *et al.*, 2008; Malito *et al.*, 2016; Welsch *et al.*, 2008). Beernink and co-workers (2008) showed that a minimum of 4 µg/ml of each mAB is needed for bactericidal activity. Strain L91543, and isolates 4, 12 and 13 showed resistance to killing (≤11%) (Fig 5.29a). This was not surprising since these isolates did not display sufficient FHbp on their surface due to poor transcription of *fHbp.* Isolate 4 which is not recognised by JAR4 (Fig 5.17) was also resistant to killing in this SBA assay, as expected.

a

	Strain	Percentage of killing (%)
Class 1	MC58	**78.1** ± 9.7
	1	**75.6** ± 5.3
	2	**70.3** ± 15.4
	3	**70.8** ± 6.3
	4	**11.4** ± 5.9
Class 3	5	**42.9** ± 29.1
	6	**52.3** ± 20.7
	7	**44.0** ± 22
	8	**69.7** ± 5.6
	9	**60.0** ± 10.8
Class 2	L91543	**8.9** ± 4.8
	10	**49.5** ± 6.4
	11	**36.9** ± 20.1
	12	**9.5** ± 3.9
	13	**11.2** ± 3.7
Class 4	14	**38.2** ± 4.6
	15	**50.5** ± 13
	16	**54.9** ± 10.9
	17	**49.6** ± 13.3
	18	**92.6** ± 2.6

b

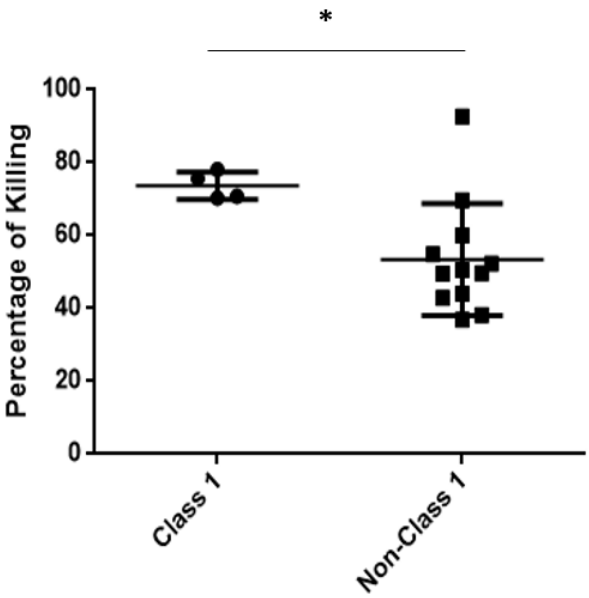


Figure 5. 29- SBA assay with Mabs JAR4 and JAR5 with the 20 isolates

a. The actual percentage killing for each isolate with Mabs JAR4, JAR5 and human complement is shown. The mean percentage killing derived from 4 independent SBA experiments, each with 2 technical replicates is shown. **b.** Pooled data for class 1 and non-class 1 isolates, excluding the outliers (isolates that do not express FHbp on the cell surface or failed to be recognised by JAR4 and JAR5). Values were analysed by unpaired T Tests with Welsch's correction. Columns represent mean ± SEM, *p<0.05 vs class 1.

Interestingly, pooled results of both experiments for class 1 and non-class 1, excluding these 4 poor expressors of FHbp, showed significant differences (Fig 5.28c and 5.29b) concerning FHbp display and bactericidal killing of antibodies. The number of FHbp molecules in class 1 had an average of 7869 compared to 4547 molecules found on average in non-class 1 isolates. The SBA data corresponded to the FACS data and showed significant reduction in killing of non-class 1 FHbp expressing isolates compared to class 1 isolates (53.4% vs 73.7%).

The domains 6 and 7 of the protein FH directly interact with the β barrels of FHbp (Schneider *et al*., 2009). To investigate if the presence of the additional 26 AAs, comprising the uncleaved SP of non-class 1 isolates, influence FHbp binding to FH, FACS was performed (Fig 5.30). Using equivalent number of cells, isolates were incubated with the same amount of purified FH and incubated with an anti-FH antibody (methods section 2.7.2). No significant differences in binding were observed between class 1 and non-class 1 isolates, indicating no detectable influence of the additional 26 FHbp AAs on the overall binding of meningococcal isolates from different FHbp SP classes to FH.

a

	Strain	FH binding as inferred by rMFI
Class 1	MC58	**228.85** ± 9.98
	1	**153.05** ± 6.92
	2	**191.42** ± 14.65
	3	**216.07** ± 27.11
	4	**201.30** ± 4.29
Class 3	5	**196.15** ± 16.72
	6	**202.45** ± 11.75
	7	**163.20** ± 18.62
	8	**184.89** ± 9.49
	9	**172.54** ± 23.67
Class 2	L91543	**176.01** ± 12.22
	10	**122.93** ± 0.89
	11	**128.58** ± 22.90
	12	**154.68** ± 22.27
	13	**200.62** ± 11.92
Class 4	14	**200.84** ± 28.32
	15	**160.44** ± 38.73
	16	**211.81** ± 10.14
	17	**187.45** ± 32.37
	18	**140.57** ± 34.47

b

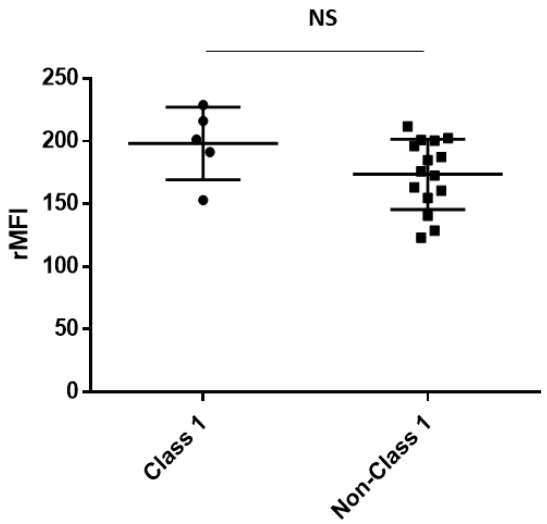


Figure 5. 30- The binding of FH to the 20 representative meningococcal isolates

a. Values show the Relative Mean of Fluorescence Intensity (rMFI) for total fluorescing cells after incubation with FH and anti-FH PE-conjugated antibody. The values were normalised against the negative control of cells alone incubated with antibody. **b.** Pooled data for class 1 and non-class 1 isolates. Values were analysed by unpaired T Tests with Welsch's correction. Columns represent mean ± SEM; NS, not significant vs class 1.

5.9- Discussion

This chapter has demonstrated the importance of the SP for translocation, processing and surface localisation of FHbp. In a series of experiments that included SDM, immuno-dot blotting and WBs, two SNPs in the SP of L91543 were shown to affect FHbp display on the surface. Moreover, both SNPs were shown to prevent lipidation in this strain (Fig. 5.18). Interestingly, the majority of the 1,895 MRF isolates investigated carry SNP2: substitution of the polar T residue with a hydrophobic A residue at position 19 (Table 5.2).

We hypothesised that substitutions in the SP influence IM translocation of FHbp by SecA. The BACTH results showed reduced affinity of L91543 FHbp for SecA compared with MC58 FHbp (Fig 5.16). We show that the reduced recognition of FHbp by SecA leads to an accumulation of FHbp in the cytoplasm particularly when the FHbp expression level is high, as shown for isolates 6 and 18, and the IPTG-induced L91543Δ*fHbp*+L*fHbp* strain (Figs 5.17 and 5.28). The reduced binding of FHbp to SecA likely allows time for folding of the nascent FHbp protein affecting its delivery to SecYEG. Since the Sec translocon specifically transports of unfolded proteins across the IM (Tsukazaki, 2019), FHbp folding could have deleterious effects on its IM translocation.

The crystal structures of Lgt and LspA have recently been solved from *E. coli* (Mao *et al*., 2016) and *Pseudomonas aeruginosa,* respectively (Vogeley *et al*., 2016). The structures provided relevant information regarding substrate recognition by these two enzymes in Gram-negative bacteria. Whilst, for the Lgt enzyme, the positioning of the preprolipoprotein

is very important for its enzymatic action, for LspA, the diacylglyceryl modification of preprolipoprotein was confirmed to be crucial for its substrate recognition as a signal peptidase (Vogeley *et al.*, 2016; Mao *et al.*, 2016). Additionally, for preprolipoproteins to access the active site of Lgt, they must be translocated properly to the periplasmic side of the membrane with the C residue orientated correctly in relation to the diacylglyceryl moiety of peptidoglycan for transacylation to occur (Mao *et al*., 2016). The reduced binding of FHbp to SecA could lead to some folding of FHbp, and of the FHbp that is translocated, its ability to access Lgt may be affected, thus preventing lipidation of the preprolipoprotein (Fig 5.18). The preprolipoprotein lacking di-acylation is consequently not processed by LspA since LspA requires the di-acylated substrate for recognition (Mao *et al.*, 2016; Fig 5.19).

This study allowed us to hypothesise about a possible auxiliary function for Lnt (Fig 5.21) regarding unprocessed lipoproteins. It seems that upon accumulation in the cytoplasm (Figs 5.25 and 5.26b), Lnt resumes the pathway helping in FHbp translocation to the OM and ultimately surface display. Lnt would be delivering non-processed FHbp to the Lol apparatus before being localised at the surface by Slam (Fig 5.24). Recent crystal structures of *E. coli* Lnt showed that Lnt possesses several trans-membrane domains with membrane-embedded cavities, a lateral opening and an exit to the periplasm (Lu *et al*., 2017). From its structure, it could be envisaged that Lnt takes part in the translocation of unprocessed FHbp. The Sec translocon would hand on the preprolipoprotein to Lnt which in its turn would deliver it to Lol or even directly to Slam to expose it at the cell

surface. Further studies are necessary to address the full role of Lnt in this process.

The deletion of Slam meant that for all isolates tested FHbp was not surface localised (Fig 5.24). Thus, we show that Slam facilitates FHbp surface localisation regardless of its acylation status. In other words, we show that Slam is a chaperone helping not only lipoproteins to be surface displayed but also other proteins. Interestingly, Hooda and collaborators (2016) showed that the Slam substrate-recognition site is within the C-terminus of lipoproteins rather than at the acylated N-terminus. Therefore, as long as the proteins contain the relevant C-terminal domain, Slam may surface localise a larger number of proteins and lipoproteins rather than just the 2 specific meningococcal lipoproteins (FHbp and TbpB) previously described.

Due to the high prevalence of SP SNPs of FHbp (91% of the isolates; table 5.2) it is possible there is an evolutionary selection pressure for expression of unprocessed FHbp. Possibly it could cost less metabolically to the cell than exploiting the entire canonical machinery to lipidate and cleave the SP. Alternatively, non-lipidated FHbp could provide a fitness benefit when trying to escape the immune system. The approximate 2-fold reduced surface exposure of the preprolipoprotein, shown in this study (Fig 5.28c), may be selected for to facilitate escape from anti-FHbp antibody-mediated killing, whilst retaining sufficient FH binding to prevent killing by the alternative complement pathway.

In addition to the influence of SP SNPs on FHbp surface localisation we show that the transcript levels of FHbp influence its surface display (Figs 5.26 and 5.27). FHbp transcription was previously shown to respond to the

presence of iron and oxygen, known to vary in the mucosal environment where Nm normally colonises (Oriente *et al.*, 2010; Sanders *et al.*, 2012). FHbp transcription could be linked to how the meningococcal cells responds to the immune system and to external signals. It is reasonable to speculate that the meningococcus can alter FHbp transcription and, consequently, its exposure on the cell surface according to its needs when responding to environment stimuli.

The findings of this chapter show that new prediction programmes need to be developed to predict whether preprolipoproteins are processed to become mature lipoproteins or remain as precursors. Our results are possibly pointing to a reduced number of fully processed lipoproteins. For FHbp, for instance, the polar residue in the h-region close to the lipobox seems to be crucial for its full maturation as a lipoprotein. Software such as DOLOP (Babu *et al*., 2006) does not include the residue close to the lipobox in its algorithm. Other putative lipoproteins with similar mutations are potentially unprocessed as shown for FHbp. This chapter also shows that not only SNP2 affects processing but also SNP1 for class 2 organisms. More experimental work in other lipoproteins is needed to correlate key residues with processing enabling new lipoprotein prediction precursors to be deployed.

It would be interesting to explore biological relevance of unprocessed FHbp further and test human sera of vaccinated individuals with a large number of FHbp that contain different SPs. The SBA experiment showed 73.7% killing for class 1 vs 53.4% killing for non-class 1 (Fig 5.29). We predict even greater observations will be seen if testing sera from Trumenba vaccinated-

individuals since, differently from the majority of isolates, Trumenba consists of cleaved lipid-modified FHbp.

Finally, this chapter added to the current knowledge available about the Trumenba and Bexsero antigen FHbp, supplying invaluable insight to consider unprocessed proteins in the vaccine formulation. A recent study from McNeil *et al.* (2018) showed that when testing the ability of human serum from vaccinated individuals to kill Nm serogroup B isolates, 11% of isolates which expressed surface FHbp above the established cut-off of their study were resistant to the bactericidal activity of vaccinated sera. It would be interesting to see if this data correlates with the presence of SNPs in the FHbp SP of these isolates.

Chapter 6

General Discussion

6- General Discussion

Nm can cause devastating invasive meningococcal septicaemia that results in severe sequalae or death. Currently, mortality by meningococcus infection remains at 10 to 15% for infected individuals in developed countries and greater than 20% in developing countries (Dretler *et al.*, 2018) accordingly this highlights the clinical significance of this organism today despite available prophylaxis programs.

FHbp is known to be a triacylated protein that is surface displayed in the meningococcus. It has been identified as a lipoprotein that binds FH protein in order to downregulate and avoid complement deposition. Due to its ability to elicit potent bactericidal antibody responses that correlate with the abundance of FHbp on the surface of Nm, FHbp is today part of two protein-based vaccines against the meningococcus B (as reviewed by Seib *et al.*, 2015). This thesis aimed to elucidate the molecular mechanisms involved in the processing and localisation of FHbp. It also aimed to analyse the impact that disruptions in these mechanisms can cause in the cell and on the biological behaviour of the meningococcus and to identify potential drug targets. In this thesis we were interested in investigating how Nm processes and localises FHbp to the cell surface which is potentially useful knowledge to those developing or improving lipoprotein-based vaccines such as Trumemba, which has FHbp as its sole antigen. In order to pursue this, a Tn library was generated (Chapter 3). As a result of screening the Tn library for mutants affected in the surface display of FHbp the role of the gene *lnt* in acylating FHbp was explored. The biological changes caused by the disruption of Lnt and its potential as a drug target were investigated

(Chapter 4). Finally, the influence of the SP in the processing and surface localisation of FHbp was researched (Chapter 5).

6.1- Main Findings

This thesis has as main findings:

i- The generation of a Tn library in Nm strain MC58 allowed the exploration of molecular mechanisms involved in the processing and localisation of the neisserial lipoprotein FHbp;

ii- The Tn insertion in *lnt* resulted in the reduced binding of whole cells to the anti-FHbp monoclonal antibody, JAR4, as shown by immuno-dot blotting, WGS and fluorescence microscopy;

iii- Likely role of Lnt in adding a third acyl group was determined by gel mobility changes in His-tagged truncated version of FHbp in strain MC58Lnt;

iv- Disruption of Lnt affected total FHbp levels as on RNA levels and protein levels as shown by qRT-PCR and WB, respectively;

v- The biological importance of Lnt in Nm and its potential as a drug target was investigated by TEM, RNASeq and *in vitro* cellular assays. While TEM did not show major phenotypic changes in MC58Lnt, RNAseq revealed a plethora of genes that had their expression affected by Lnt disruption including adhesins. *In vitro* cellular assays with HCECs and HUVECs corroborated these predicted biological changes in adhesion and invasion of the mutant. No difference in fitness was observed for the mutant in THP-1 cell assays. Indicators of inflammation, IL-8 and IL-6 levels were

investigated, and differences observed with MC58Lnt causing more expression of IL-8 and IL-6 in HUVECs and more IL-6 expression in THP-1 cells;

vi- The biological importance of Lnt in Nm and its potential as drug target was also investigated by comparing abilities of MC58 and MC58Lnt to form biofilm. Biofilm assays indicated the mutant to be affected in its ability to form biofilm. *In vivo* tests in *G. mellonella* (performed for the first time to our knowledge with Nm) showed reduced virulence of the mutant in this model. MICs supported the candidacy of Lnt as a drug target due to increased susceptibility to killing by several different antibiotics in MC58Lnt;

vii- Amino acid changes resulted from SNPs in the FHbp sequence of L91543FHbp SP were investigated for their influence in processing and localisation of FHbp in Nm by SDM, bacterial two-hybrid and comparing different isolates of Nm with variant SPs for their surface expression of FHbp. While SDM confirmed SNPs to influence FHbp surface display, bacterial two-hybrid experiments confirmed decreased ability of SecA to recognise and bind to the SP of L91543 FHbp. Different isolates of Nm with variant SPs were compared by WC immuno-dot blot and the data corroborated SNPs to be affecting processing and surface localisation;

viii- To investigate influence of the SNPs in lipidation of FHbp, click-chemistry and globomycin studies were performed confirmed non-lipidation of FHbp with SNPs in the SP;

ix- RT-PCR and investigation of the sub-cellular distributions of FHbp by WB confirmed translocation with subsequent surface exposure;

x- Knockouts of Lnt and Slam indicate these proteins to be involved in translocation of unprocessed FHbp and in its surface display, respectively.

The role of Lnt in post-translationally modifying FHbp by adding a third acyl group was explored first (Chapter 3). Initially a random Tn library was created in Nm strain MC58 and the first 100 mutants were screened by WC immuno-dot blotting. One mutant showed reduced binding to the anti-FHbp monoclonal antibody, JAR4. After WGS, the Tn was found to be inserted in gene NMB0713, disrupting the annotated gene, *lnt.* Fluorescence microscopy confirmed a remarkable reduction of FHbp on the surface of MC58Lnt that was restored when Lnt was complemented (strain MC58LntC). To investigate the role of Lnt in adding a third acyl group to FHbp, a truncated version of *fHbp* His-tagged at the new C-terminus was cloned and expressed in MC58 and MC58Lnt. Based on the higher gel mobility of MC58Lnt equivalent to the expected size of one fatty acid difference and the lack of any other possible homologue responsible for N-acylation of apolipoproteins in MC58, it was inferred Lnt is the enzyme responsible for adding a third acyl group onto FHbp. Kurokawa *et al.* (2012) performed a similar truncation experiment in their Lnt mutant of *S. aureus* which they subsequently confirmed by MALDI-TOF MS analysis.

Lnt disruption in MC58 resulted in dramatically reduced total FHbp levels and transcription alone could not explain the 10-fold decrease in total FHbp.

This decrease is likely to be a periplasmic protease mechanism to combat the accumulation of diacylated proteins in the IM of Nm which for *E. coli* is known to be lethal (Zückert, 2014). In addition, comparative immuno-dot blotting of MC58Lnt and strain L91543 suggested that MC58Lnt surface localises some of the diacylated FHbp supporting similar findings in an Lnt mutant of Ng (LoVullo *et al.,* 2015). There is the same organisation of the Lol apparatus in Nm and Ng (LolFD compared to LolCDE of *E. coli*) which appear to be more 'promiscuous' with affinity for diacylated lipoproteins as well as triacylated lipoproteins, which could explain non-lethality of the MC58Lnt mutant.

Next in Chapter 4, we investigated global biological changes in the Lnt mutant. While TEM did not show dramatic changes in the phenotype of MC58Lnt, RNAseq revealed 183 genes to be DE with a global metabolic change in this mutant. Genes related to RNA biology, survival and adhesion of MC58Lnt were among the ones most affected. Eighteen percent of DE genes were related to cell metabolism. The greatest proportion of these genes (86 out of 183 DE genes) is hypothetical genes. The fact that Nm can cope with disruption of Lnt which is expected to cause envelope stress as shown for *E. coli*, suggests the activation of mechanisms to combat stress. Two-component systems in bacteria have been reported to support bacterial adaptation to different challenges that are faced in diverse niches (Breland *et al.*, 2017). To date, only 4 two-component systems have been reported in the meningococcus that combat cell stress. One of these is employed specifically against cationic antimicrobial peptides (Tzeng *et al*., 2004). This is a small number of two-component systems in comparison to

62 identified in *E. coli* (Breland *et al.*, 2017). Thus, due to the number of DE hypothetical genes, this represents an opportunity for the discovery of new systems that are potentially being employed by the meningococcus when Lnt is disrupted. Since the disruption of Lnt in MC58Lnt is likely affecting all of its lipoproteins, a closer look into the predicted lipoproteins in MC58Lnt showed several of them to be DE. Some of the above hypothetical proteins that were affected may well be lipoproteins that have not yet been identified as such, in which case this study may be useful in the identification of novel lipoproteins.

From the *in vitro* assays with HCECs and HUVECs, Lnt showed a reduced ability to invade HCECs whilst it showed a decreased ability to adhere to HUVECs and an increased ability to invade these cells. From the RNAseq, several adhesins were downregulated such as pili and Opc, but not Opa-encoding genes. From this observation, we hypothesised that Opa-CEACAM is mediating adhesion and invasion. Inflammatory proteins IL-6 and IL-8 were expressed at greater levels in HUVECs in MC58Lnt-infected cells compared to MC58 suggesting that the greater ability to invade increases the propensity for inflammatory proteins to be secreted. However, this hypothesis needs confirmation with the use Cytochalasin D which blocks bacterial invasion. MC58Lnt showed no alteration in its ability to survive and replicate in THP-1 cells but did show reduced ability to form biofilms which could influence its capacity to colonise *in vivo.* MC58Lnt was also found to be less virulent in the *G. mellonella in vivo* model. These reduced virulence phenotypes displayed by MC58Lnt seem to indicate a degree of attenuation.

Finally, altered MICs in MC58Lnt also support the potential for Lnt as a novel drug target. Due to possible membrane disturbances caused by Lnt disruption, we hypothesised MC58Lnt could be more permeable and susceptible to antibiotics. MC58Lnt showed increased susceptibility to seven of the tested antibiotics which suggests that that a combined therapy of Lnt inhibitor with lower dose of antibiotic could help in the fight against antimicrobial resistance which is particularly a problem in combating Ng infections.

In Chapter 5, we set out to test the hypothesis that that 2 non-synonymous SNPs in the h-region of FHbp SP of L91543 could be affecting processing and localisation of FHbp to the cell surface (Karlyshev *et al*., 2015). Leucine (L) substituted by phenylalanine (F) at position 15 (SNP1) and threonine (T) substituted by alanine (A) at position 19 (SNP2) were the SNPs identified. Initially an *in silico* evaluation of all the genes involved in the canonical lipoprotein pathway was conducted in L91543 and compared to MC58. No aberrations were found. The gene with greatest divergence between the two strains was FHbp. Transformation of L91543 with FHbp of MC58 restored processing and surface localisation of FHbp proving that differences in the sequence of FHbp are responsible the lack of surface exposure of FHbp in this strain. It was then important to test the involvement of the SNPs in FHbp retention in the cell of L91543 by reversing the SNPs back to the amino acids found in the SP of MC58 that efficiently displays FHbp. The individual SNPs and the two combined were tested by SDM. By fixing each SNP separately, FHbp was surface displayed in this strain. However, this surface exposure was not as great as when both correct

SNPs are present in the SP. Moreover, their electrophoretic mobility was not restored, indicating that fixing these SNPs individually is not sufficient to restore SP cleavage. We questioned if these SNPs are a common feature of FHbp among circulating invasive serogroup B strains. From 1,895 isolates analysed in the MRF database, only 9% were found to possess a SP with no SNPs compared to the reference strain, MC58. This *in silico* analysis also allowed us to classify the SPs in 4 main groups. Five isolates from each group were randomly selected for further analysis. All 15 isolates with SNPs in their SP displayed lower gel mobility indicating SP retention since the difference in size corresponds to the size of the SP. Important to note that overall non-class 1 expressed less of FHbp on the cell surface, and this could have implications in the coverage offered by Trumemba.

We next investigated the specific deleterious effect SP SNPs have on the canonical pathway for FHbp processing and surface localisation. One isolate from each SP class was investigated. Click-chemistry revealed non-class 1 FHbps to lack lipid, which suggests Lgt is not diacylating these species of FHbp. It could well be due to incorrect positioning and delivery by Sec translocon of non-class 1 FHbp to Lgt. Structural data of Lgt indicates that correct positioning of Lgt substrate is crucial for Lgt enzymatic role. Next, following treatment of these isolates with Globomycin, WB confirmed SP retention and lack of activity of LspA over non-class 1 FHbp (Mao *et al.,* 2016). This was expected since, from Vogeley *et al*. (2016) studies, diacylation of LspA substrates was shown to be crucial for its enzymatic activity. Interestingly, our Lnt and Slam knockout studies suggest that these two proteins are involved in surface display of unprocessed FHbp

since both knockouts affected surface localisation. This represents a novel role for both proteins with Lnt acting as a chaperone facilitating transfer of the FHbp precursor to Lol or to Slam, and Slam flipping FHbp to face the exterior surface.

Bacterial two-hybrid experiments with MC58 FHbp and L91543 FHbp and MC58 SecA showed lower ability of SecA to bind to L91543 FHbp, this suggested there could be reduced translocation rate and a possible accumulation of L91543 FHbp in the cytoplasm. Analysis of subcellular compartments of one representative of each FHbp class confirmed cytoplasmic retention of FHbp in non-class 1 isolates except for L91543 with has poor transcription rate as was shown to accumulate FHbp in the IM and periplasm. We hypothesised that the lower transcription rate of *fHbp* allows these cells to cope with the initial translocation made by SecA despite their lower affinity. For isolates 6 and 18, this lower affinity by SecA was more impactful due to their higher transcription rate of *fHbp* and therefore greater level of total FHbp. The accumulation of FHbp in the cytoplasm was observed for these two isolates.

We tested the ability of our panel of 20 isolates to bind FH. Interestingly, all classes retained their ability to bind FH despite having or not having their SP removed. Since the localisation of FHbp to the surface is critical for targeting by FHbp-based vaccine, first we rigorously evaluated surface exposure of FHbp by FACs. Overall, non-class 1 isolates expressed about 2-fold less FHbp on the cell surface compared with class 1 isolates. We then compared all 20 isolates for their susceptibility to killing by FHbp-specific antibodies. The SBA performed showed significantly reduced killing

by FHbp antibodies, JAR4 and JAR5, for non-class 1 isolates (53.4% killing vs 73.7% killing for class 1). Since Trumemba only uses cleaved lipid-modified FHbp, it would be interesting to test whether Trumemba vaccinated-individuals sera present same killing properties for class 1 and non-class 1 FHbp.

6.2- Future directions

Future work could be to exploit strain MC58Lnt to identify novel lipoproteins and elucidate membrane stress mechanisms by testing candidate genes that were shown to be significantly up or downregulated by RNAseq analysis. Further work investigating adhesion and invasion by MC58Lnt is needed using other cell models. Of particular interest would be to test by blocking invasion the expression of IL-6 and IL-8. Other in vivo models would be worth testing for altered virulence of MC58Lnt. Future work could also explore the effect of SP SNPs in other lipoproteins in Nm and in other organisms and deploying new lipoproteins prediction tools accordingly.

Glossary

1- **Adaptive immune system:** composed of highly specialised, systemic cells and processes that eliminate pathogens or prevent their growth.
2- **Firmicutes:** a phylum of bacteria, most of which have gram-positive cell wall structure.
3- **Homologous recombination:** a type of genetic recombination in which nucleotide sequences are exchanged between two similar or identical molecules of DNA.
4- **Innate immune system:** it is the first-line host defence specified to confine infection in the early hours after exposure to the microbial infection.
5- **Leptomeninges:** the inner two meninges, the arachnoid and the pia mater, between which circulates the cerebrospinal fluid.
6- **Opsonisation:** the coating/labelling by the complement system of a pathogen or particle with proteins that facilitate phagocytosis of the pathogen/particle by phagocytic cells such as macrophages.
7- **Orthologue gene:** are homologous genes where a gene diverges after a speciation event, but the gene and its main function are conserved.
8- **Phase variation:** the on and off switching of protein expression in bacteria as a way of responding to rapidly changing environments.
9- **Shear Force:** unaligned forces pushing one part of a body in one specific direction, and another part of the body in the opposite direction.
10- **Transposon:** class of genetic elements that can “jump” to different locations within a genome, sometimes creating or reversing mutations and altering the cell's genetic identity and genome size.

Appendix I- Commonly used solutions and reagents

Agarose gel: 1.0 % agarose was prepared by dissolving 1 g of agarose powder (Sigma), 2 ml 50 × TAE buffer and made up to 100 ml with dH2O; melted and Sybr Safe (10,000x diluted) added.

SDS-sample buffer (2 x): 100mM Tris HCL pH 6.8, 2% SDS, 10% glycerol, 10% DTT, 0.10% Bromophenol blue and dH_2O added up to 10 ml, pH at 6.8.

Resolving (separating) gel: 2.5 ml SDS-resolving buffer, 2.52 ml Acrylamide/BisAcrylamide (30%), 5 ml dH_2O, 20 µl Saturated Ammonium persulfate (APS) and 10 µl Tetramethyl ethylenediamine (TEMED).

Stacking gel: 1.25 ml SDS-stacking buffer, 0.75 ml Acrylamide/Bis-Acrylamide (30%), 3 ml dH_2O, 8 µl APS and 10 µl TEMED.

Semi-dry blotting buffer: 5.82 g Tris base, 2.93 g Glycine, 3.75ml 10% SDS, 200 ml methanol made up to 1000 ml with dH_2O.

Phosphate buffered saline solution (PBS): prepared by dissolving 1 tablet of Phosphate buffered saline (Dulbaco A, Oxoid) in 100 ml dH_2O and autoclaved, to give sodium chloride 0.16 mol, potassium chloride 0.003 mol, disodium hydrogen phosphate 0.008 mol and potassium dihydrogen phosphate 0.001 mol at pH 7.3.

BSA (Albumin from bovine serum, Sigma): as lyophilized powder (MW ca. 66 kDa) was prepared in sterile PBS according to the concentration needed.

LB (Luria-Bertani) agar: Tryptone 10 g, Yeast extract 5 g, Sodium chloride 10g, Microbial tested agar 15 g made up to 1000 mL with dH_2O, pH 7.0 ± 0.2 at 25°C.

GC agar (Oxoid): Special peptone 15 g, corn starch 1 g, Sodium chloride 5 g, Dipotassium hydrogen phosphate 4 g, Potassium dihydrogen phosphate 1 g, Agar 10 g made up to 1000 mL with dH_2O , pH 7.2 ± 0.2 at 25°C.

GC Broth medium: Protease peptone 15 g, Potassium phosphate dibasic 4 g, Potassium phosphate monobasic 1 g, Sodium chloride 5 g made up to 1000 mL with dH_2O, pH 7.2 ± 0.2 at 25°C.

Iron Supplement: Ferric nitrate ($Fe(NO_3)_3$) 1.25 g in 250 ml dH_2O. Filter sterilised and then stored at 4°C.

D-Glucose Supplement: D-Glucose 400 g in 700 ml dH_2O dissolved by stirring on warm plate. After cooled, 10 g L-glutamine and 20 mg Co-carboxylase (Thiamine pyrophosphate) were added. Volume brought to 1 L. Filter sterilized and then stored at 4 °C.

IPTG (Isopropyl-β-D thiogalactopyranoside): (FW 238.8) for 1 M solution, 0.23 g of IPTG dissolved in 1 ml dH2O, sterilized by filtration and stored at -20˚C.

TAE buffer (Tris-Acetate-EDTA buffer, 1x): 40 mM Tris base, 40 mM Acetic acid and 1 mM EDTA.

TBS-T (Tris Buffered Saline-Tween, 1 x): 10mM Tris/HCl, pH 7.4, 75mM NaCl, 0.05% Tween-20 (v/v).

TAE buffer (50 × stock): 242 g Tris base (Life Technologies), 57.1 ml glacial acetic acid (Fisher chemicals), 100 ml 500 mM EDTA (pH 8.0) made up to 1 L with dH2O.

Appendix II- Vector maps and list of generated plasmids

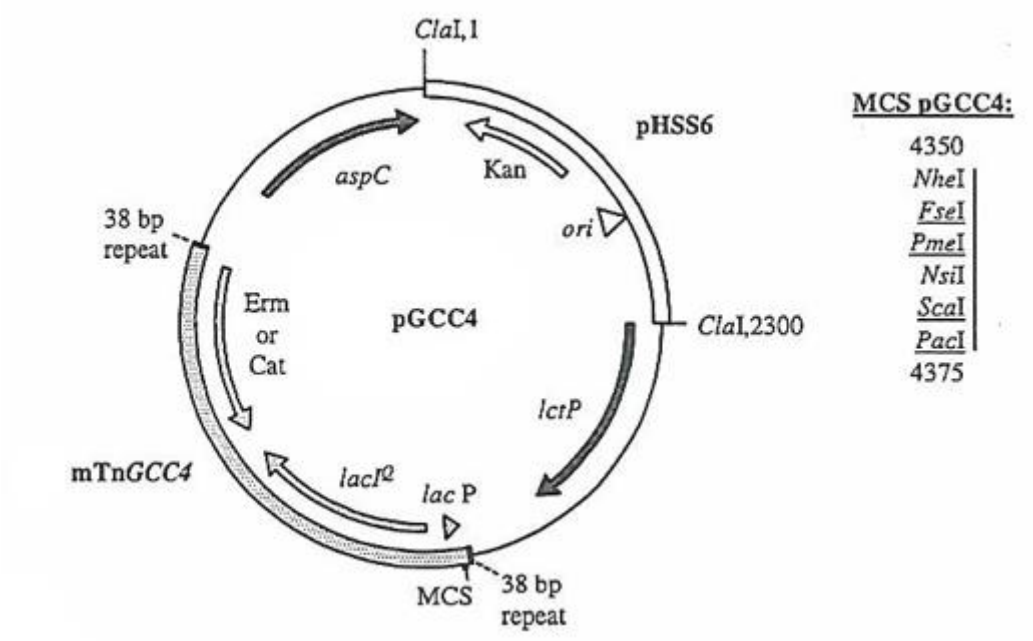


Figure- pGCC4 vector map. Multiple cloning site (MCS) is shown on the right (vector map from Addgene website)

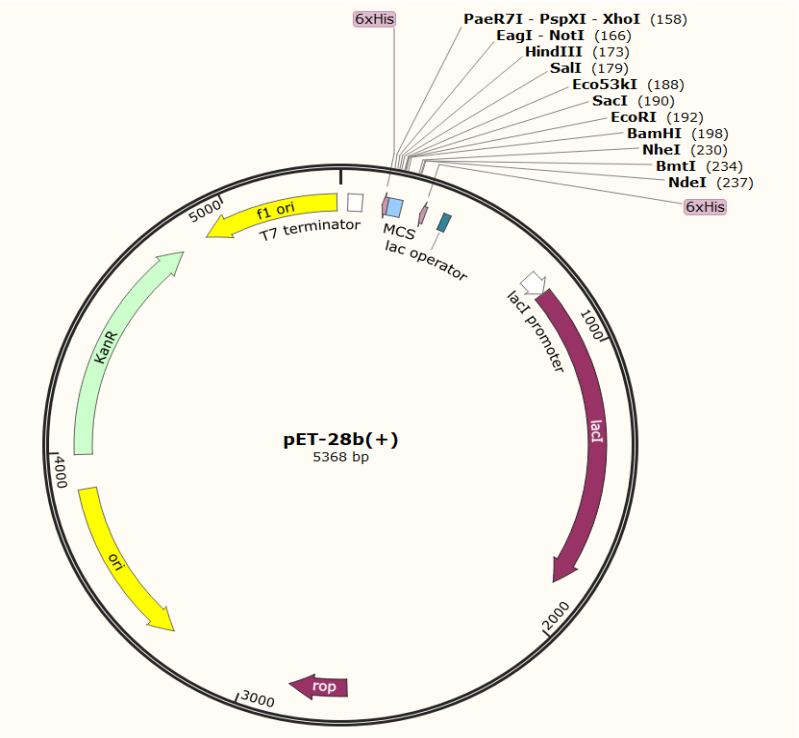


Figure- pET-28b(+) vector map. Multiple cloning site (MCS) is shown along with 6x His tag encoding region (vector map from SnapGene website)

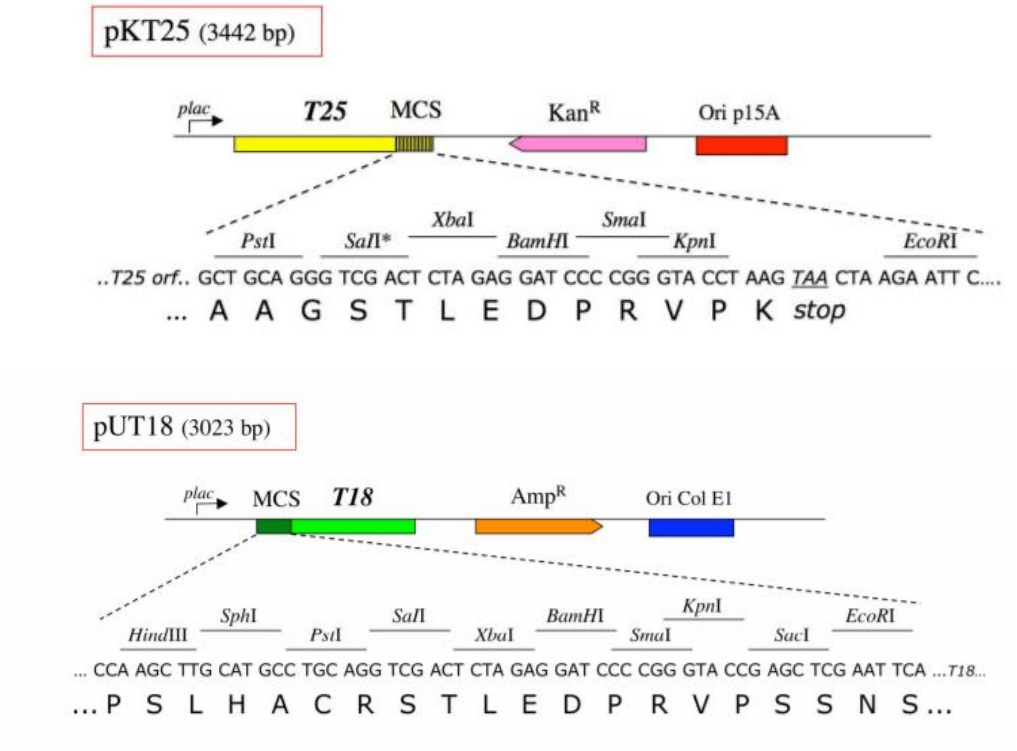


Figure- BACTH vector maps (image from Euromedex BACTH manual)

Table- List of generated plasmids

Chapter 3	**Chapter 5**
pMOD$_{TM}$-2<Tn5KAN-2DUS>	pGCC4*fHbp*MC58
pET28b_S*fHbp*His	pGCC4*fHbp*L91543
pGCC4S*fHbp*His	pGCC4L*fHbp*SNP1
	pGCC4L*fHbp*SNP2
	pGCC4L*fHbp*SNP1+2
	pUT18_*fHbp*MC58
	pUT18_*fHbp*L91543
	pKT25_*secA*

Appendix III- Alignment of pGCC4S*fHbpHIS* against MC58 *fHbp.*

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50
FHbp_MC58       ---------- ---------- ---------- ---------- ----------
Fwd             GCGTCAGGCA GCCATCGGAA GCTGTGGTAT GGCTGTGCAG GTCGTAAATC
Rev             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
FHbp_MC58       ---------- ---------- ---------- ---------- ----------
Fwd             ACTGCATAAT TCGTGTCGCT CAAGGCGCAC TCCCGTTCTG GATAATGTTT
Rev             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
FHbp_MC58       ---------- ---------- ---------- ---------- ----------
Fwd             TTTGCGCCGA CATCATAACG GTTCTGGCAA ATATTCTGAA ATGAGCTGTT
Rev             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
FHbp_MC58       ---------- ---------- ---------- ---------- ----------
Fwd             GACAATTAAT CATCGGCTCG TATAATGTGT GGAATTGTGA GCGGATAACA
Rev             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
FHbp_MC58       ---------- ---------- ---------- ---------- ----------
Fwd             ATTTCACACA GGAAACTAGG CACCCCAGGC TTTACACTTT ATGCTTCCGG
Rev             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       260        270        280        290        300
FHbp_MC58       ---------- ---------- ---------- ---------- ----------
Fwd             CTCGTATGTT GTGTGGAATT GTGAGCGGAT AACAATTTCA CACAGGAAAC
Rev             ---------- ---------- ---------- ---------- -ACAGGAA-C

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       310        320        330        340        350
FHbp_MC58       ---------- ---------- ---------T TAATTAAATG CCGTCTGAAA
Fwd             AGCTATGACC ATGATTACGA ATTCCCGGAT TAATTAAATG CCGTCTGAAA
Rev             AGCTATGACC ATGATTACGA ATTCCCGGAT TAATTAAATG CCGTCTGAAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       360        370        380        390        400
FHbp_MC58       TGCCGTCTGA ACCGCCGTTC GGACGACATT TGATTTTTGC TTCTTTGACC
Fwd             TGCCGTCTGA ACCGCCGTTC GGACGACATT TGATTTTTGC TTCTTTGACC
Rev             TGCCGTCTGA ACCGCCGTTC GGACGACATT TGATTTTTGC TTCTTTGACC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       410        420        430        440        450
FHbp_MC58       TGCCTCATTG ATGCGGTATG CAAAAAAAGA TACCATAACC AAAATGTTTA
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Fwd          TGCCTCATTG ATGCGGTATG CAAAAAAAGA TACCATAACC AAAATGTTTA
Rev          TGCCTCATTG ATGCGGTATG CAAAAAAAGA TACCATAACC AAAATGTTTA


             ....|....| ....|....| ....|....| ....|....| ....|....|
                     460        470        480        490        500
FHbp_MC58    TATATTATCT ATTCTGCGTA TGACTAGGAG TAAACCTGTG AATCGAACTG
Fwd          TATATTATCT ATTCTGCGTA TGACTAGGAG TAAACCTGTG AATCGAACTG
Rev          TATATTATCT ATTCTGCGTA TGACTAGGAG TAAACCTGTG AATCGAACTG


             ....|....| ....|....| ....|....| ....|....| ....|....|
                     510        520        530        540        550
FHbp_MC58    CCTTCTGCTG CCTTTCTCTG ACCACTGCCC TGATTCTGAC CGCCTGCAGC
Fwd          CCTTCTGCTG CCTTTCTCTG ACCACTGCCC TGATTCTGAC CGCCTGCAGC
Rev          CCTTCTGCTG CCTTTCTCTG ACCACTGCCC TGATTCTGAC CGCCTGCAGC


             ....|....| ....|....| ....|....| ....|....| ....|....|
                     560        570        580        590        600
FHbp_MC58    AGCGGAGGGG GTGGTGTCGC CGCCGACATC GGTGCGGGGC TTGCCGATGC
Fwd          AGCGGAGGGG GTGGTGTCGC CGCCGACATT GGTGCGGGGC TTGCCGATGC
Rev          AGCGGAGGGG GTGGTGTCGC CGCCGACATT GGTGCGGGGC TTGCCGATGC


             ....|....| ....|....| ....|....| ....|....| ....|....|
                     610        620        630        640        650
FHbp_MC58    ACTAACCGCA CCGCTCGACC ATAAAGACAA AGGTTTGCAG TCTTTGACGC
Fwd          ACTAACCGCA CCGCTCGACC ATAAAGACAA AGGTTTGCAG TCTTTGACGC
Rev          ACTAACCGCA CCGCTCGACC ATAAAGACAA AGGTTTGCAG TCTTTGACGC


             ....|....| ....|....| ....|....| ....|....| ....|....|
                     660        670        680        690        700
FHbp_MC58    TGGATCAGTC CGTCAGGAAA AACGAGAAAC TGAAGCTGGC GGCACAAGGT
Fwd          TGGATCAGTC CGTCAGGAAA AACGAGAAAC TGAAGCTGGC GGCACAAGGT
Rev          TGGATCAGTC CGTCAGGAAA AACGAGAAAC TGAAGCTGGC GGCACAAGGT


             ....|....| ....|....| ....|....| ....|....| ....|....|
                     710        720        730        740        750
FHbp_MC58    GCGGAAAAAA CTTATGGAAA CGGTGACAGC CTCAATACGG GCAAATTGAA
Fwd          GCGGAAAAAA CTTATGGAAA CGGTGACAGC CTCAATACGG GCAAATTGAA
Rev          GCGGAAAAAA CTTATGGAAA CGGTGACAGC CTCAATACGG GCAAATTGAA


             ....|....| ....|....| ....|....| ....|....| ....|....|
                     760        770        780        790        800
FHbp_MC58    GAACGACAAG GTCAGCCGTT TCGACTTTAT CCGCCAAATC GAAGTGGACG
Fwd          GAACGACAAG GTCAGCCGTT TCGACTTTAT CCGCCAAATC GAAGTGGACG
Rev          GAACGACAAG GTCAGCCGTT TCGACTTTAT CCGCCAAATC GAAGTGGACG


             ....|....| ....|....| ....|....| ....|....| ....|....|
                     810        820        830        840        850
FHbp_MC58    GGCAGCTCAT TACCTTGGAG AGTGGAGAGT TCCAAGTATA CAAACAAAGC
Fwd          GGCAGCTCAT TACCTTGGAG AGTGGAGAGT TCCAAGTATA CAAACTCGAG
Rev          GGCAGCTCAT TACCTTGGAG AGTGGAGAGT TCCAAGTATA CAAACTCGAG


             ....|....| ....|....| ....|....| ....|....| ....|....|
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                 860        870        880        890        900
FHbp_MC58    CATTCCGCCT TAACCGCCTT TCAGACCGAG CAAATACAAG ATTCGGAGCA
Fwd          CA--CCACCA CCACCACCAC TGAGATCCGG CTGCTAACAA AGTTTAAACG
Rev          CA--CCACCA CCACCACCAC TGAGATCCGG CTGCTAACAA AGTTTAAACG

             ....|....| ....|....| ....|....| ....|....| ....|....|
                 910        920        930        940        950
FHbp_MC58    TTCCGGGAAG ATGGTTGCGA AACGCCAGTT CAGAATCGGC GACATAGCGG
Fwd          GCCGGCCCTA GTGCTAGCGG ATCCCC---- ---------- --CTTAACG-
Rev          GCCGGCCCTA GTGCTAGCGG ATCCCC---- ---------- --CTTAACG-

             ....|....| ....|....| ....|....| ....|....| ....|....|
                 960        970        980        990        1000
FHbp_MC58    GCGAACATAC ATCTTTTGAC AAGCTTCCCG AAGGCGGCAG GGCGACATAT
Fwd          -TGAGTTT-- -TCGTTCCAC TGAGCGTCAG ACCCCGAAAC G---------
Rev          -TGAGTTT-- -TCGTTCCAC TGAGCGTCAG ACCCCGAAAC GAGGGTATAG

             ....|....| ....|....| ....|....| ....|....| ....|....|
                 1010       1020       1030       1040       1050
FHbp_MC58    CGCGGGACGG --CGTTCGGT TCAGACGATG CCGGCGGAAA ACTGACCTAC
Fwd          ---------- ---------- ---------- ---------- ----------
Rev          AGCAGAACGG ATGGTTCTTG TTGGCGGATG TCTTCAGGAA GGGTAAGCGC

             ....|....| ....|....| ....|....| ....|....| ....|....|
                 1060       1070       1080       1090       1100
FHbp_MC58    ACCATAGATT TCGCCGCCAA GCAGGGAAAC GGCAAAATCG AACATTTGAA
Fwd          ---------- ---------- ---------- ---------- ----------
Rev          AGTCATGGTA T-GCCGTCTG AAAAGTGGGG ATTATAGCGG ATTGCGGCTT

             ....|....| ....|....| ....|....| ....|....| ....|....|
                 1110       1120       1130       1140       1150
FHbp_MC58    ATCGCCAGAA CTCAATGTCG ACCTGGCCGC CGCCGATATC AAGCCGGATG
Fwd          ---------- ---------- ---------- ---------- ----------
Rev          TGCGCCGAAA ----ATATCC TTTAGCCTGC CGATGGCGTA AAATGGGCGC

             ....|....| ....|....| ....|....| ....|....| ....|....|
                 1160       1170       1180       1190       1200
FHbp_MC58    GAAAACGCCA TGCCGTCATC AGCGGTTCCG TCCTTTACAA CCAAGCCGAG
Fwd          ---------- ---------- ---------- ---------- ----------
Rev          ACGCCAACCA CGCAAAGGAA AATCAAATGG ACAATCTGAA TCCG--CAGG

             ....|....| ....|....| ....|....| ....|....| ....|....|
                 1210       1220       1230       1240       1250
FHbp_MC58    AAAGGCAGTT ACTCCCTCGG TATCTTTGGC GGAAAAGCCC AGGAAGTTGC
Fwd          ---------- ---------- ---------- ---------- ----------
Rev          AAATTTCCGT GTTGCCGGAA AATCTGCCGC TGTATTGCTC GGGACCGGAC

             ....|....| ....|....| ....|....| ....|....| ....|....|
                 1260       1270       1280       1290       1300
FHbp_MC58    CGGCAGCGCG GAAGTGAAAA CCGTAAACGG CATACGCCAT ATCGGCCTTG
Fwd          ---------- ---------- ---------- ---------- ----------
Rev          AACGAGCATT GGAACGGGCA TCCGAGGGT- ---------- ----------
```

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                ....|....| ....
                        1310
FHbp_MC58       CCGCCAAGCA ATAA
Fwd             ---------- ----
Rev             ---------- ----
```

Alignment of pGCC4*fHbp*MC58 against MC58 *fHbp*

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
MC58            ---------- ---------- ---------- ---------- ----------
Fwd             NNNANNTTTN NNANAGGAAA CAGCTATGAC CATGATTACG AATTCCCGGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90        100
MC58            ---------- ---------- ---------- ---------- ----------
Fwd             TTAATTAATT GCTTCTTTGA CCTGCCCGCC AAGCAATAAC CATTGTTTAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
MC58            ---------- -ATGCCGTCT GAACCGCCGT TCGGACGACA TTTGATTTTT
Fwd             ACAATGGTTA TATGCCGTCT GAACCGCCGT TCGGACGACA TTTGATTTTT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        160        170        180        190        200
MC58            GCTTCTTTGA CCTGCCTCAT TGATGCGGTA TGCAAAAAAA GATACCATAA
Fwd             GCTTCTTTGA CCTGCCTCAT TGATGCGGTA TGCAAAAAAA GATACCATAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        210        220        230        240        250
MC58            CCAAAATGTT TATATATTAT CTATTCTGCG TATGACTAGG AGTAAACCTG
Fwd             CCAAAATGTT TATATATTAT CTATTCTGCG TATGACTAGG AGTAAACCTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        260        270        280        290        300
MC58            TGAATCGAAC TGCCTTCTGC TGCCTTTCTC TGACCACTGC CCTGATTCTG
Fwd             TGAATCGAAC TGCCTTCTGC TGCCTTTCTC TGACCACTGC CCTGATTCTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350
MC58            ACCGCCTGCA GCAGCGGAGG GGGTGGTGTC GCCGCCGACA TCGGTGCGGG
Fwd             ACCGCCTGCA GCAGCGGAGG GGGTGGTGTC GCCGCCGACA TCGGTGCGGG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        360        370        380        390        400
MC58            GCTTGCCGAT GCACTAACCG CACCGCTCGA CCATAAAGAC AAAGGTTTGC
Fwd             GCTTGCCGAT GCACTAACCG CACCGCTCGA CCATAAAGAC AAAGGTTTGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        410        420        430        440        450
MC58            AGTCTTTGAC GCTGGATCAG TCCGTCAGGA AAAACGAGAA ACTGAAGCTG
Fwd             AGTCTTTGAC GCTGGATCAG TCCGTCAGGA AAAACGAGAA ACTGAAGCTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        460        470        480        490        500
MC58            GCGGCACAAG GTGCGGAAAA AACTTATGGA AACGGTGACA GCCTCAATAC
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Fwd          GCGGCACAAG GTGCGGAAAA AACTTATGGA AACGGTGACA GCCTCAATAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       510        520        530        540        550
MC58         GGGCAAATTG AAGAACGACA AGGTCAGCCG TTTCGACTTT ATCCGCCAAA
Fwd          GGGCAAATTG AAGAACGACA AGGTCAGCCG TTTCGACTTT ATCCGCCAAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       560        570        580        590        600
MC58         TCGAAGTGGA CGGGCAGCTC ATTACCTTGG AGAGTGGAGA GTTCCAAGTA
Fwd          TCGAAGTGGA CGGGCAGCTC ATTACCTTGG AGAGTGGAGA GTTCCAAGTA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       610        620        630        640        650
MC58         TACAAACAAA GCCATTCCGC CTTAACCGCC TTTCAGACCG AGCAAATACA
Fwd          TACAAACAAA GCCATTCCGC CTTAACCGCC TTTCAGACCG AGCAAATACA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       660        670        680        690        700
MC58         AGATTCGGAG CATTCCGGGA AGATGGTTGC GAAACGCCAG TTCAGAATCG
Fwd          AGATTCGGAG CATTCCGGGA AGATGGTTGC GAAACGCCAG TTCAGAATCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                     710        720        730        740        750
MC58         GCGACATAGC GGGCGAACAT ACATCTTTTG ACAAGCTTCC CGAAGGCGGC
Fwd          GCGACATAGC GGGCGAACAT ACATCTTTTG ACAAGCTTCC CGAAGGCGGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       760        770        780        790        800
MC58         AGGGCGACAT ATCGCGGGAC GGCGTTCGGT TCAGACGATG CCGGCGGAAA
Fwd          AGGGCGACAT ATCGCGGGAC GGCGTTCGGT TCAGACGATG CCGGCGGAAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       810        820        830        840        850
MC58         ACTGACCTAC ACCATAGATT TCGCCGCCAA GCAGGGAAAC GGCAAAATCG
Fwd          ACTGACCTAC ACCATAGATT TCGCCGCCAA GCAGGGAAAC GGCAAAATCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       860        870        880        890        900
MC58         AACATTTGAA ATCGCCAGAA CTCAATGTCG ACCTGGCCGC CGCCGATATC
Fwd          AACATTTGAA ATCGCCAGAA CTCAATGTCG ACCTGGCCGC CGCCGATATC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       910        920        930        940        950
MC58         AAGCCGGATG GAAAACGCCA TGCCGTCATC AGCGGTTCCG TCCTTTACAA
Fwd          AAGCCGGATG GAAAACGCCA TGCCGTCATC AGCGGTTCCG TCCTTTACAA

               ....|....| ....|....| ....|....| ....|....| ....|....|
                  960        970        980        990       1000
MC58         CCAAGCCGAG AAAGGCAGTT ACTCCCTCGG TATCTTTGGC GGAAAAGCCC
Fwd          CCAAGCCGAG AAAGGCAGTT ACTCCCTCGG TATCTTTGGC GGAAAAGCCC
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                  1010       1020       1030       1040       1050
MC58            AGGAAGTTGC CGGCAGCGCG GAAGTGAAAA CCGTAAACGG CATACGCCAT
Fwd             AGGAAGTTGC CGGCAGCGCG GAAGTGAAAA CCGTAAACGG CATACGCCAT

                ....|....| ....|....| ....
                        1060       1070
MC58            ATCGGCCTTG CCGCCAAGCA ATAA
Fwd             ATCGGCCTTG CCGCCAAGCA ATAA
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Alignment of pGCC4*fHbp*L91543 against L91543 *fHbp*

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
L91543          ---------- ---------- ---------- ---------- ----------
Fwd             TNNNANAGGA AACAGCTATG ACCATGATTA CGAATTCCCG GATTAATTAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90        100
L91543          ---------- ---------- ---------- ---------- ----------
Fwd             TTGCTTCTTT GACCTGCCCG CCAAGCAATA ACCATTGTTT AAACAATGGT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
L91543          ---------- ---------- ---------- ---------- ----------
Fwd             TATTGCTTGG CGGCAAGGCC GATATGGCGT ATGCCGTTTA CGGTTTTCAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        160        170        180        190        200
L91543          ---------- ---------- ---------- --TTACTGCT TGGCGGCAAG
Fwd             TTCCGCGCTG CCGGCAACTT CCTGGGCTTT TCTTACTGCT TGGCGGCAAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        210        220        230        240        250
L91543          ACCGATATGG TGTATGCCGT TTGCGGTTTC CACTTCCGCG CTGCCGGCAA
Fwd             ACCGATATGG TGTATGCCGT TTGCGGTTTC CACTTCCGCG CTGCCGGCAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        260        270        280        290        300
L91543          CTTCCTGGGC TTGCCCGCCA AAGATACCGA GGGAGTAACT GCCTTTCTCG
Fwd             CTTCCTGGGC TTGCCCGCCA AAGATACCGA GGGAGTAACT GCCTTTCTCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350
L91543          TCTTGGTTGT AAAGGACGGA ACCGCTGATA ACGGCATGGC GTTTTTCATC
Fwd             TCTTGGTTGT AAAGGACGGA ACCGCTGATA ACGGCATGGC GTTTTTCATC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        360        370        380        390        400
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L91543      CGGCTTGATA TAGGCGGTGG CAAGCTCGAC ATTGAGTTCG GGCGATTTCA
Fwd         CGGCTTGATA TAGGCGGTGG CAAGCTCGAC ATTGAGTTCG GGCGATTTCA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       410        420        430        440        450
L91543      AATGTTCGAT TTTGCCGTGT CCCTGCTTGG CGGCGAAATC TATAGTATAG
Fwd         AATGTTCGAT TTTGCCGTGT CCCTGCTTGG CGGCGAAATC TATAGTATAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       460        470        480        490        500
L91543      GTCAGTTTTC CGCCAGCATC GTCTGAACCG AACGCCGTCC CGCGATATGT
Fwd         GTCAGTTTTC CGCCAGCATC GTCTGAACCG AACGCCGTCC CGCGATATGT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       510        520        530        540        550
L91543      CGCACTGCCG CCTTTGGGAA GCTTGTCAAA AGATGTATGT TCGCCCGCTA
Fwd         CGCACTGCCG CCTTTGGGAA GCTTGTCAAA AGATGTATGT TCGCCCGCTA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       560        570        580        590        600
L91543      TGTCGCCGAT TCTGAACTGG CGTTTCGCAA CCATCTTCCC GGAATCCTCC
Fwd         TGTCGCCGAT TCTGAACTGG CGTTTCGCAA CCATCTTCCC GGAATCCTCC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       610        620        630        640        650
L91543      GAGTCTTGTA CTTGCTCGGT CTGAAGGGCG GTTAAGGCGG AATGGCTTTG
Fwd         GAGTCTTGTA CTTGCTCGGT CTGAAGGGCG GTTAAGGCGG AATGGCTTTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       660        670        680        690        700
L91543      TTTGTACACT TGGAACTCTC CGCTCTCCAA GGTAATGAGC TTCCCGTCCA
Fwd         TTTGTACACT TGGAACTCTC CGCTCTCCAA GGTAATGAGC TTCCCGTCCA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       710        720        730        740        750
L91543      CTTCGATTTG ACGGATAAAG TCGAAGCGGC TGACCTTGTC GTTCTTCAAT
Fwd         CTTCGATTTG ACGGATAAAG TCGAAGCGGC TGACCTTGTC GTTCTTCAAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       760        770        780        790        800
L91543      TTGCCCGTAT TAAGGCTGTC GCCGTTTCCA TAAGTTTTTT CCGCACCTTG
Fwd         TTGCCCGTAT TAAGGCTGTC GCCGTTTCCA TAAGTTTTTT CCGCACCTTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       810        820        830        840        850
L91543      TGCCGCCAGC TTCAGTTTCT CGTTTTTCCT GACGGACTGA TCCAGCGTTA
Fwd         TGCCGCCAGC TTCAGTTTCT CGTTTTTCCT GACGGACTGA TCCAGCGTTA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       860        870        880        890        900
L91543      AAGACTGCAA ACCTTTGTCT TTATGGTCGA GCGGTGCGGT TAGTGCATCG
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Fwd          AAGACTGCAA ACCTTTGTCT TTATGGTCGA GCGGTGCGGT TAGTGCATCG


             ....|....| ....|....| ....|....| ....|....| ....|....|
                     910        920        930        940        950
L91543       GCAAGCCCCG CACCGATGTC GGCGGCGACA CCGCCCCCTC CGCTGCTGCA
Fwd          GCAAGCCCCG CACCGATGTC GGCGGCGACA CCGCCCCCTC CGCTGCTGCA


             ....|....| ....|....| ....|....| ....|....| ....|....|
                     960        970        980        990       1000
L91543       GGCGGTCAGA ATCAGGGCGG CGGTCAGAGA AAAGCAGCAG AAGGCAGTTC
Fwd          GGCGGTCAGA ATCAGGGCGG CGGTCAGAGA AAAGCAGCAG AAGGCAGTTC


             ....|....| ....|....| ....|....| ....|....| ....|....|
                    1010       1020       1030       1040       1050
L91543       GGTTCACAGG TTTACTCCTA GTCATACGCA GAATAGATAA TATATAAACA
Fwd          GGTTCACAGG TTTACTCCTA GTCATACGCA GAATAGATAA TATATAAACA


             ....|....| ....|....| ....|....| ....|....| ....|....|
                    1060       1070       1080       1090       1100
L91543       TTTTGGTTAT GGTATCTTTT TTTGCATACT GCATCAATGA GGCAGGTCAA
Fwd          TTTTGGTTAT GGTATCTTTT TTTGCATACT GCATCAATGA GGCAGGTCAA


             ....|....| ....|....| ....|....| ....|....| ....|....|
                    1110       1120       1130       1140       1150
L91543       AGAAGCAAAA ATCAAATGCC GTCCGAACAA CGGTTCAGAC GGCAT-----
Fwd          AGAAGCAAAA ATCAAATGCC GTCCGAACAA CGGTTCAGAC GGCATTAAGC


             ....|...

L91543       --------
Fwd          GTAGCTGC
```

Alignment of pGCC4L*fHbp*SNP2 against L91543 *fHbp*

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
L91543          ---------- ---------- ---------- ---------- ----------
Fwd             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90        100
L91543          ---------- ---------- ---------- ---------- ----------
Fwd             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
L91543          ---------- ---------- ---------- ---------- ----------
Fwd             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        160        170        180        190        200
L91543          ---------- ---------- ---------- --TTACTGCT TGGCGGC-AA
Fwd             -NNNAAANNT NNNGAAAAGC CCAGGAAGTT GCCGGCAGCG CGGAAGTGAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        210        220        230        240        250
L91543          GACCG-ATAT GGTGTATGCC GTTTGCGGTT TCCACTTCCG CGCTGCCGGC
Fwd             AACCGTAAAC GGCATACGCC ATATCGGCCT TGCCGCCAAG CAATAACCAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        260        270        280        290        300
L91543          AACTT---CC TGGGCT--TG CCCGCCAAAG ATACCGAGGG AGTAACTGCC
Fwd             TGTTTAAACA ATGGTTATTG CTTGGCGGGC AGGTCAAAGA AGCAATTAAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350
L91543          T--TTCTCGT CTTGGTTGTA AAGG----AC GGAACCGCTG ATAACGGCAT
Fwd             TAATCCGGGA ATTCGTAATC ATGGTCCCAT NNTNTNNNAN NTNNNNNNNN

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        360        370        380        390        400
L91543          GGCGTTTTTC ATCCGGCTTG ATATAGGCGG ---TGGCAAG CTCGACATTG
Fwd             NNNNNNNNNN TCCTGCCTTC ATTTGGANCG GTATGCAAAA AAAGATACCA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        410        420        430        440        450
L91543          -AGTTCGGGC GATTTCAAAT GTTCGATTTT GC-CGTGTCC CTGCTTGGCG
Fwd             TAACCAAAAT GTTTATATAT TATCTATTCT GCGTATGACT AGGAGTAAAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        460        470        480        490        500
L91543          GCG-AAATCT A--TAGTATA GGTCAGTTTT CCGCCAGCAT CGTC-TGAAC
Fwd             CTGTGAATCG AACTGCCTTC TGCTGCTTTT CTCTGACCGC CGCCCTGATT
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        510        520        530        540        550
L91543          CGAACGCCGT CCCGCGAT-- ATGTCGCACT GCCGCCTTTG GGAAGCTTGT
Fwd             CTGACCGCCT GCAGCAGCGG AGGGGGTGGT GTCGCCGCCG ACATCGGTG-

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        560        570        580        590        600
L91543          CAAAAGATGT ATGTTCGCCC GCTATGTCGC -CGATTCTGA AC-TGGCGTT
Fwd             CGGGGCTTGC CGATGCACTA ACCGCACCGC TCGACCATAA AGACAAAGGT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        610        620        630        640        650
L91543          TCGCAACCAT CTTCCCGGAA TCCTCCGAGT CTTGTACTTG CTCGGTCTGA
Fwd             TTGCAGTCTT TGACGCTGGA TCAGTCC-GT CAGGAAAAAC GAGAAACTGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        660        670        680        690        700
L91543          AG--GGCGGT -TAAGG--CG GAATGGCTTT GTTTGTACAC TTGGAACTCT
Fwd             AGCTGGCGGC ACAAGGTGCG GAAAAAACTT ATGGAAACGG TGACAGCCTC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        710        720        730        740        750
L91543          CCGCTCTCCA AGGTAATGAG CTTC----CC GTCCACTTCG ATTTGACGGA
Fwd             AATACGGGCA AATTGAAGAA CGACAAGGTC AGCCGTTTCG ACTTTATCCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        760        770        780        790        800
L91543          TAAAGTCGAA GCGGCTGACC TTGTCGTTCT TC----AATT TGCCCGTATT
Fwd             CCAAATCGAA GTGGACGGGC AGCTCATTAC CTTGGAGAGT GGAGAGTTCC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        810        820        830        840        850
L91543          AAGGCTGTCG CCGTTTCCAT AA-GTTTTTT CCGCACCTTG TGCCG--CCA
Fwd             AAGTATACAA ACAAAGCCAT TCCGCCTTAA CCGCCTTTCA GACCGAGCAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        860        870        880        890        900
L91543          GCTTCAGTTT CTCGTTTTTC CTGACGGA-- ---------- -------CTG
Fwd             ATACAAGATT CGGAGCATTC CGGGAAGATG GTTGCGAAAC GCCAGTTCAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        910        920        930        940        950
L91543          ATCCAGCGTT AAAGACTGCA AACCTTTGTC TTTA------ -TGGTCGAGC
Fwd             AATCGGCGAC ATAGCGGGCG AACATACATC TTTTGACAAG CTTCCCGAAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        960        970        980        990       1000
L91543          GGTGC-GGTT AGTGCATCGG CAAGCCCCGC AC-------- CGATGTCGGC
Fwd             GCGGCAGGGC GACATATCGC GGGACGGCGT TCGGTTCAGA CGATGCCGGC
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1010       1020       1030       1040       1050
L91543          GGCGACACCG CCCCCTCCGC TGCTGCAGGC GGTCAGAATC AGGGCGGCGG
Fwd             GGAAAACTGA CCTACACCAT AGATTTCGCC GCCAAGCAGG GAAACGGCAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1060       1070       1080       1090       1100
L91543          --TCAGAGA- ---AAAGCAG CAGAAGGCAG TTCGGTTCAC AGGTTTACTC
Fwd             AATCGAACAT TTGAAATCGC CAGAACTCAA T---GTCGAC CTGGCCGCCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1110       1120       1130       1140       1150
L91543          CTAGTCATAC GCAGAATAGA TAATATATAA ACATTTTGGT TATGGTATCT
Fwd             CCGATATCAA GCCGGATGGA AAACGCCATG CCGTCATCA- -GCGGTTCCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1160       1170       1180       1190       1200
L91543          TTTTTTGCAT ACTGCATCAA TGAGGCAG-- ---------- -GTCAAAGAA
Fwd             TCCTTTACAA CCAAGCCGAG AAAGGCAGTT ACTCCCTCGG TATCTTTGGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1210       1220       1230       1240       1250
L91543          GCAAAAATCA AA-----TGC CGTCCGAACA ACGGTTCAGA CGGCAT----
Fwd             GGAAAAGCCC AGGAAGTTGC CGGCAGCGCG GAAGTGAAAA CCGTAAACGG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1260       1270       1280       1290       1300
L91543          ---------- ---------- ---------- ---------- ----------
Fwd             CATACGCCAT ATCGGCCTTG CCGCCAAGCA ATAACCATTG TTTAAACAAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1310       1320       1330       1340       1350
L91543          ---------- ---------- ---------- ---------- ----------
Fwd             GGTTATTGCT TGGCGGGCAG GTCAAAGAAG CAATTAATTA ATCCGGGAAT

                ....|....| ....|....| ....|....
                        1360       1370
L91543          ---------- ---------- ---------
Fwd             TCGTAATCAT GGTCATAGCT GTTTCCTNT
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Alignment of pGCC4L*fHbp*SNP1 against L91543 *fHbp*

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       560        570        580        590        600
L91543_FHb      GGGCGGTTAA GGCGGAATGG CTTTGTTTGT ACACTTGGAA CTCTCCGCTC
Rev             AGGCGGTTAA GGCGGAATGG CTTTGTTTGT ATACTTGGAA CTCTCCACTC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       610        620        630        640        650
L91543_FHb      TCCAAGGTAA TGAGCTTCCC GTCCACTTCG ATTTGACGGA TAAAGTCGAA
Rev             TCCAAGGTAA TGAGCTGCCC GTCCACTTCG ATTTGGCGGA TAAAGTCGAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       660        670        680        690        700
L91543_FHb      GCGGCTGACC TTGTCGTTCT TCAATTTGCC CGTATTAAGG CTGTCGCCGT
Rev             ACGGCTGACC TTGTCGTTCT TCAATTTGCC CGTATTGAGG CTGTCACCGT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       710        720        730        740        750
L91543_FHb      TTCCATAAGT TTTTTCCGCA CCTTGTGCCG CCAGCTTCAG TTTCTCGTTT
Rev             TTCCATAAGT TTTTTCCGCA CCTTGTGCCG CCAGCTTCAG TTTCTCGTTT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       760        770        780        790        800
L91543_FHb      TTCCTGACGG ACTGATCCAG CGTTAAAGAC TGCAAACCTT TGTCTTTATG
Rev             TTCCTGACGG ACTGATCCAG CGTCAAAGAC TGCAAACCTT TGTCTTTATG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       810        820        830        840        850
L91543_FHb      GTCGAGCGGT GCGGTTAGTG CATCGGCAAG CCCCGCACCG ATGTCGGCGG
Rev             GTCGAGCGGT GCGGTTAGTG CATCGGCAAG CCCCGCACCG ATGTCGGCGG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       860        870        880        890        900
L91543_FHb      CGACACCGCC CCCTCCGCTG CTGCAGGCGG TCAGAATCAG GGCGGCGGTC
Rev             CGACACCACC CCCTCCGCTG CTGCAGGCGG TCAGAATCAG GGCAGTGGTC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       910        920        930        940        950
L91543_FHb      AGAGAAAAGC AGCAGAAGGC AGTTCGGTTC ACAGGTTTAC TCCTAGTCAT
Rev             AGAGAAAGGC AGCAGAAGGC AGTTCGATTC ACAGGTTTAC TCCTAGTCAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       960        970        980        990        1000
L91543_FHb      ACGCAGAATA GATAATATAT AAACATTTTG GTTATGGTAT CTTTTTTTGC
Rev             ACGCAGAATA GATAATATAT AAACATTTTG GTTATGGTAT CTTTTTTTGC

                     ....|....| ....|....| ....|....| ....|....| ....|....|
                       1010       1020       1030       1040       1050
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L91543_FHb   ATACTGCATC AAT--GAGGC AGGTCAAAGA AGCAAAAATC AAATGCCGTC
Rev          ATACCGNTCC AAATGAAGGC AGG------- ---------- ----------


             ....|....| ....|....| ..
                     1060       1070
L91543_FHb   CGAACAACGG TTCAGACGGC AT
Rev          ---------- ---------- --
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Alignment of *fHbp* SOE final product against *kan* gene sequence flanked by *fHbp* regions

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50
FHbp_SOE        CGTCCGCAGC GGCGAGTATG CAGCTTTGCG GCGGCGGCAG CGAGGCGGCG
Fwd             ---------- ---------- ---------N NNNNNCNNNN NNNNGCGGCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
FHbp_SOE        GCATCGAGCG TGGGGATGCC GATTAAAGGC GTATCAAACG GCGTTGCCAA
Fwd             GCATCGAGCG TGGGGATGCC GATTAAAGGC GTATCAAACG GCGTTGCCAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
FHbp_SOE        ACCTTGCGCC ACGCCGATGC CGATACGCAG TCCCGTAAAC GCGCCGGGGC
Fwd             ACCTTGCGCC ACGCCGATGC CGATACGCAG TCCCGTAAAC GCGCCGGGGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
FHbp_SOE        CTTTCGCATA AACAATCGCC CCCAAATCGG CGGCGGTAAT GCCCGCATTT
Fwd             CTTTCGCATA AACAATCGCC CCCAAATCGG CGGCGGTAAT GCCCGCATTT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
FHbp_SOE        CGGAATAGGG TGCGGATTTC CGGCAGAATC AGTTCGGATT GGCGGCTGCC
Fwd             CGGAATAGGG TGCGGATTTC CGGCAGAATC AGTTCGGATT GGCGGCTGCC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       260        270        280        290        300
FHbp_SOE        GGCCTCTTG- ATGGAACAGG TGGGTTTCGC CGTCGGCGCG CAGCGCGAGC
Fwd             GGCCTCTTG- ATGGAACAGG TGGGTTTCGC CGTCGGCGCG CAGCGCGAGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       310        320        330        340        350
FHbp_SOE        GACAAATAGG AAGTACCGGT ATCGATGGCC AGGACGGGAC GGTTAAAATC
Fwd             GACAAATAGN NAGTACCGGT ATCGATGGCC AGGACGGGAC GGTTAAAATC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       360        370        380        390        400
FHbp_SOE        AGCTTGCATG GTATGGTTCT CGTT-GGTTC AGACGGCATT ATATAGTGAA
Fwd             AGCTTGCATG GTATGGTTCT CGTT-GGTTC AGACGGCATT ATATAGTGAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       410        420        430        440        450
FHbp_SOE        ATCAGCTTGC CTGCCGTGCC GTCGTGTCCT AGGGCGGTAT GGCGCAAAAA
Fwd             ATCAGCTTGC CTGCCGTGCC GTCGTGTCCT AGGGCGGTAT GGCGCAAAAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       460        470        480        490        500
FHbp_SOE        TGCCGTCCGA ACGGTAAATT ATTGTGTT-C GGACGGCATT TTCACAATGG
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Fwd          TGCCGTCCGA ACGGTAAATT ATTGTGTT-C GGACGGCATT TTCACAATGG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       510        520        530        540        550
FHbp_SOE     TTACTGCTTG GCGGCTGTCT CTTATACACA TCTCAACCAT CATC--GATG
Fwd          ---------- ---------- ---------- ---CAACCAT CATC--GATG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       560        570        580        590        600
FHbp_SOE     A-ATTGTGTC TCAAAATCTC TGA--TGTTA CATTG-CACA AGATA-AAAA
Fwd          A-ATTGTGTC TCAAAATCTC TGA--TGTTA CATTG-CACA AGATA-AAAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       610        620        630        640        650
FHbp_SOE     TATATCATCA TGAACAATAA AACTGTCTGC TTACATAAAC AGTAATACAA
Fwd          TATATCATCA TGAACAATAA AACTGTCTGC TTACATAAAC AGTAATACAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       660        670        680        690        700
FHbp_SOE     GGGGTGTTAT GAGCCATATT CAACGGGAAA CGTCTTGCTC GAGGCCGCGA
Fwd          GGGGTGTTAT GAGCCATATT CAACGGGAAA CGTCTTGCTC GAGGCCGCGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       710        720        730        740        750
FHbp_SOE     TTAAATTCCA ACATGGATGC TGATTTATAT GGGT-ATAAA TGGGCTCGCG
Fwd          TTAAATTCCA ACATGGATGC TGATTTATAT GGGT-ATAAA TGGGCTCGCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       760        770        780        790        800
FHbp_SOE     ATAATGTCGG GCAATCAGGT GCGACAATCT ATCGATTGTA TGGGAAGCCC
Fwd          ATAATGTCGG GCAATCAGGT GCGACAATCT ATCGATTGTA TGGGAAGCCC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       810        820        830        840        850
FHbp_SOE     GATGCGCCAG AGTTGTTTCT GAAACATGGC AAAGGTAGCG TTGCCAATGA
Fwd          GATGCGCCAG AGTTGTTTCT GAAACATGGC AAAGGTAGCG TTGCCAATGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       860        870        880        890        900
FHbp_SOE     TGTTACAGAT GAGATGGTCA GACTAAACTG GCTGACGGAA TTTATGCCTC
Fwd          TGTTACAGAT GAGATGGTCA GACTAAACTG GCTGACGGAA TTTATGCCTC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       910        920        930        940        950
FHbp_SOE     TTCCGACCAT CAAGCATTTT ATCCGTACTC CTGATGATGC ATGGTTACTC
Fwd          TTCCGACCAT CAAGCATTTT ATCCGTACTC CTGATGATGC ATGGTTACTC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       960        970        980        990       1000
FHbp_SOE     ACCACTGCGA TCCCCGGAAA AACAGCATTC CAGGTATTAG AAGAATATCC
Fwd          ACCACTGCGA TCCCCGGAAA AACAGCATTC CAGGTATTAG AAGAATATCC
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1010       1020       1030       1040       1050
FHbp_SOE        TGATTCAGGT GAAAATATTG TTGATGCGCT GGCAGTGTTC CTGCGCCGGT
Fwd             TGATTCAGGT GAAAATATTG TTGATGCGCT GGCAGTGTTC CTGCGCCGGT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1060       1070       1080       1090       1100
FHbp_SOE        TG--CATTCG ATTCCTGTTT GTAATTGTCC TTTTAACAGC GATCGCGTAT
Fwd             TG--CATTCN ATTCCTGTTT GTAATTGTCC TTTTAACAGC GATCGCGTAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1110       1120       1130       1140       1150
FHbp_SOE        TTCGTCTCGC TCAGGCGCAA TCACGAATGA ATAACGGTTT GGT-TGATGC
Fwd             TTCGTCTCGC TCAGGCGCAA TCACGAATGA ATAACGGTTT GGT-TGATGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1160       1170       1180       1190       1200
FHbp_SOE        GAGTGATTTT GATGACGAGC GTAATGGCTG G-CCTGTTGA ACAAGTCTGG
Fwd             GAGTGATTTT GATGACGAGC GTAATGGCNN GGCCTGTTGA ACAAGTCTGG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1210       1220       1230       1240       1250
FHbp_SOE        AAAGAAATGC ATAAACTTTT G-CCATTCTC ACCGG-ATTC AGTCGTCACT
Fwd             AAAGAAATGC ATAAACTTTT NGCCATTCTC ACCNGGATTC AGTCGTCACT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1260       1270       1280       1290       1300
FHbp_SOE        CATGGTGAT- TTCT-CACTT GATAACCTTA TTTTT-GACG A-GGGGAAAT
Fwd             CATGGTGAT- TNCNTNNCTT GATAACCTTA TTTTTTGACN AAGGGNAANT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1310       1320       1330       1340       1350
FHbp_SOE        TAATAGGTTG TATT-GATGT TGGACGAGTC GGAATCGCAG ACCGATACCA
Fwd             TAATAGGTTG NNNTTGNTGT TGGNN----- ---------- --CANNTCCN

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1360       1370       1380       1390       1400
FHbp_SOE        GGATCTTGCC ATCCTATGGA ACTGCCTCGG TGAGTTTTCT CCTTCATTAC
Fwd             NANTCCCNNN NCC------- GATACCNNGG ---ATNTTNN CCNNNNNNNG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1410       1420       1430       1440       1450
FHbp_SOE        AGAAACGGCT TTTTCAAAAA TATGGTATTG ATAATCCTGA TATGAATAAA
Fwd             GNAACTGNC- --CTCGGGNA NGTTTTNNNC CTTNNNTTNN NGAANCCGGN

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1460       1470       1480       1490       1500
FHbp_SOE        TTGCAGTTTC ATTTGATGCT CGATGAGTTT TTCTAATCAG AATTGGTTAA
Fwd             NTTTNNTNAA ANNNNNNGNN NNTTGNNTAN NCCNGGNNNG NNNNNAA-AN
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1510       1520       1530       1540       1550
FHbp_SOE        TTGGTTGTAA CACTGGCAGA GCATTACGCT GACTTGACGG GACGGCGGCT
Fwd             TTGGCNGTTN CNNTTNGAAN NNCCNNNNNN GTTTTNCCNA ANNNNNAANT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1560       1570       1580       1590       1600
FHbp_SOE        TTGTTGAATA AATCGAACTT TT---GCTGA GTTGAAGGAT CAGATCACGC
Fwd             TNGGTNA--A ANTTGGNTTN --------GA ANCNNNNGGN NANNNNNNNN

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1610       1620       1630       1640       1650
FHbp_SOE        ATCTTCCCGA CAACGCAGAC CGTTCCGTGG CAAAGCAAAA GTTCAAAATC
Fwd             NNNNNNCNNN NNA--NAANN NNTTNACNNN NNANNNNNAA NNNNGAANNN

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1660       1670       1680       1690       1700
FHbp_SOE        ACCAACTGGT CCACCTACAA CAAAGCTCTC ATCAACC--G TGGCGGGGAT
Fwd             NNNAANNNAN NNGNAAA-AA NNNNTNNNNN NTNNNNN--G GNAAANNGNT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1710       1720       1730       1740       1750
FHbp_SOE        CCTCTAGA-G TCGACCTGCA GGCATGCAAG CTTCAGGGTT GAGATGTGTA
Fwd             TTTNNAGGCN NNGGGNNNNN NNNNNNNNNN NNNTGGNANN ANNNNNNNAN

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1760       1770       1780       1790       1800
FHbp_SOE        TAAGAGACAG CAGTTCGGTT CACAGGTTTA CTCCTAGTCA TACGCAGAAT
Fwd             NAANNNNNAN NNNNNNNNNN NNNAANNNAA NNNNNNNTTT NNNNNNNNNN

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1810       1820       1830       1840       1850
FHbp_SOE        AGATAATATA TAAACATTTT GGTTATGGTA TCTTTTTTTG CATACTGCAT
Fwd             NAANNNTT-- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1860       1870       1880       1890       1900
FHbp_SOE        CAATGAGGCA GGTCAAAGAA GCAAAAATCA AATGCCGTCC GAACAACGGT
Fwd             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1910       1920       1930       1940       1950
FHbp_SOE        TCAGACGGCA TTTTGTTTAC AAGCAACCTG TTATTTGACG ATTTGGTTCA
Fwd             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1960       1970       1980       1990       2000
FHbp_SOE        ATTCGCCCTT GGCATAACGG TTTGCCATTT TTTCCAAGGA AACCGGTTTG
Fwd             ---------- ---------- ---------- ---------- ----------
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2010       2020       2030       2040       2050
FHbp_SOE        ATTTTGCCTG CCTGACCTTC GCAACCGAAC GCGAGGTAGC GGTCGAGGCA
Fwd             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2060       2070       2080       2090       2100
FHbp_SOE        GATTTGCTTC ATCGCTTCAA TGGTTTTGCT CAAATATTTG CGCGGATCGA
Fwd             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2110       2120       2130       2140       2150
FHbp_SOE        AGTCGGACGG GTTTTCGGCA AGGTAGCGGC GTACCGCGCC GGTGGAAGCA
Fwd             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2160       2170       2180       2190       2200
FHbp_SOE        AGGCGCAAGT CGGTATCGAT GTTGACTTTG CGCACGCCGT GTTTGATGCC
Fwd             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2210       2220       2230       2240       2250
FHbp_SOE        TTCGACGATT TCTTCAACCG GCACGCCGTA GGTTTCACCG ATATTGCCGC
Fwd             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2260       2270       2280       2290       2300
FHbp_SOE        CGTATTCGTT GATGACTTTC AGCCATTCTT GCGGAACGGA GCTGGAACCG
Fwd             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2310       2320       2330       2340       2350
FHbp_SOE        TGCATCACGA TGTGTGTATT GGGCAGGGCT TGGTGGATTT CTTTGATGCG
Fwd             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2360       2370       2380       2390       2400
FHbp_SOE        GTCGATACGC AATACGTCGC CTGTGGGCGG ACGGGTGAAT TTGTATGCGC
Fwd             ---------- ---------- ---------- ---------- ----------

                ....|....| .
                       2410
FHbp_SOE        CGTGGCTGGT G
Fwd             ---------- -
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Alignment of *fHbp* SOE final product against *kan* gene sequence flanked by *Slam* regions

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
Slam_SOE        AGGCGCAGTT TACCTACTTG GGCGTAAACG GCGGCTTTAC CGACAGCGAG
Fwd             AGGCGCAGTT TACCTACTTG GGCGTAAACG GCGGCTTTAC CGACAGCGAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90        100
Slam_SOE        GGGACGGCGG TCGGACTGCT CGGCAGCGGT CAGTGGCAAA GCCGCGCCGG
Fwd             GGGACGGCGG TCGGACTGCT CGGCAGCGGT CAGTGGCAAA GCCGCGCCGG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
Slam_SOE        CATTCGGGCA AAAACCCGTT TTGCTTTGCG TAACGGTGTC AATCTTCAGC
Fwd             CATTCGGGCA AAAACCCGTT TTGCTTTGCG TAACGGTGTC AATCTTCAGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        160        170        180        190        200
Slam_SOE        CTTTTGCCGC TTTTAATGTT TTGCACAGGT CAAAATCTTT CGGCGTGGAA
Fwd             CTTTTGCCGC TTTTAATGTT TTGCACAGGT CAAAATCTTT CGGCGTGGAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        210        220        230        240        250
Slam_SOE        ATGGACGGCG AAAAACAGAC GCTGGCAGGC AGGACGGCAC TCGAAGGGCG
Fwd             ATGGACGGCG AAAAACAGAC GCTGGCAGGC AGGACGGCAC TCGAAGGGCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        260        270        280        290        300
Slam_SOE        GTTCGGTATT GAAGCCGGTT GGAAAGGCCA TATGTCCGCA CGCATCGGAT
Fwd             GTTCGGTATT GAAGCCGGTT GGAAAGGCCA TATGTCCGCA CGCATCGGAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350
Slam_SOE        ATGGCAAAAG GACGGACGGC GACAAAGAAG CCGCATTGTC GCTCAAATGG
Fwd             ATGGCAAAAG GACGGACGGC GACAAAGAAG CCGCATTGTC GCTCAAATGG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        360        370        380        390        400
Slam_SOE        CTGTTTTGAT GCGTCGGGAA ATGTTTTGAC GCACAGGCGG TACACCGGCA
Fwd             CTGTTTTGAT GCGTCGGGAA ATGTTTTGAC GCACAGGCGG TACACCGGCA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        410        420        430        440        450
Slam_SOE        CGGCACCGCG CGCCGCCCCG CAAACCAATC CGAACCCTGC CGCCCCGAAG
Fwd             CGGCACCGCG CGCCGCCCCG CAAACCAATC CGAACCCTGC CGCCCCGAAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        460        470        480        490        500
Slam_SOE        GGCGGGGCAT AATGATGAAA CCGGCGGAAA ACCGCCGGTT TTTTGCCGCC
Fwd             GGCGGGGCAT AATGATGAAA CCGGCGGAAA ACCGCCGGTT TTTTGCCGCC
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        510        520        530        540        550
Slam_SOE        GTTTGAAACC CGATTCTGGC TTCAGACGGC ATTGTCGCGG CATCGGGCGG
Fwd             GTTTGAAACC CGATTCTGGC TTCAGACGGC ATTGTCGCGG CATCGGGCGG


                ....|....| ....|....| ....|....| ....|....| ....|....|
                        560        570        580        590        600
Slam_SOE        CAGGGTTTGG AACAGCGGCA TAAAAAACTG ATACAATCCG CCGATTGATA
Fwd             CAGGGTTTGG AACAGCGGCA TAAAAAACTG ATACAATCCG CCGATTGATA


                ....|....| ....|....| ....|....| ....|....| ....|....|
                        610        620        630        640        650
Slam_SOE        ATGGTTATTT TTTATTTTTG TGCTGTCTCT TATACACATC TCAACCATCA
Fwd             ATGGTTATTT TTTATTTTTG TGCTGTCTCT TATACACATC TCAACCATCA


                ....|....| ....|....| ....|....| ....|....| ....|....|
                        660        670        680        690        700
Slam_SOE        TCGATGAATT GTGTCTCAAA ATCTCTGATG TTACATTGCA CAAGATAAAA
Fwd             TCGATGAATT GTGTCTCAAA ATCTCTGATG TTACATTGCA CAAGATAAAA


                ....|....| ....|....| ....|....| ....|....| ....|....|
                        710        720        730        740        750
Slam_SOE        ATATATCATC ATGAACAATA AAACTGTCTG CTTACATAAA CAGTAATACA
Fwd             ATATATCATC ATGAACAATA AAACTGTCTG CTTACATAAA CAGTAATACA


                ....|....| ....|....| ....|....| ....|....| ....|....|
                        760        770        780        790        800
Slam_SOE        AGGGGTGTTA TGAGCCATAT TCAACGGGAA ACGTCTTGCT CGAGGCCGCG
Fwd             AGGGGTGTTA TGAGCCATAT TCAACGGGAA ACGTCTTGCT CGAGGCCGCG


                ....|....| ....|....| ....|....| ....|....| ....|....|
                        810        820        830        840        850
Slam_SOE        ATTAAATTCC AACATGGATG CTGATTTATA TGGGTATAAA TGGGCTCGCG
Fwd             ATTAAATTCC AACATGGATG CTGATTTATA TGGGTATAAA TGGGCTCGCG
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Alignment of pUT18_*fHbp*MC58, pUT18_*fHbp*L91543 and pKT25_*secA* against fHbp MC58, fHbp L91543 and secA MC58

-pUT18_*fHbp*MC58

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50
MC58            ---------- ---------- ---------- ---------- ----------
Fwd             GANTTNNANN NAACTGCGAC ATACGCCGAC TCGGGCTGAC CCAAAGTCAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
MC58            ---------- ---------- ---------- ---------- ----------
Fwd             CACAATGAGC TGCGTAAAAT CCGCACCGCC TTCAAAATGG CGGGCGACAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
MC58            ---------- ---------- ---------- ---------- ----------
Fwd             GGCGCGTTTG AAGGTTATGC ATTCCGAACA CAGCCGCCGC CGGTCTGTCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
MC58            ---------- ---------- ---------- ---------- ----------
Fwd             TCGAAATCAT TTCCTCGGAT GTTTTTAATC GGAACGAGGC GCGCGATTAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
MC58            ---------- ---------- ---------- ---------- ----------
Fwd             GTCGAAAGCC GCTATTTGTC CGGTATGGAT TTTGCGGTGG ACGAATTGGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       260        270        280        290        300
MC58            ---------- ---------- ---------- ---------- ----------
Fwd             AATCCAACAC CGGTTCTTCC ATATCCTCAC ACCGCAACAG CAGCAAATGT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       310        320        330        340        350
MC58            ---------- ---------- ---------- ---------- ----------
Fwd             GGCTTTCTTC CTGCCTCAAA GGATCCCCGG GTACCGAGCT CGAATTCATC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       360        370        380        390        400
MC58            ---------- ---------- ---------- ---------- ----------
Fwd             GATATAACTA AGTAATATGG TGCACTCTCA GTACAATCTG CTCTGATGCC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       410        420        430        440        450
MC58            ---------- ---------- ---------- ---------- ----------
Fwd             GCATAGTTAA GCCAGCCCCG ACACCCGCCA ACACCCGCTG ACGCGCCCTG
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       460        470        480        490        500
MC58            ---------- ---------- ---------- ---------- ----------
Fwd             ACGGGCTTGT CTGCTCCCGG CATCCGCTTA CAGACAAGCT GTGACCGTCT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       510        520        530        540        550
MC58            ---------- ---------- ---------- ---------- ----------
Fwd             CCGGGAGCTG CATGTGTCAG AGGTTTTCAC CGTCATCACC GAAACGCGCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       560        570        580        590        600
MC58            ---------- ---------- ---------- ---------- ----------
Fwd             AGACGAAAGG GCCTCGTGAT ACGCCTATTT TTATAGGTTA ATGTCATGAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       610        620        630        640        650
MC58            ---ATGCCGT CTGAACCGCC GTTCGGACGA CATTTGATTT TTGCTTCTTT
Fwd             AATATGCCGT CTGAACCGCC GTTCGGACGA CATTTGATTT TTGCTTCTTT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       660        670        680        690        700
MC58            GACCTGCCTC ATTGATGCGG TATGCAAAAA AAGATACCAT AACCAAAATG
Fwd             GACCTGCCTC ATTGATGCGG TATGCAAAAA AAGATACCAT AACCAAAATG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       710        720        730        740        750
MC58            TTTATATATT ATCTATTCTG CGTATGACTA GGAGTAAACC TGTGAATCGA
Fwd             TTTATATATT ATCTATTCTG CGTATGACTA GGAGTAAACC TGTGAATCGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       760        770        780        790        800
MC58            ACTGCCTTCT GCTGCCTTTC TCTGACCACT GCCCTGATTC TGACCGCCTG
Fwd             ACTGCCTTCT GCTGCCTTTC TCTGACCACT GCCCTGATTC TGACCGCCTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       810        820        830        840        850
MC58            CAGCAGCGGA GGGGGTGGTG TCGCCGCCGA CATCGGTGCG GGGCTTGCCG
Fwd             CAGCAGCGGA GGGGGTGGTG TCGCCGCCGA CATCGGTGCG GGGCTTGCCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       860        870        880        890        900
MC58            ATGCACTAAC CGCACCGCTC GACCATAAAG ACAAAGGTTT GCAGTCTTTG
Fwd             ATGCACTAAC CGCACCGCTC GACCATAAAG ACAAAGGTTT GCAGTCTTTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       910        920        930        940        950
MC58            ACGCTGGATC AGTCCGTCAG GAAAAACGAG AAACTGAAGC TGGCGGCACA
Fwd             ACGCTGGATC AGTCCGTCAG GAAAAACGAG AAACTGAAGC TGGCGGCACA
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       960        970        980        990       1000
MC58            AGGTGCGGAA AAAACTTATG GAAACGGTGA CAGCCTCAAT ACGGGCAAAT
Fwd             AGGTGCGGAA AAAACTTATG GAAACGGTGA CAGCCTCAAT ACGGGCAAAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1010       1020       1030       1040       1050
MC58            TGAAGAACGA CAAGGTCAGC CGTTTCGACT TTATCCGCCA AATCGAAGTG
Fwd             TGAAGAACGA CAAGGTCAGC CGTTTCGACT TTATCCGCCA AATCGAAGTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1060       1070       1080       1090       1100
MC58            GACGGGCAGC TCATTACCTT GGAGAGTGGA GAGTTCCAAG TATACAAACA
Fwd             GACGGGCAGC TCATTACCTT GGAGAGTGGA GAGTTCCAAG TATACAAACA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1110       1120       1130       1140       1150
MC58            AAGCCATTCC GCCTTAACCG CCTTTCAGAC CGAGCAAATA CAAGATTCGG
Fwd             AAGCCATTCC GCCTTAACCG CCTTTCAGAC CGAGCAAATA CAAGATTCGG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1160       1170       1180       1190       1200
MC58            AGCATTCCGG GAAGATGGTT GCGAAACGCC AGTTCAGAAT CGGCGACATA
Fwd             AGCATTCCGG GAAGATGGTT GCGAAACGCC AGTTCAGAAT CGGCGACATA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1210       1220       1230       1240       1250
MC58            GCGGGCGAAC ATACATCTTT TGACAAGCTT CCCGAAGGCG GCAGGGCGAC
Fwd             GCGGGCGAAC ATACATCTTT TGACAAGCTT CCCGAAGGCG GCAGGGCGAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1260       1270       1280       1290       1300
MC58            ATATCGCGGG ACGGCGTTCG GTTCAGACGA TGCCGGCGGA AAACTGACCT
Fwd             ATATCGCGGG ACGGCGTTCG GTTCAGACGA TGCCGGCGGA AAACTGACCT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1310       1320       1330       1340       1350
MC58            ACACCATAGA TTTCGCCGCC AAGCAGGGAA ACGGCAAAAT CGAACATTTG
Fwd             ACACCATAGA TTTCGCCGCC AAGCAGGGAA ACGGCAAAAT CGAACATTTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1360       1370       1380       1390       1400
MC58            AAATCGCCAG AACTCAATGT CGACCTGGCC GCCGCCGATA TCAAGCCGGA
Fwd             AAATCGCCAG AACTCAATGT CGACCTGGCC GCCGCCGATA TCAAGCCGGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1410       1420       1430       1440       1450
MC58            TGGAAAACGC CATGCCGTCA TCAGCGGTTC CGTCCTTTAC AACCAAGCCG
Fwd             TGGAAAACGC CATGCCGTCA TCAGCGGTTC CGTCCTTTAC AACCAAGCCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
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                  1460       1470       1480       1490       1500
MC58          AGAAAGGCAG TTACTCCCTC GGTATCTTTG GCGGAAAAGC CCAGGAAGTT
Fwd           AGAAAGGCAG TTACTCCCTC GGTATCTTTG GCGGAAAAGC CCAGGAAGTT

              ....|....| ....|....| ....|....| ....|....| ....|....|
                  1510       1520       1530       1540       1550
MC58          GCCGGCAGCG CGGAAGTGAA AACCGTAAAC GGCATACGCC ATATCGGCCT
Fwd           GCCGGCAGCG CGGAAGTGAA AACCGTAAAC GGCATACGCC ATATCGGCCT

              ....|....| ....|.
                  1560
MC58          TGCCGCCAAG CAATAA
Fwd           TGCCGCCAAG CAATAA
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pUT18_*fHbp*L91543

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               ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50
L91543         ---------- ---------- ---------- ---------- ----------
Fwd            NNNNNACGNN NNANNNAGCA AATGTGGCTT TCTTCCTGCC TCAAAGGATC

               ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
L91543         ---------- ---------- ---------- ---------- ----------
Fwd            CCCGGGTACC GAGCTCGAAT TCATCGATAT AACTAAGTAA TATGGTGCAC

               ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
L91543         ---------- ---------- ---------- ---------- ----------
Fwd            TCTCAGTACA ATCTGCTCTG ATGCCGCATA GTTAAGCCAG CCCCGACACC

               ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
L91543         ---------- ---------- ---------- ---------- ----------
Fwd            CGCCAACACC CGCTGACGCG CCCTGACGGG CTTGTCTGCT CCCGGCATCC

               ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
L91543         ---------- ---------- ---------- ---------- ----------
Fwd            GCTTACAGAC AAGCTGTGAC CGTCTCCGGG AGCTGCATGT GTCAGAGGTT

               ....|....| ....|....| ....|....| ....|....| ....|....|
                       260        270        280        290        300
L91543         ---------- ---------- ---------- ---------- ----------
Fwd            TTCACCGTCA TCACCGAAAC GCGCGAGACG AAAGGGCCTC GTGATACGCC

               ....|....| ....|....| ....|....| ....|....| ....|....|
                       310        320        330        340        350
L91543         ---------- ---------- --------TT ACTGCTTGGC GGCAAGACCG
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Fwd          TATTTTTATA GGTTAATGTC ATGATAATTT ACTGCTTGGC GGCAAGACCG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            360        370        380        390        400
L91543       ATATGGTGTA TGCCGTTTGC GGTTTCCACT TCCGCGCTGC CGGCAACTTC
Fwd          ATATGGTGTA TGCCGTTTGC GGTTTCCACT TCCGCGCTGC CGGCAACTTC

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            410        420        430        440        450
L91543       CTGGGCTTGC CCGCCAAAGA TACCGAGGGA GTAACTGCCT TTCTCGTCTT
Fwd          CTGGGCTTGC CCGCCAAAGA TACCGAGGGA GTAACTGCCT TTCTCGTCTT

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            460        470        480        490        500
L91543       GGTTGTAAAG GACGGAACCG CTGATAACGG CATGGCGTTT TTCATCCGGC
Fwd          GGTTGTAAAG GACGGAACCG CTGATAACGG CATGGCGTTT TTCATCCGGC

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            510        520        530        540        550
L91543       TTGATATAGG CGGTGGCAAG CTCGACATTG AGTTCGGGCG ATTTCAAATG
Fwd          TTGATATAGG CGGTGGCAAG CTCGACATTG AGTTCGGGCG ATTTCAAATG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            560        570        580        590        600
L91543       TTCGATTTTG CCGTGTCCCT GCTTGGCGGC GAAATCTATA GTATAGGTCA
Fwd          TTCGATTTTG CCGTGTCCCT GCTTGGCGGC GAAATCTATA GTATAGGTCA

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            610        620        630        640        650
L91543       GTTTTCCGCC AGCATCGTCT GAACCGAACG CCGTCCCGCG ATATGTCGCA
Fwd          GTTTTCCGCC AGCATCGTCT GAACCGAACG CCGTCCCGCG ATATGTCGCA

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            660        670        680        690        700
L91543       CTGCCGCCTT TGGGAAGCTT GTCAAAAGAT GTATGTTCGC CCGCTATGTC
Fwd          CTGCCGCCTT TGGGAAGCTT GTCAAAAGAT GTATGTTCGC CCGCTATGTC

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            710        720        730        740        750
L91543       GCCGATTCTG AACTGGCGTT TCGCAACCAT CTTCCCGGAA TCCTCCGAGT
Fwd          GCCGATTCTG AACTGGCGTT TCGCAACCAT CTTCCCGGAA TCCTCCGAGT

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            760        770        780        790        800
L91543       CTTGTACTTG CTCGGTCTGA AGGGCGGTTA AGGCGGAATG GCTTTGTTTG
Fwd          CTTGTACTTG CTCGGTCTGA AGGGCGGTTA AGGCGGAATG GCTTTGTTTG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            810        820        830        840        850
L91543       TACACTTGGA ACTCTCCGCT CTCCAAGGTA ATGAGCTTCC CGTCCACTTC
Fwd          TACACTTGGA ACTCTCCGCT CTCCAAGGTA ATGAGCTTCC CGTCCACTTC
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        860        870        880        890        900
L91543          GATTTGACGG ATAAAGTCGA AGCGGCTGAC CTTGTCGTTC TTCAATTTGC
Fwd             GATTTGACGG ATAAAGTCGA AGCGGCTGAC CTTGTCGTTC TTCAATTTGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        910        920        930        940        950
L91543          CCGTATTAAG GCTGTCGCCG TTTCCATAAG TTTTTTCCGC ACCTTGTGCC
Fwd             CCGTATTAAG GCTGTCGCCG TTTCCATAAG TTTTTTCCGC ACCTTGTGCC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        960        970        980        990       1000
L91543          GCCAGCTTCA GTTTCTCGTT TTTCCTGACG GACTGATCCA GCGTTAAAGA
Fwd             GCCAGCTTCA GTTTCTCGTT TTTCCTGACG GACTGATCCA GCGTTAAAGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1010       1020       1030       1040       1050
L91543          CTGCAAACCT TTGTCTTTAT GGTCGAGCGG TGCGGTTAGT GCATCGGCAA
Fwd             CTGCAAACCT TTGTCTTTAT GGTCGAGCGG TGCGGTTAGT GCATCGGCAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1060       1070       1080       1090       1100
L91543          GCCCCGCACC GATGTCGGCG GCGACACCGC CCCCTCCGCT GCTGCAGGCG
Fwd             GCCCCGCACC GATGTCGGCG GCGACACCGC CCCCTCCGCT GCTGCAGGCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1110       1120       1130       1140       1150
L91543          GTCAGAATCA GGGCGGCGGT CAGAGAAAAG CAGCAGAAGG CAGTTCGGTT
Fwd             GTCAGAATCA GGGCGGCGGT CAGAGAAAAG CAGCAGAAGG CAGTTCGGTT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1160       1170       1180       1190       1200
L91543          CACAGGTTTA CTCCTAGTCA TACGCAGAAT AGATAATATA TAAACATTTT
Fwd             CACAGGTTTA CTCCTAGTCA TACGCAGAAT AGATAATATA TAAACATTTT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1210       1220       1230       1240       1250
L91543          GGTTATGGTA TCTTTTTTTG CATACTGCAT CAATGAGGCA GGTCAAAGAA
Fwd             GGTTATGGTA TCTTTTTTTG CATACTGCAT CAATGAGGCA GGTCAAAGAA

                ....|....| ....|....| ....|....| ....|....| .
                       1260       1270       1280       1290
L91543          GCAAAAATCA AATGCCGTCC GAACAACGGT TCAGACGGCA T
Fwd             GCAAAAATCA AATGCCGTCC GAACAACGGT TCAGACGGCA T
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-pKT25_*secA*

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
SecA            ---------- ---------- ---------- ---------- ----------
Fwd             CNNNNTTTTT TTNNNTTNNN NAGNAAAAGC GCATCCGCAG ACCGTTCGGT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90        100
SecA            ---------- ---------- ---------- ---------- ----------
Fwd             TTGATGTTTT CGGCGATGAT TTTGCCGCAG TGCTGTTCAA TAATATGTTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
SecA            ---------- ---------- ---------- ---------- ----------
Fwd             GGTCAATGCA AGCCCCAGTC CTGTTCCGGG TTTGTTGGCA CTGGAGTCTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        160        170        180        190        200
SecA            ---------- ---------- ---------- ---------- ----------
Fwd             CACGGTAGAA AGCGGTGAAG ATGTGCGGGA GCTGCATTTC GTCCACGCCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        210        220        230        240        250
SecA            ---------- ---------- ---------- ---------- ----------
Fwd             GGGCCGTTGT CGGTAACGTC GATTATCCAG TGTTTGTGGT CTTGTCCGAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        260        270        280        290        300
SecA            ---------- ---------- ---------- ---------- ----------
Fwd             GTTGATCAGG ATGGTGCTGC CTTCGGGACT GTAGTTGACG GCGTTGCGGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350
SecA            ---------- ---------- ---------- ---------- ----------
Fwd             TGACGTTGTC GAAGGCGCGG TACAGGTAGC TTTCGTTGGC AAGGATGGTT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        360        370        380        390        400
SecA            ---------- ---------- ---------- ---------- ----------
Fwd             GTGTTTTCGG GGATTTTTCC GTCGGCAGAC AGGGTAGCCG TTTGTCCGTT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        410        420        430        440        450
SecA            ---------- ---------- ---------- ---------- ----------
Fwd             TTTCTGGGCA ATGCTTTGAT TGTCTTCTAC CAGGTTGCCC AGGAAGGGCA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        460        470        480        490        500
SecA            ---------- ---------- ---------- ---------- ----------
Fwd             GGAGTTTCAG GCTTTCTTTT TCCAAAGCCA TATTGGAAGT TTCGAGACGG
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        510        520        530        540        550
SecA            ---------- -------ATG CTGACAAACA TTGCCAAGAA AATCTTCGGC
Fwd             GACAGGGTTA ACAGTTCATG CTGACAAACA TTGCCAAGAA AATCTTCGGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        560        570        580        590        600
SecA            AGCCGCAACG ACCGCTTGCT GAAACAATAC CGTAAATCCG TTGCCAGAAT
Fwd             AGCCGCAACG ACCGCTTGCT GAAACAATAC CGTAAATCCG TTGCCAGAAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        610        620        630        640        650
SecA            CAACGCGCTC GAAGAACAGA TGCAAGCCCT AAGCGATGCT GATCTGCAAG
Fwd             CAACGCGCTC GAAGAACAGA TGCAAGCCCT AAGCGATGCT GATCTGCAAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        660        670        680        690        700
SecA            CCAAAACTGC CGAATTCAAA CAACGCCTCG CCGACGGTCA GACTTTGGAC
Fwd             CCAAAACTGC CGAATTCAAA CAACGCCTCG CCGACGGTCA GACTTTGGAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        710        720        730        740        750
SecA            GGCATTTTGC CCGAAGCCTT CGCCGTCTGC CGCGAAGCGT CCCGCCGCAC
Fwd             GGCATTTTGC CCGAAGCCTT CGCCGTCTGC CGCGAAGCGT CCCGCCGCAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        760        770        780        790        800
SecA            CCTCGGTATG CGCCACTTCG ACGTGCAGCT TATCGGCGGT ATGGTGCTGC
Fwd             CCTCGGTATG CGCCACTTCG ACGTGCAGCT TATCGGCGGT ATGGTGCTGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        810        820        830        840        850
SecA            ACGACGGCAA AATCGCCGAA ATGCGTACCG GCGAAGGCAA AACCTTGGTC
Fwd             ACGACGGCAA AATCGCCGAA ATGCGTACCG GCGAAGGCAA AACCTTGGTC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        860        870        880        890        900
SecA            GCCACCCTCG CCGTCTATCT CAACGCGCTG GCCGGCAAAG GCGTACACGT
Fwd             GCCACCCTCG CCGTCTATCT CAACGCGCTG GCCGGCAAAG GCGTACACGT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        910        920        930        940        950
SecA            CGTTACCGTC AACGACTACC TCGCCTCACG CGATGCGGGC ATTATGGAGC
Fwd             CGTTACCGTC AACGACTACC TCGCCTCACG CGATGCGGGC ATTATGGAGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        960        970        980        990       1000
SecA            CGCTCTACAA TTTCCTCGGC CTTACCGTGG GCGTGATTAT TTCAGATATG
Fwd             CGCTCTACAA TTTCCTCGGC CTTACCGTGG GCGTGATTAT TTCAGATATG
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1010       1020       1030       1040       1050
SecA            CAGCCGTTCG ACCGTCAAAA CGCCTATGCC GCCGATATCA CCTACGGCAC
Fwd             CAGCCGTTCG ACCGTCAAAA CGCCTATGCC GCCGATATCA CCTACGGCAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1060       1070       1080       1090       1100
SecA            CAATAATGAA TTCGGCTTCG ACTACCTGCG CGACAATATG GTTACCGACC
Fwd             CAATAATGAA TTCGGCTTCG ACTACCTGCG CGACAATATG GTTACCGACC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1110       1120       1130       1140       1150
SecA            AATACGACAA AGTGCAGCGC GAATTGAATT TTGCCGTTGT CGATGAAGTG
Fwd             AATACGACAA AGTGCAGCGC GAATTGAATT TTGCCGTTGT CGATGAAGTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1160       1170       1180       1190       1200
SecA            GATTCCATCT TGATTGACGA AGCGCGCACT CCGCTGATTA TCTCCGGTCA
Fwd             GATTCCATCT TGATTGACGA AGCGCGCACT CCGCTGATTA TCTCCGGTCA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1210       1220       1230       1240       1250
SecA            GGCGGATGAC AACATCCAGT TGTACCAAAT CATGAACACC GTTCCGCCCC
Fwd             GGCGGATGAC AACATCCAGT TGTACCAAAT CATGAACACC GTTCCGCCCC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1260       1270       1280       1290       1300
SecA            ACCTCGTCCG TCAAGAGACA GAAGAAGGCG AAGGCGACTA TTGGGTCGAC
Fwd             ACCTCGTCCG TCAAGAGACA GAAGAAGGCG AAGGCGACTA TTGGGTCGAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1310       1320       1330       1340       1350
SecA            GAAAAGGCAC ATCAGGTCAT CCTGAGCGAA GCAGGTCACG AACACGCCGA
Fwd             GAAAAGGCAC ATCAGGTCAT CCTGAGCGAA GCAGGTCACG AACACGCCGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1360       1370       1380       1390       1400
SecA            GCAAATCCTG ACCCAAATGG GATTGCTGGC AGAAAACGAC TCCCTCTATT
Fwd             GCAAATCCTG ACCCAAATGG GATTGCTGGC AGAAAACGAC TCCCTCTATT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1410       1420       1430       1440       1450
SecA            CCGCCGCCAA TATCGCCCTG ATGCACCACC TTATGGCGGC ATTGCGCGCG
Fwd             CCGCCGCCAA TATCGCCCTG ATGCACCACC TTATGGCGGC ATTGCGCGCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1460       1470       1480       1490       1500
SecA            CATTCCCTCT TCCACAAAGA CCAACATTAC GTCATCCAAG ACGGCGAAAT
Fwd             CATTCCCTCT TCCACAAAGA CCAACATTAC GTCATCCAAG ACGGCGAAAT
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1510       1520       1530       1540       1550
SecA            CGTCATCGTG GACGAATTCA CCGGCCGGCT GATGTCCGGC CGCCGCTGGT
Fwd             CGTCATCGTG GACGAATTCA CCGGCCGGCT GATGTCCGGC CGCCGCTGGT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1560       1570       1580       1590       1600
SecA            CGGAGGGTCT GCATCAAGCC GTCGAAGCCA AAGAAGGCGT GGAAATCAAA
Fwd             CGGAGGGTCT GCATCAAGCC GTCGAAGCCA AAGAAGGCGT GGAAATCAAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1610       1620       1630       1640       1650
SecA            CGCGAAAACC AAACGCTTGC ATCTATTACC TTCCAAAACT ATTTCCGCCT
Fwd             CGCGAAAACC AAACGCTTGC ATCTATTACC TTCCAAAACT ATTTCCGCCT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1660       1670       1680       1690       1700
SecA            GTACACCAAG CTCTCCGGCA TGACCGGCAC AGCCGATACC GAAGCCTTCG
Fwd             GTACACCAAG CTCTCCGGCA TGACCGGCAC AGCCGATACC GAAGCCTTCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1710       1720       1730       1740       1750
SecA            AGTTCCAAAG CATCTACAAC CTCGAAACCG TCATCATTCC GACCAACCGC
Fwd             AGTTCCAAAG CATCTACAAC CTCGAAACCG TCATCATTCC GACCAACCGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1760       1770       1780       1790       1800
SecA            CCCGTACAGC GCAAAGACTT CAACGACCAG ATTTTCCGTT CCGCCGAAGA
Fwd             CCCGTACAGC GCAAAGACTT CAACGACCAG ATTTTCCGTT CCGCCGAAGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1810       1820       1830       1840       1850
SecA            AAAATTCGAA GCCGTCGTTA AAGACATTGA GGAATGCCAC AAACGCGGGC
Fwd             AAAATTCGAA GCCGTCGTTA AAGACATTGA GGAATGCCAC AAACGCGGGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1860       1870       1880       1890       1900
SecA            AGCCCGTCCT CGTCGGCACC ACCAGCATTG AAAACTCCGA ACTGGTATCC
Fwd             AGCCCGTCCT CGTCGGCACC ACCAGCATTG AAAACTCCGA ACTGGTATCC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1910       1920       1930       1940       1950
SecA            AAGCTGCTGA CCCAAGCCGG ACTGCCGCAC AACGTCCTCA ACGCCAAAGA
Fwd             AAGCTGCTGA CCCAAGCCGG ACTGCCGCAC AACGTCCTCA ACGCCAAAGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1960       1970       1980       1990       2000
SecA            ACACGAACGC GAAGCCCTGA TTGTCGCCCA AGCCGGCAAA GTCGGCGCGA
Fwd             ACACGAACGC GAAGCCCTGA TTGTCGCCCA AGCCGGCAAA GTCGGCGCGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
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                2010       2020       2030       2040       2050
SecA       TTACCGTTGC CACCAATATG GCGGGACGCG GTACGGACAT CGTTTTAGGC
Fwd        TTACCGTTGC CACCAATATG GCGGGACGCG GTACGGACAT CGTTTTAGGC

           ....|....| ....|....| ....|....| ....|....| ....|....|
                2060       2070       2080       2090       2100
SecA       GGCAACCTGA AGCACCAAAC CGATGCCATC CGCGCCGACG AAACCTTGAG
Fwd        GGCAACCTGA AGCACCAAAC CGATGCCATC CGCGCCGACG AAACCTTGAG

           ....|....| ....|....| ....|....| ....|....| ....|....|
                2110       2120       2130       2140       2150
SecA       CGACGAAGAG AAACAGGCAC AAATCGCCGC ACTCGAAGAC GGCTGGCAGG
Fwd        CGACGAAGAG AAACAGGCAC AAATCGCCGC ACTCGAAGAC GGCTGGCAGG

           ....|....| ....|....| ....|....| ....|....| ....|....|
                2160       2170       2180       2190       2200
SecA       CGGAACACGA CAAAGTGATG GAAGCAGGCG GTTTGCACAT CATCGGTACG
Fwd        CGGAACACGA CAAAGTGATG GAAGCAGGCG GTTTGCACAT CATCGGTACG

           ....|....| ....|....| ....|....| ....|....| ....|....|
                2210       2220       2230       2240       2250
SecA       GAACGCCACG AAAGCCGCCG CATCGACAAC CAATTGCGCG GACGTTCCGG
Fwd        GAACGCCACG AAAGCCGCCG CATCGACAAC CAATTGCGCG GACGTTCCGG

           ....|....| ....|....| ....|....| ....|....| ....|....|
                2260       2270       2280       2290       2300
SecA       CCGTCAGGGC GACCCCGGAT CCAGCCGCTT CTATCTCTCC TTTGAAGACC
Fwd        CCGTCAGGGC GACCCCGGAT CCAGCCGCTT CTATCTCTCC TTTGAAGACC

           ....|....| ....|....| ....|....| ....|....| ....|....|
                2310       2320       2330       2340       2350
SecA       CATTGCTGCG CTTATTCGCA CTCGACCGCG CCGCCGCCAT CCTCAACCGC
Fwd        CATTGCTGCG CTTATTCGCA CTCGACCGCG CCGCCGCCAT CCTCAACCGC

           ....|....| ....|....| ....|....| ....|....| ....|....|
                2360       2370       2380       2390       2400
SecA       CTCGCCCCCG AACGCGGCGT CGCCATCGAA CACAACCTGC TGACGCGCCA
Fwd        CTCGCCCCCG AACGCGGCGT CGCCATCGAA CACAACCTGC TGACGCGCCA

           ....|....| ....|....| ....|....| ....|....| ....|....|
                2410       2420       2430       2440       2450
SecA       AATCGAAGGG GCGCAACGCA AAGTCGAAGG CAGAAACTTC GATATGCGCA
Fwd        AATCGAAGGG GCGCAACGCA AAGTCGAAGG CAGAAACTTC GATATGCGCA

           ....|....| ....|....| ....|....| ....|....| ....|....|
                2460       2470       2480       2490       2500
SecA       AACAGGTTTT GGAATACGAC GACGTTGCCA ACGAACAGCG CAAAGTCATT
Fwd        AACAGGTTTT GGAATACGAC GACGTTGCCA ACGAACAGCG CAAAGTCATT

           ....|....| ....|....| ....|....| ....|....| ....|....|
                2510       2520       2530       2540       2550
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SecA        TACAGCCAGC GCAACGAAAT TCTGACCAGC AAAGACATCA GCGACCTGAT
Fwd         TACAGCCAGC GCAACGAAAT TCTGACCAGC AAAGACATCA GCGACCTGAT


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2560       2570       2580       2590       2600
SecA        GCAGGAAATC CGTTCTGATG TCGTCAGCGA CCTCGTGGAT ACCTATATGC
Fwd         GCAGGAAATC CGTTCTGATG TCGTCAGCGA CCTCGTGGAT ACCTATATGC


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2610       2620       2630       2640       2650
SecA        CGCCCGACAG CATGGAAGAA CAATGGGACA TCCCGACTTT GGAGAACCGT
Fwd         CGCCCGACAG CATGGAAGAA CAATGGGACA TCCCGACTTT GGAGAACCGT


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2660       2670       2680       2690       2700
SecA        CTGGCTGCCG AATTCAGACT GCACGAAGAC ATCCAATCCT GGCTGAAGGC
Fwd         CTGGCTGCCG AATTCAGACT GCACGAAGAC ATCCAATCCT GGCTGAAGGC


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2710       2720       2730       2740       2750
SecA        GGACAATGCG ATTGACGGTC AAGACATCAA AGAACGCCTG ATCGAACGCA
Fwd         GGACAATGCG ATTGACGGTC AAGACATCAA AGAACGCCTG ATCGAACGCA


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2760       2770       2780       2790       2800
SecA        TCGAAAACGA ATATGCCGCC AAAACCGAAC TGGTCGGCAA GCAGGCAATG
Fwd         TCGAAAACGA ATATGCCGCC AAAACCGAAC TGGTCG---- ----------


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2810       2820       2830       2840       2850
SecA        GCCGATTTCG AGCGCAACGT GATGTTGCAG GTCATCGACA ACCAATGGCG
Fwd         ---------- ---------- ---------- ---------- ----------


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2860       2870       2880       2890       2900
SecA        CGAACACCTC GCCGCTATGG ACTACCTGCG ACAAGGCATA CACCTGCGCA
Fwd         ---------- ---------- ---------- ---------- ----------


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2910       2920       2930       2940       2950
SecA        GCTATGCCCA AAAAAATCCG AAGCAGGAAT ACAAACGTGA AGCCTTTACC
Fwd         ---------- ---------- ---------- ---------- ----------


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       2960       2970       2980       2990       3000
SecA        ATGTTCCAAG ACCTGTGGAA CGGCATCAAA TTCCATATTG CCTCCCTGCT
Fwd         ---------- ---------- ---------- ---------- ----------


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       3010       3020       3030       3040       3050
SecA        TACCTCGGTT CAAATCGAAC AAAACCCTGT CGCGGTGGTT GAAGAGCAAC
```

```
Fwd          ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        3060       3070       3080       3090       3100
SecA         CCATCGGCAA CATCCAGTCC ATCCATTCCG AATCGCCCGA TATGGAAGAA
Fwd          ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        3110       3120       3130       3140       3150
SecA         CTTTTGGGTC AGTCGCAAAC CGATCTGGTT ACCGAAGCCT TTAATCCCGA
Fwd          ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        3160       3170       3180       3190       3200
SecA         TGGGACAGAT TTCAGCCCCG AAGCCTTGGA AGCGCGGGGG CAAATCGTCC
Fwd          ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        3210       3220       3230       3240       3250
SecA         ACCGCAACGA CCCCTGCCCC TGCGGCAGCG GTTTGAAATA CAAACAATGC
Fwd          ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|...
                        3260
SecA         CACGGCAAAC TGGCTTAA
Fwd          ---------- --------
```

Alignment pMOD$_{TM}$-2<Tn5KAN-2DUS> against *kan* gene

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                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
Kan          CTGTCTCTTA TACACATCTC AACCATCATC GATGAATTGT GTCTCAAAAT
rev          ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90        100
Kan          CTCTGATGTT ACATTGCACA AGATAAAAAT ATATCATCAT GAACAATAAA
rev          ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
Kan          ACTGTCTGCT TACATAAACA GTAATACAAG GGGTGTTATG AGCCATATTC
rev          ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        160        170        180        190        200
Kan          AACGGGAAAC GTCTTGCTCG AGGCCGCGAT TAAATTCCAA CATGGATGCT
rev          ---------- -------TCG AGGCCGCGAT TAAATTCCAA CATGGATGCT
```

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        210        220        230        240        250
Kan             GATTTATATG GGTATAAATG GGCTCGCGAT AATGTCGGGC AATCAGGTGC
rev             GATTTATATG GGTATAAATG GGCTCGCGAT AATGTCGGGC AATCAGGTGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        260        270        280        290        300
Kan             GACAATCTAT CGATTGTATG GGAAGCCCGA TGCGCCAGAG TTGTTTCTGA
rev             GACAATCTAT CGATTGTATG GGAAGCCCGA TGCGCCAGAG TTGTTTCTGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350
Kan             AACATGGCAA AGGTAGCGTT GCCAATGATG TTACAGATGA GATGGTCAGA
rev             AACATGGCAA AGGTAGCGTT GCCAATGATG TTACAGATGA GATGGTCAGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        360        370        380        390        400
Kan             CTAAACTGGC TGACGGAATT TATGCCTCTT CCGACCATCA AGCATTTTAT
rev             CTAAACTGGC TGACGGAATT TATGCCTCTT CCGACCATCA AGCATTTTAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        410        420        430        440        450
Kan             CCGTACTCCT GATGATGCAT GGTTACTCAC CACTGCGATC CCCGGAAAAA
rev             CCGTACTCCT GATGATGCAT GGTTACTCAC CACTGCGATC CCCGGAAAAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        460        470        480        490        500
Kan             CAGCATTCCA GGTATTAGAA GAATATCCTG ATTCAGGTGA AAATATTGTT
rev             CAGCATTCCA GGTATTAGAA GAATATCCTG ATTCAGGTGA AAATATTGTT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        510        520        530        540        550
Kan             GATGCGCTGG CAGTGTTCCT GCGCCGGTTG CATTCGATTC CTGTTTGTAA
rev             GATGCGCTGG CAGTGTTCCT GCGCCGGTTG CATTCGATTC CTGTTTGTAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        560        570        580        590        600
Kan             TTGTCCTTTT AACAGCGATC GCGTATTTCG TCTCGCTCAG GCGCAATCAC
rev             TTGTCCTTTT AACAGCGATC GCGTATTTCG TCTCGCTCAG GCGCAATCAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        610        620        630        640        650
Kan             GAATGAATAA CGGTTTGGTT GATGCGAGTG ATTTTGATGA CGAGCGTAAT
rev             GAATGAATAA CGGTTTGGTT GATGCGAGTG ATTTTGATGA CGAGCGTAAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        660        670        680        690        700
Kan             GGCTGGCCTG TTGAACAAGT CTGGAAAGAA ATGCATAAAC TTTTGCCATT
rev             GGCTGGCCTG TTGAACAAGT CTGGAAAGAA ATGCATAAAC TTTTGCCATT
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       710        720        730        740        750
Kan             CTCACCGGAT TCAGTCGTCA CTCATGGTGA TTTCTCACTT GATAACCTTA
rev             CTCACCGGAT TCAGTCGTCA CTCATGGTGA TTTCTCACTT GATAACCTTA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       760        770        780        790        800
Kan             TTTTTGACGA GGGGAAATTA ATAGGTTGTA TTGATGTTGG ACGAGTCGGA
rev             TTTTTGACGA GGGGAAATTA ATAGGTTGTA TTGATGTTGG ACGAGTCGGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       810        820        830        840        850
Kan             ATCGCAGACC GATACCAGGA TCTTGCCATC CTATGGAACT GCCTCGGTGA
rev             ATCGCAGACC GATACCAGGA TCTTGCCATC CTATGGAACT GCCTCGGTGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       860        870        880        890        900
Kan             GTTTTCTCCT TCATTACAGA AACGGCTTTT TCAAAAATAT GGTATTGATA
rev             GTTTTCTCCT TCATTACAGA AACGGCTTTT TCAAAAATAT GGTATTGATA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       910        920        930        940        950
Kan             ATCCTGATAT GAATAAATTG CAGTTTCATT TGATGCTCGA TGAGTTTTTC
rev             ATCCTGATAT GAATAAATTG CAGTTTCATT TGATGCTCGA TGAGTTTTTC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       960        970        980        990       1000
Kan             TAATCAGAAT TGGTTAATTG GTTGTAACAC TGGCAGAGCA TTACGCTGAC
rev             TAATCAGAAT TGGTTAATTG GTTGTAACAC TGGCAGAGCA TTACGCTGAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1010       1020       1030       1040       1050
Kan             TTGACGGGAC GGCGGCTTTG TTGAATAAAT CGAACTTTTG CTGAGTTGAA
rev             TTGACGGGAC GGCGGCTTTG TTGAATAAAT CGAACTTTTG CTGAGTTGAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1060       1070       1080       1090       1100
Kan             GGATCAGATC ACGCATCTTC CCGACAACGC AGACCGTTCC GTGGCAAAGC
rev             GGATCAGATC ACGCATCTTC CCGACAACGC AGACCGTTCC GTGGCAAAGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1110       1120       1130       1140       1150
Kan             AAAAGTTCAA AATCACCAAC TGGTCCACCT ACAACAAAGC TCTCATCAAC
rev             AAAAGTTCAA AATCACCAAC TGGTCCACCT ACAACAAAGC TCTCATCAAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1160       1170       1180       1190       1200
Kan             CGTGGCGGGG ATCCTCTAGA GTCGACCTGC AGGCATGCAA GCTTCAGGGT
rev             CGTGGCGGGG ATCCTCTAGA GTCGACCTGC AGGCATGCAA GCTTCAGGGT

                ....|....| ....|....| ....|....| ....|....| ....|....|
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```
                1210       1220       1230       1240       1250
Kan          TGAGATGTGT ATAAGAGACA G--------- ---------- ----------
rev          TGAGATGTGT ATAAGAGACA GATGCCGTCT GAANNNTTCG GAAANNTNNN


             ....|....| ....|....| ....|....| ....|....| ....|....|
                1260       1270       1280       1290       1300
Kan          ---------- ---------- ---------- ---------- ----------
rev          GAAAAGCCCA GGAAGTTGCC GGCAGCGCGG AAGTGAAAAC CGTAAACGGC


             ....|....| ....|....| ....|....| ....|....| ....|....|
                1310       1320       1330       1340       1350
Kan          ---------- ---------- ---------- ---------- ----------
rev          ATACGCCATA TCGTNNTNAA ATCNNNNNNN NNNNNTCCTG CCTTCATTTG


             ...

Kan          ---
rev          GAN
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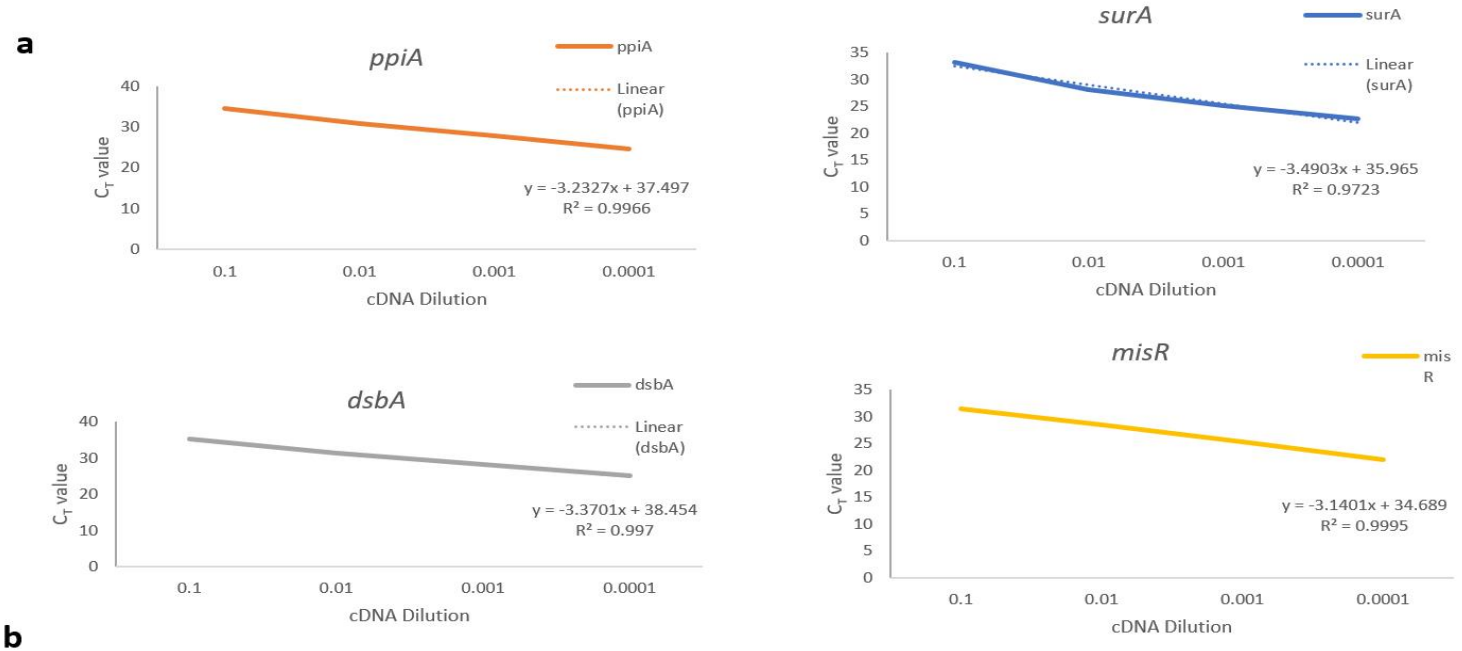
Appendix IV- Figure- a. qPCR reaction efficiency and b. primer optimisation and of *ppiA* (NMB1262), *surA* (NMB0281), *dsbA* (NMB0294) and *misR* (NMB0595)



Appendix V- Gel of pGCC4L*fHbp*SNP1+2 construction and alignment pGCC4L*fHbp*SNP1+2 against L91543 *fHbp*

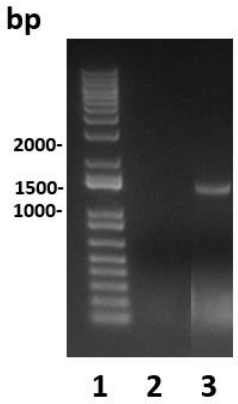


Figure- Verification of pGCC4L*fHbp*SNP1+2 by PCR. Lanes; 1, ladder; 2, no DNA negative control; 3, pGCC4L*fHbp*SNP1+2.

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50
FHbp            ---------- ---------- ---------- ------TTAC TGCTTGGCGG
Fwd             NNNTTCGGAA ANNTNNNGAA AAGCCCAGGA AGTTGCCGGC AGCGCGGAAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
FHbp            C-AAGACCG- ATATGGTGTA TGCCGT-TTG CGGTT---TC CACTTCCGCG
Fwd             TGAAAACCGT AAACGGCATA CGCCATATCG TNNTNAAATC NNNNNNNNNN
```

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
FHbp            CTGCCGGCAA CTTCCTGGGC TTGCCCGCCA AA---GATAC CG-----AGG
Fwd             NNTCCTGCCT TCATTTGGAN CGGTATGCAA AAAAAGATAC CATAACCAAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        160        170        180        190        200
FHbp            GAGTAACTGC CTTTCTCGTC TTGGTTGTAA --AGGACGGA ACCGCTGAT-
Fwd             ATGTTTATAT ATTATCTATT CTGCGTATGA CTAGGAGTAA ACCTGTGAAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        210        220        230        240        250
FHbp            --AACGGCAT ---GGCGTTT TTCAT----- -CCGGCTTGA T----ATAGG
Fwd             CGAACTGCCT TCTGCTGCCT TTCTCTGACC ACTGCCCTGA TTCTGACCGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        260        270        280        290        300
FHbp            CGGTGGCAAG CTCGACATTG AGTTCG--GG CGATTTCAAA TGTTCGATTT
Fwd             CTGCAGCAGC GGAGGGGGTG GTGTCGCCGC CGACATCGG- TGCGGGGCTT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350
FHbp            TGCCGTGTCC CTGCTTGGCG GCGAAATC-T ATAG-TATAG GT---CAGTT
Fwd             GCCGATGCAC TAACCGCACC GCTCGACCAT AAAGACAAAG GTTTGCAGTC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        360        370        380        390        400
FHbp            TTC--CGCCA GCATCGTCTG AACCGAACGC CGTCCCGCGA TATGTCGCAC
Fwd             TTTGACGCTG GATCAGTCCG TCAGGAAAAA CGAGAAACTG AAGCTGGCGG

                ....|....| ....|....| ....|....| ....|....| ....|....|
```

```
                  410        420        430        440        450
FHbp        TGCCGCCTTT GGGAAG--CT TGTCAAAAGA TGTATGTTCG C----CCGCT
Fwd         CACAAGGTGC GGAAAAAACT TATGGAAACG GTGACAGCCT CAATACGGGC

                  ....|....| ....|....| ....|....| ....|....| ....|....|
                  460        470        480        490        500
FHbp        ATGTCGCCGA TTCTGAA--- CTGGCGTTTC GCAACCATCT TCCCG---GA
Fwd         AAATTGAAGA ACGACAAGGT CAGCCGTTTC GACTTTATCC GCCAAATCGA

                  ....|....| ....|....| ....|....| ....|....| ....|....|
                  510        520        530        540        550
FHbp        ATCCTCCGAG TCTTGTACTT GCTCGGTCTG AAGGGCGGTT AAGGCGGAAT
Fwd         AGTGGACGGG CAGCTCATTA CCTTGGAGAG TGGAGAGTTC CAAGTATACA

                  ....|....| ....|....| ....|....| ....|....| ....|....|
                  560        570        580        590        600
FHbp        GGCTTTGTTT GTACACTTGG AACTCTCCGC TCTCCAAGGT AATG-AGCTT
Fwd         AACAAAGCCA TTCCGCCTTA ACCGCCTTTC AGACCGAGCA AATACAAGAT

                  ....|....| ....|....| ....|....| ....|....| ....|....|
                  610        620        630        640        650
FHbp        CCCGTCCACT TCGATTTGAC GGAT------ ---------A AAGTCGA---
Fwd         TCGGAGCATT CCGGGAAGAT GGTTGCGAAA CGCCAGTTCA GAATCGGCGA

                  ....|....| ....|....| ....|....| ....|....| ....|....|
                  660        670        680        690        700
FHbp        ---AGCGGCT GACCTTGTCG TTCTT--CAA TTTGCCCGTA T----TAAGG
Fwd         CATAGCGGGC GAACATACAT CTTTTGACAA GCTTCCCGAA GGCGGCAGGG

                  ....|....| ....|....| ....|....| ....|....| ....|....|
                  710        720        730        740        750
FHbp        C----TGTCG C-------CG TT---TCCAT AAGTTTTTC CGCACCTTGT
Fwd         CGACATATCG CGGGACGGCG TTCGGTTCAG ACGATGCCGG CGGAAAACTG
```

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                       760        770        780        790        800
FHbp            GCCGCCAGCT TCAGTTTCTC GTTTTTCCTG ACGGACTG-- --ATCCAGCG
Fwd             ACCTACACCA TAGATTTCGC CGCCAAGCAG GGAAACGGCA AAATCGAACA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       810        820        830        840        850
FHbp            TTAAAGACTG C---AAACCT TTGTCTTTAT GGTCGAGCGG TGCGGTTAGT
Fwd             TTTGAAATCG CCAGAACTCA ATGTCGACCT GGCCGCCGCC GATATCAAGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       860        870        880        890        900
FHbp            GCATCGGCAA GCCCCGCACC GATGTCGGCG GCGACACCGC CCCCTCC---
Fwd             CGGATGGAAA ACGCCATGCC GTCATCAGCG GTTCCGTCCT TTACAACCAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       910        920        930        940        950
FHbp            GCTGCTGCAG GCGGTCAGAA TCAGGGCGGC GGTCAGAGAA AAGCAGCAGA
Fwd             GCCGAGAAAG GCAGTTACTC CCTCGGTATC TTTGGCGGAA AAGCC-CAGG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       960        970        980        990       1000
FHbp            AGGCAGTTCG GTTCACAGGT TTACTCCTAG TCATACGCAG AATAGATAAT
Fwd             AAGTTGCCGG CAGCGCGGA- --AGTGAAAA CCGTAAACGG CATA--CGCC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1010       1020       1030       1040       1050
FHbp            ATATAAACAT TTTGGTTATG GTAT---CTT TTTTTGCATA CTG--CATCA
Fwd             ATATCGGCCT TGCCGCCAAG CAATAACCAT TGTTTAAACA ATGGTTATTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
```

```
                  1060       1070       1080       1090       1100
FHbp          ATGAG---GC AGGTCAAAGA AGCAAAAATC AAATGCCG-- --TCCGAACA
Fwd           CTTGGCGGGC AGGTCAAAGA AGCAATTAAT TAATCCGGGA ATTCGTAATC


              ....|....| ....|....| ....|..
                     1110       1120
FHbp          ACGGTTCAGA CGGCAT---- -------
Fwd           ATGGTCATAG CTGTTTCTAT GCNNTNT
```

Alignment *Int* KO against *Int* gene

```
          ....|....| ....|....| ....|....| ....|....| ....|....|
                    10         20         30         40         50
Lnt           ---------- ---------- ---------- ---------- ----------
Fwd           NNNTTCGGAA ANNTNNNGAA AAGCCCAGGA AGTTGCCGGC AGCGCGGAAG


              ....|....| ....|....| ....|....| ....|....| ....|....|
                    60         70         80         90        100
Lnt           ---------- ---------- ---------- ---------- ----------
Fwd           TGAAAACCGT AAACGGCATA CGCCATATCG TNNTNAAATC NNNNNAATTG


              ....|....| ....|....| ....|....| ....|....| ....|....|
                    110        120        130        140        150
Lnt           ---------- ---------- ---------- --ATGTTCAG ACGGTATCTT
Fwd           CGTCAANNNN NNNNTCCTGC CTTCATTTGG ANATGTTCAG ACGGTATCTT


              ....|....| ....|....| ....|....| ....|....| ....|....|
                    160        170        180        190        200
Lnt           CCGAACAGAC AGATGAATAT GGTTTCCAAA CTGGACAAAT ACTGGCAGCA
Fwd           CCGAACAGAC AGATGAATAT GGTTTCCAAA CTGGACAAAT ACTGGCAGCA


              ....|....| ....|....| ....|....| ....|....| ....|....|
                    210        220        230        240        250
Lnt           CCCCGCCCTC TACTGGCCTT TGCTCATCCT TTTTGCCGCC GCCACCCCCT
```

```
Fwd          CCCCGCCCTC TACTGGCCTT TGCTCATCCT TTTTGCCGCC GCCACCCCCT


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       260        270        280        290        300
Lnt          TTACCTTCGC ACCCTACTAC CACTTTTGGC TGATGCCCTT GATTTTCGGT
Fwd          TTACCTTCGC ACCCTACTAC CACTTTTGGC TGATGCCCTT GATTTTCGGT


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       310        320        330        340        350
Lnt          GCCTTCGTCC GCCTCATCGA ACTGCGTCCG CGTTTTGCTG TCTCTTCCGC
Fwd          GCCTTCGTCC GCCTCATCGA ACTGCGTCCG CGTTTTGCTG TCTCTTCCGC


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       360        370        380        390        400
Lnt          CTACCTGTTC GGCCTGACCG CATACACGAC ACAGTTCTAC TGGATACACA
Fwd          CTACCTGTTC GGCCTGACCG CATACACGAC ACAGTTCTAC TGGATACACA


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       410        420        430        440        450
Lnt          CCGCCCTGCA CGACGTTTCC GGCCTGCCCG ACCTCTATGC CGTACCGCTG
Fwd          CCGCCCTGCA CGACGTTTCC GGCCTGCCCG ACCTCTATGC CGTACCGCTG


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       460        470        480        490        500
Lnt          ACCTTCCTAC TCCCCGCCTA CCTTGCCCTT TATCCGGCAC TGTGTTTCTG
Fwd          ACCTTCCTAC TCCCCGCCTA CCTTGCCCTT TATCCGGCAC TGTGTTTCTG


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       510        520        530        540        550
Lnt          GCTGTGGAAA AAATTTACCC TGCCTCGGGG CATAAAAATC GGTTTGGTAC
Fwd          GCTGTGGAAA AAATTTACCC TGCCTCGGGG CATAAAAATC GGTTTGGTAC
```

```
            ....|....| ....|....| ....|....| ....|....| ....|....|
                    560        570        580        590        600
Lnt         TGCCCATCCT GTGGACGCTG ACCGAGTTTG CCCGCGAACG TTTCCTGACC
Fwd         TGCCCATCCT GTGGACGCTG ACCGAGTTTG CCCGCGAACG TTTCCTGACC

            ....|....| ....|....| ....|....| ....|....| ....|....|
                    610        620        630        640        650
Lnt         GGATTCGGCT GGGGCGCAAT CGGCTACTCC CAAATCACCC CGGACAGCCC
Fwd         GGATTCGGCT GGGGCGCAAT CGGCTACTCC CAAATCACCC CGGACAGCCC

            ....|....| ....|....| ....|....| ....|....| ....|....|
                    660        670        680        690        700
Lnt         GCTCGCCGGC TTTGCCCCAT TGGGCGGCAT CCACATGGTT ACACTGGCAA
Fwd         GCTCGCCGGC TTTGCCCCAT TGGGCGGCAT CCACATGGTT ACACTGGCAA

            ....|....| ....|....| ....|....| ....|....| ....|....|
                    710        720        730        740        750
Lnt         CCGCCTTTCT CGGTGTCTGG CTGGTTTTGG CGAGTAATAA CACCGCACGT
Fwd         CCGCCTTTCT CGGTGTCTGG CTGGTTTTGG CGAGTAATAA CACCGCACGT

            ....|....| ....|....| ....|....| ....|....| ....|....|
                    760        770        780        790        800
Lnt         TCGGGCAAAC GCCTGCTTCC GATTATCCTG ATTGCCGCCC TGCTTGCCGC
Fwd         TCGGGCAAAC GCCTGCTTCC GATTATCCTG ATTGCCGCCC TGCTTGCCGC

            ....|....| ....|....| ....|....| ....|....| ....|....|
                    810        820        830        840        850
Lnt         AGGCTACACC GCCCGACAAA CCGACTTCAC CCGCCCCGAC GGCAGCCGCA
Fwd         AGGCTACACC GCCCGACAAA CCGACTTCAC CCGCCCCGAC GGCAGCCGCA

            ....|....| ....|....| ....|....| ....|....| ....|....|
                    860        870        880        890        900
Lnt         GCACCGTCGC CCTGCTTCAA GGCAACATCG ACCAAACCCT CAAATGGCGT
```

```
Fwd          GCACCGTCGC CCTGCTTCAA GGCAACATCG ACCAAACCCT CAAATGGCGT


             ....|....| ....|....| ....|....| ....|....| ....|....|
                     910        920        930        940        950
Lnt          GAAGACCAAG TTATCCCGAC CATACAGAAA TATTACGAAC AAGTCGGCAA
Fwd          GAAGACCAAG TTATCCCGAC CATACAGAAA TATTCTGTCT C--TTATACA


             ....|....| ....|....| ....|....| ....|....| ....|....|
                     960        970        980        990       1000
Lnt          AACCACCGCC GACATCGTCA TCCTGCCCGA AACCGCCATC CCCGTTATGC
Fwd          CATCTCAACC ATCATCGATG AATTGTGTCT CAAAATCTCT GATGTTACAT


             ....|....| ....|....| ....|....| ....|....| ....|....|
                    1010       1020       1030       1040       1050
Lnt          GCCAAAACCT GCCGGAAAAC ATACTGGCGA AATTTGCCGA ACAGGCGCAA
Fwd          TGCACAAGAT A----AAAAT ATATCATCAT GAACAATAAA ACTGTCTGCT


             ....|....| ....|....| ....|....| ....|....| ....|....|
                    1060       1070       1080       1090       1100
Lnt          AACAACGGCA GCGCGCTCGC CGTCGGCATC AGCCAATACA CTTCGGACGG
Fwd          TACATAAACA GTAATACAAG GGGTGTTATG AGCCA-TA-- -TTCAACGGG


             ....|....| ....|....| ....|....| ....|....| ....|....|
                    1110       1120       1130       1140       1150
Lnt          CAACGGTTAC GAAAACGCCG TCATCAACCT GACCGGTTAT CAGGAAAACA
Fwd          AAACGTCTTG CTCGAGGCCG CGATTAAATT ---CCAACAT --GGATGCTG


             ....|....| ....|....| ....|....| ....|....| ....|....|
                    1160       1170       1180       1190       1200
Lnt          ATCAGGACGG TATCCCCTAC TACGCCAAAA ACCACCTCGT CCCCTTCGGC
Fwd          ATTTATATGG G---TATAAA TGGGCTCGCG ATAATGTCGG GCAATCAGGT
```

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1210       1220       1230       1240       1250
Lnt             GAATACAAAC CGCTGCCTTT CCTGACCACG CCGCTTTACA AAATGATGGA
Fwd             GCG-ACAATC TATCGATTGT A-TGGGAAGC CCGATGCGCC AGA-------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1260       1270       1280       1290       1300
Lnt             TATGCCCCTT TCCGACTTCC GCAAAGGCGG CGGCAAGCAA TCCGCCCTGC
Fwd             ---GTTGTTT CTGAAACATG GCAAAGGTAG CGTT--GCCA ATGATGTTAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1310       1320       1330       1340       1350
Lnt             TGATGAAA-A ACCAAA--AA ATCGCCTTCA ACATCTGTTA CGAAGACGGA
Fwd             AGATGAGATG GTCAGACTAA ACTGGCTGAC GGAATTTATG CCTCTTCCGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1360       1370       1380       1390       1400
Lnt             TTCGGCGACG AACTGATTGC CGCCGCAAAA GATGCCACCC TGCTTGCCAA
Fwd             C-CATCAAGC ATTTTATCCG TACTCCTGAT GATGCATGGT TACTCACCAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1410       1420       1430       1440       1450
Lnt             TGCCAGCAAT ATGGCGTGGT ACGGAAAATC CAACGCCATG TACCAGCACC
Fwd             TGCGATCCCC G--------- --GAAAAACA GCATTCCAGG TATTAGAAGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1460       1470       1480       1490       1500
Lnt             TCCAACAATC GCAGGCGCGG GCTATGGAAC TCGGACGCTA TATGGTCCGC
Fwd             ATATCCTGAT TCAGGTGAAA ATATTGTTGA TGCGCTGGCA GTGTTCCTGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1510       1520       1530       1540       1550
Lnt             GCCACCAACA CCGGCGCAAC CGCCATCATC TCCCCCAAAG GCAACATCAT
```
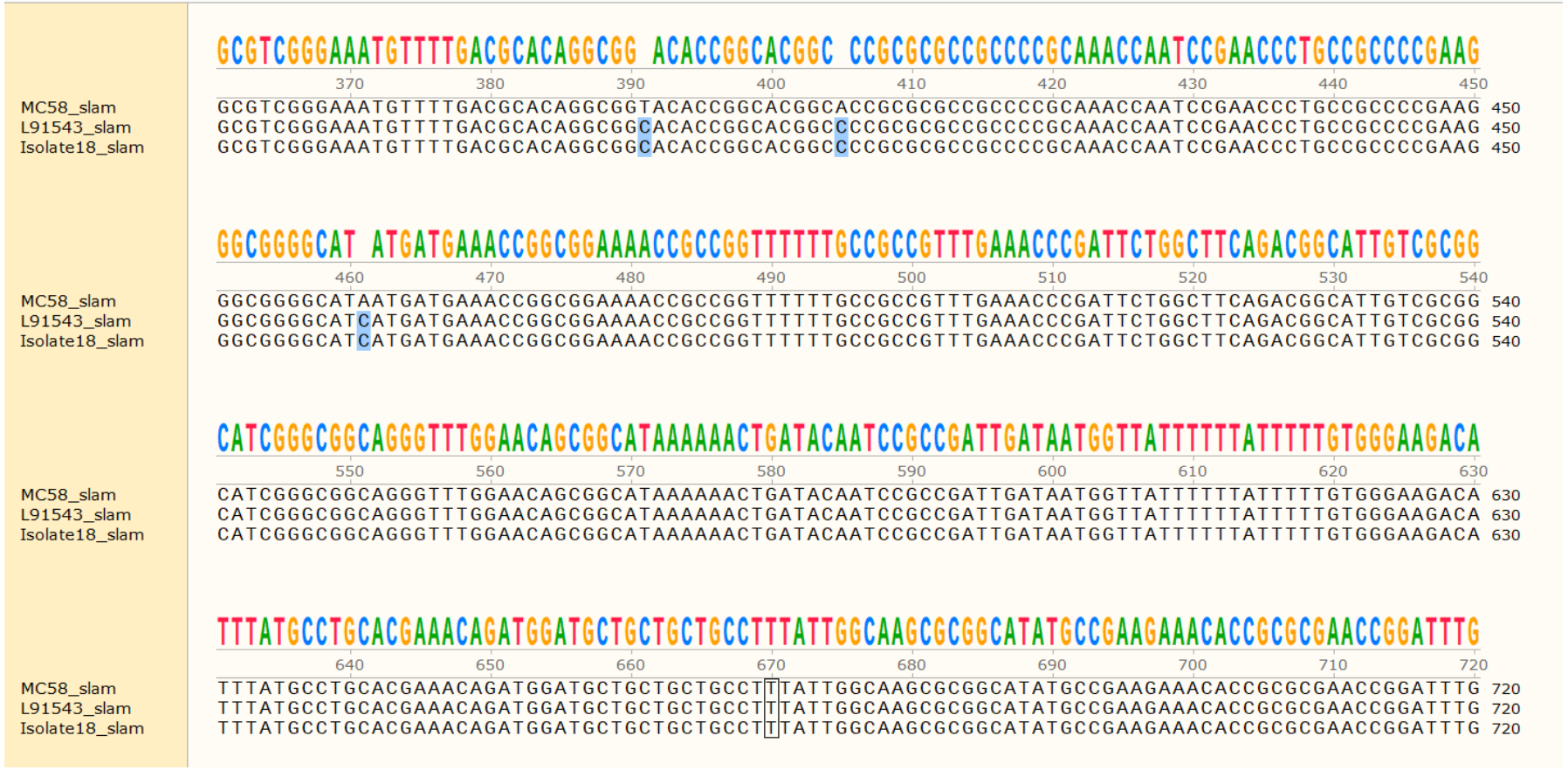

```
Fwd          GCCGGTTGCA TTC------- ---------- ---------- ----------


             ....|....| ....|....| ....|....| ....|....| ....|....|
                     1560       1570       1580       1590       1600
Lnt          CGCCCAAGCC CAACCCGATA CGGAAACCGT ATTGGAAGGA CACATCAAAG
Fwd          ---------- ---------- ---------- ---------- ----------


             ....|....| ....|....| ....|....| ....|....| ....|....|
                     1610       1620       1630       1640       1650
Lnt          GCTATGTCGG CGAAACGCCC TATATGAAAA CCGGCAGTTC ATGGTGGTTG
Fwd          ---------- ---------- ---------- ---------- ----------


             ....|....| ....|....| ....|....| ....|....| ....|....|
                     1660       1670       1680       1690       1700
Lnt          ATGGGCATAT TGGCCCTAGC CGCACTGATT CTTTTCATCT TCCGAAACAA
Fwd          ---------- ---------- ---------- ---------- ----------


             ....|....|
                     1710
Lnt          AGAACACTGA
Fwd          ----------
```

Appendix VI- *Slam* and *Slam* flanking regions (600bp up and downstream) alignment by SnapGene v4.3.11 of L91543 and isolate 18 vs MC58. Consensus sequences showed at the top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L91543_slam GCGTCGGGAAATGTTTTGACGCACAGGCGGCACACCGGCACGGCCCCGCGCGCCGCCCCGCAAACCAATCCGAACCCTGCCGCCCCGAAG 450
Isolate18_slam GCGTCGGGAAATGTTTTGACGCACAGGCGGCACACCGGCACGGCCCCGCGCGCCGCCCCGCAAACCAATCCGAACCCTGCCGCCCCGAAG 450
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L91543_slam CATCGGGCGGCAGGGTTTGGAACAGCGGCATAAAAAACTGATACAATCCGCCGATTGATAATGGTTATTTTTTATTTTTGTGGGAAGACA 630
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L91543_slam GTTTTGCAGATTGACGGAGAAACCCTGCTGAAAAATCCCGAATTGTTGTCCCGCGCGATGTATTCCGCAGTGGTCTCAAACAATATTGCC 900
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Isolate18_slam GACGGTAGGGTGAAAGAAGCGATTTCCCATTACCGGGAATTGATTGCCGCCCAACCCGACGCGCCCGCCGTCCGTATGCGTTTGGCGGCG 1080


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Isolate18_slam GAATGGGGCGGCAGCGGCCTGTCTTCGCTGTTGCGCCTCGGCGCGGCGAAACGGCATTATGAAAAACCCGGCTTTTTCAGCGGTTTTAAA 1890
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L91543_slam CTGTTCCTTTTCGGAGGAAACCCTGCCGGCGGCGGTATCACGGCGGGCATCGGCGGCTTTCGGGCGGTGCTTTGCGTGCCGCCGCGTGTG 2160
Isolate18_slam CTGTTCCTTTTCGGAGGAAACCCTGCCGGCGGCGGTATCACGGCGGGCATCGGCGGCTTTCGGGCGGTGCTTTGCGTGCCGCCGCGTGTG 2160


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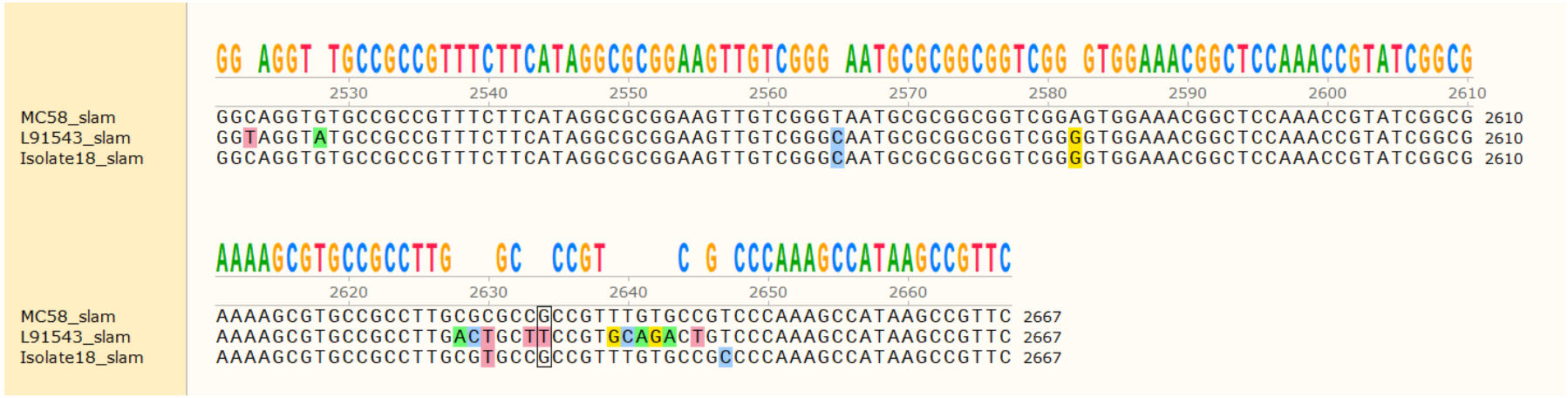
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2620
2630
2640
2650
2660
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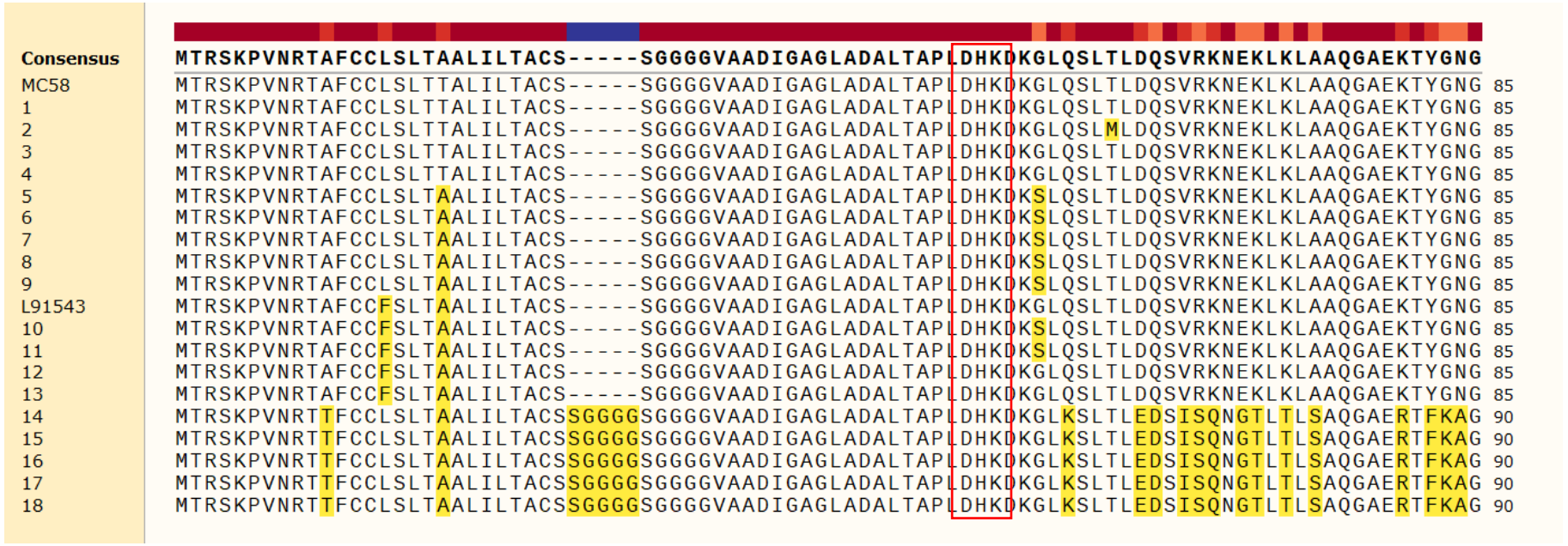
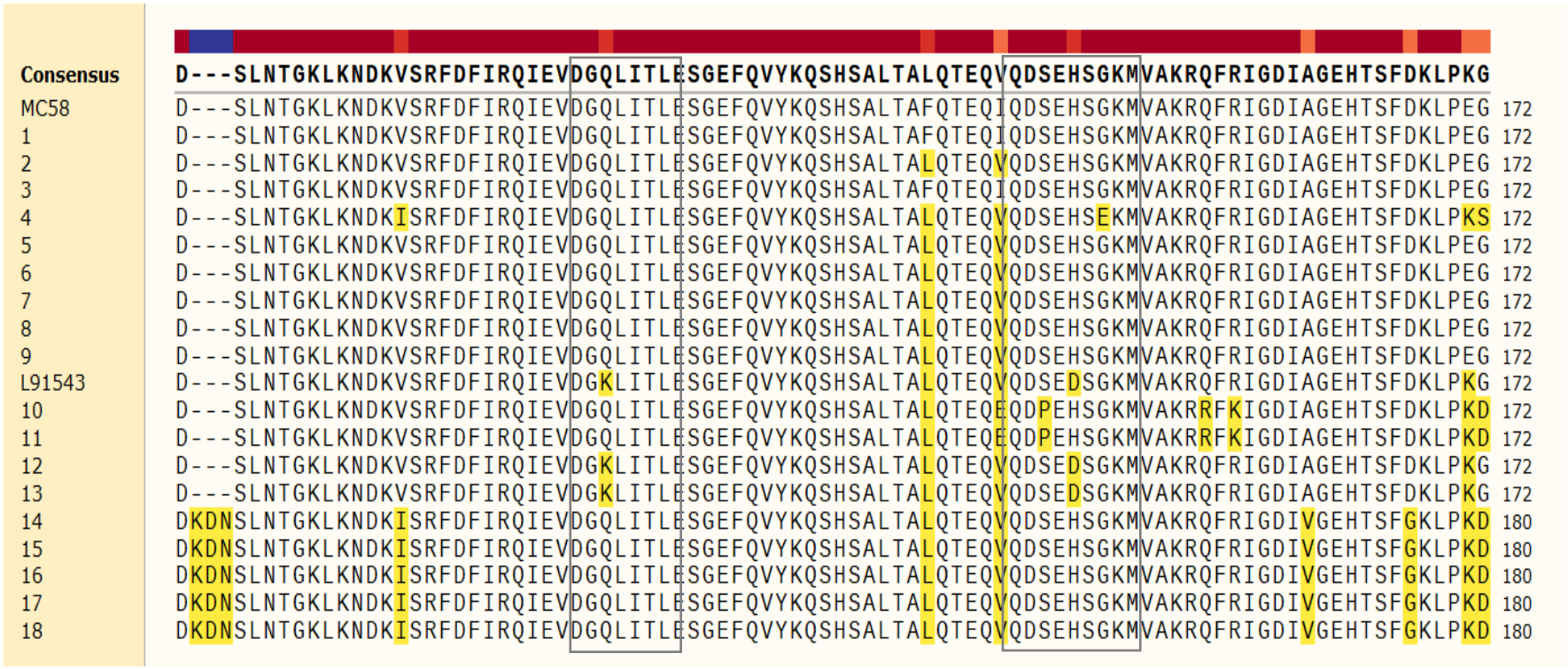
Isolate18_slam AAAAGCGTGCCGCCTTGCGTGCCGCCGTTTGTGCCGCCCCAAAGCCATAAGCCGTTC 2667

Appendix VII- Multiple alignment of FHbp of isolates used in this study and amino acid identities across whole protein relative to MC58. Framed in red and grey boxes are AA residues in the epitopes recognised by JAR4 and JAR5, respectively.

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1 D---SLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQVYKQSHSALTAFQTEQIQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEG 172
2 D---SLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQVYKQSHSALTALQTEQVQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEG 172
3 D---SLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQVYKQSHSALTAFQTEQIQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEG 172
4 D---SLNTGKLKNDKISRFDFIRQIEVDGQLITLESGEFQVYKQSHSALTALQTEQVQDSEHSEKMVAKRQFRIGDIAGEHTSFDKLPKS 172
5 D---SLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQVYKQSHSALTALQTEQVQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEG 172
6 D---SLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQVYKQSHSALTALQTEQVQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEG 172
7 D---SLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQVYKQSHSALTALQTEQVQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEG 172
8 D---SLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQVYKQSHSALTALQTEQVQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEG 172
9 D---SLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQVYKQSHSALTALQTEQVQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEG 172
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10 D---SLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQVYKQSHSALTALQTEQEQDPEHSGKMVAKRRFKIGDIAGEHTSFDKLPKD 172
11 D---SLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQVYKQSHSALTALQTEQEQDPEHSGKMVAKRRFKIGDIAGEHTSFDKLPKD 172
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15 DKDNSLNTGKLKNDKISRFDFIRQIEVDGQLITLESGEFQVYKQSHSALTALQTEQVQDSEHSGKMVAKRQFRIGDIVGEHTSFGKLPKD 180
16 DKDNSLNTGKLKNDKISRFDFIRQIEVDGQLITLESGEFQVYKQSHSALTALQTEQVQDSEHSGKMVAKRQFRIGDIVGEHTSFGKLPKD 180
17 DKDNSLNTGKLKNDKISRFDFIRQIEVDGQLITLESGEFQVYKQSHSALTALQTEQVQDSEHSGKMVAKRQFRIGDIVGEHTSFGKLPKD 180
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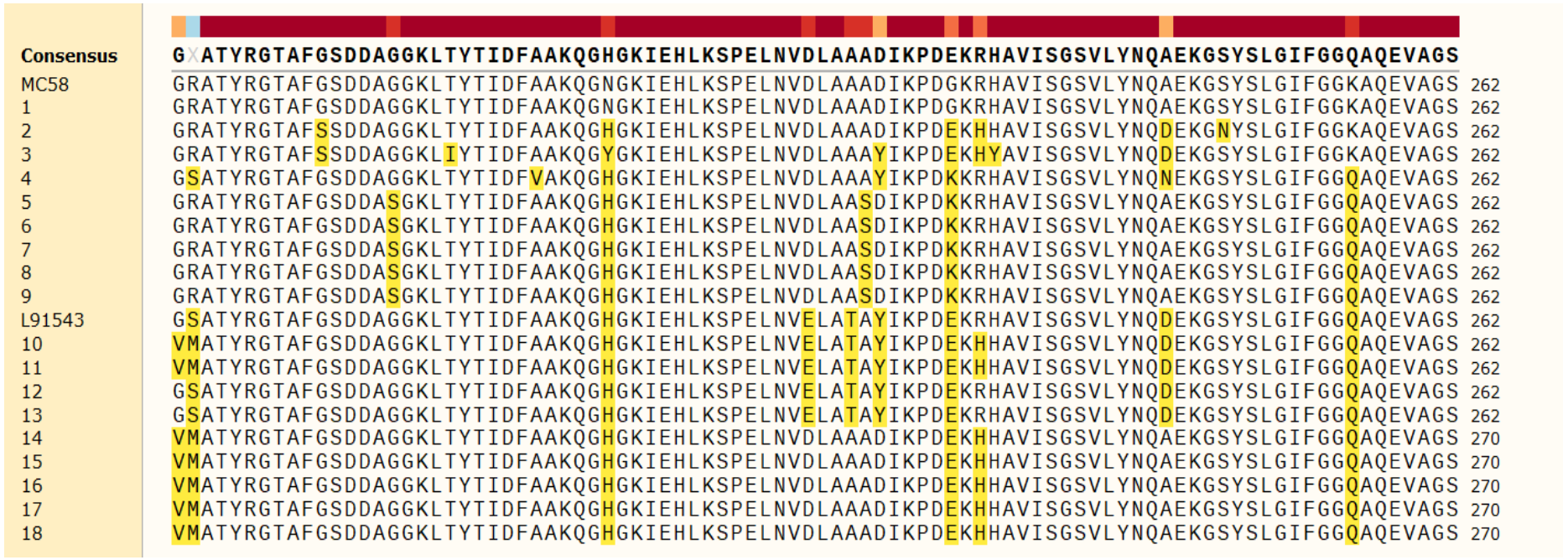
Consensus GXATYRGTAFGSDDAGGKLTYTIDFAAKQGHGKIEHLKSPELNVDLAAADIKPDEKRHAVISGSVLYNQAEKGSYSLGIFGGQAQEVAGS
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1 GRATYRGTAFGSDDAGGKLTYTIDFAAKQGNGKIEHLKSPELNVDLAAADIKPDGKRHAVISGSVLYNQAEKGSYSLGIFGGKAQEVAGS 262
2 GRATYRGTAFSSDDAGGKLTYTIDFAAKQGHGKIEHLKSPELNVDLAAADIKPDEKHHAVISGSVLYNQDEKGNYSLGIFGGKAQEVAGS 262
3 GRATYRGTAFSSDDAGGKLIYTIDFAAKQGYGKIEHLKSPELNVDLAAAYIKPDEKHYAVISGSVLYNQDEKGSYSLGIFGGKAQEVAGS 262
4 GSATYRGTAFGSDDAGGKLTYTIDFVAKQGHGKIEHLKSPELNVDLAAAYIKPDKKRHAVISGSVLYNQNEKGSYSLGIFGGQAQEVAGS 262
5 GRATYRGTAFGSDDASGKLTYTIDFAAKQGHGKIEHLKSPELNVDLAASDIKPDKKRHAVISGSVLYNQAEKGSYSLGIFGGQAQEVAGS 262
6 GRATYRGTAFGSDDASGKLTYTIDFAAKQGHGKIEHLKSPELNVDLAASDIKPDKKRHAVISGSVLYNQAEKGSYSLGIFGGQAQEVAGS 262
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8 GRATYRGTAFGSDDASGKLTYTIDFAAKQGHGKIEHLKSPELNVDLAASDIKPDKKRHAVISGSVLYNQAEKGSYSLGIFGGQAQEVAGS 262
9 GRATYRGTAFGSDDASGKLTYTIDFAAKQGHGKIEHLKSPELNVDLAASDIKPDKKRHAVISGSVLYNQAEKGSYSLGIFGGQAQEVAGS 262
L91543 GSATYRGTAFGSDDAGGKLTYTIDFAAKQGHGKIEHLKSPELNVELATAYIKPDEKRHAVISGSVLYNQDEKGSYSLGIFGGQAQEVAGS 262
10 VMATYRGTAFGSDDAGGKLTYTIDFAAKQGHGKIEHLKSPELNVELATAYIKPDEKHHAVISGSVLYNQDEKGSYSLGIFGGQAQEVAGS 262
11 VMATYRGTAFGSDDAGGKLTYTIDFAAKQGHGKIEHLKSPELNVELATAYIKPDEKHHAVISGSVLYNQDEKGSYSLGIFGGQAQEVAGS 262
12 GSATYRGTAFGSDDAGGKLTYTIDFAAKQGHGKIEHLKSPELNVELATAYIKPDEKRHAVISGSVLYNQDEKGSYSLGIFGGQAQEVAGS 262
13 GSATYRGTAFGSDDAGGKLTYTIDFAAKQGHGKIEHLKSPELNVELATAYIKPDEKRHAVISGSVLYNQDEKGSYSLGIFGGQAQEVAGS 262
14 VMATYRGTAFGSDDAGGKLTYTIDFAAKQGHGKIEHLKSPELNVDLAAADIKPDEKHHAVISGSVLYNQAEKGSYSLGIFGGQAQEVAGS 270
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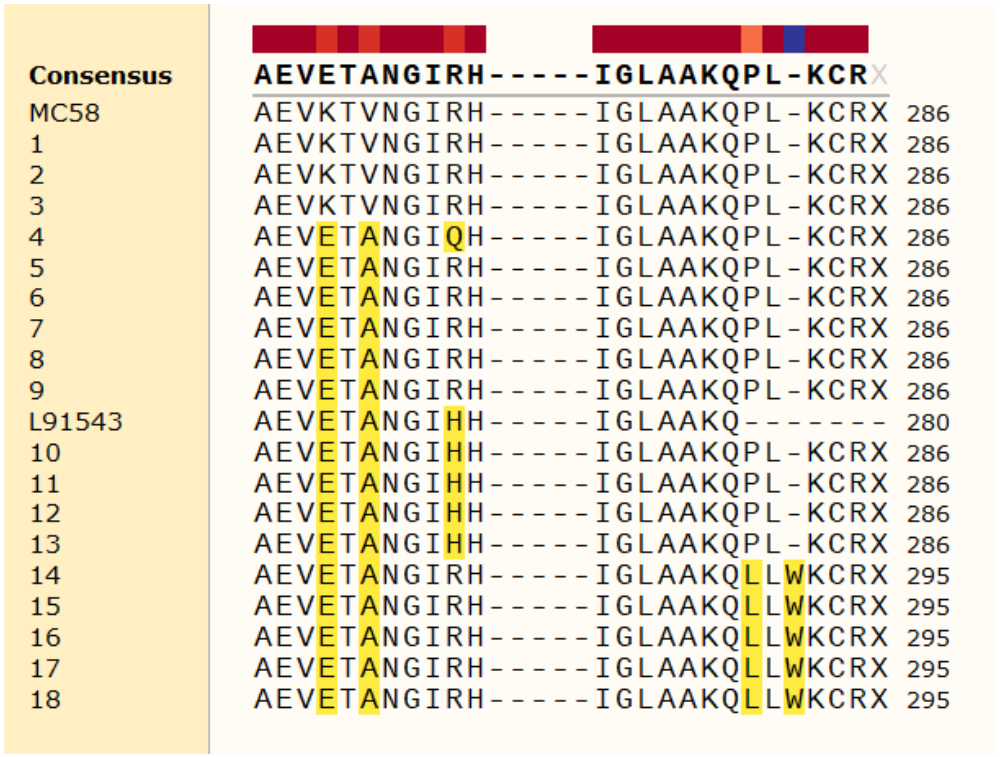

Strain	Similarity (%)
1	100
2	97
3	97
4	94
5	96
6	96
7	96
8	96
9	96
L91543	93
10	92
11	92
12	94
13	94
14	86
15	86
16	86
17	86
18	86

Appendix VIII- Crude WC immunodot blotting to test JAR4 and JAR5 reactivity to strains used in this study

Appendix IX- RT-PCR primers aligning regions

Appendix X- Gating strategy and standard curve (number of antibody vs fluorescence)

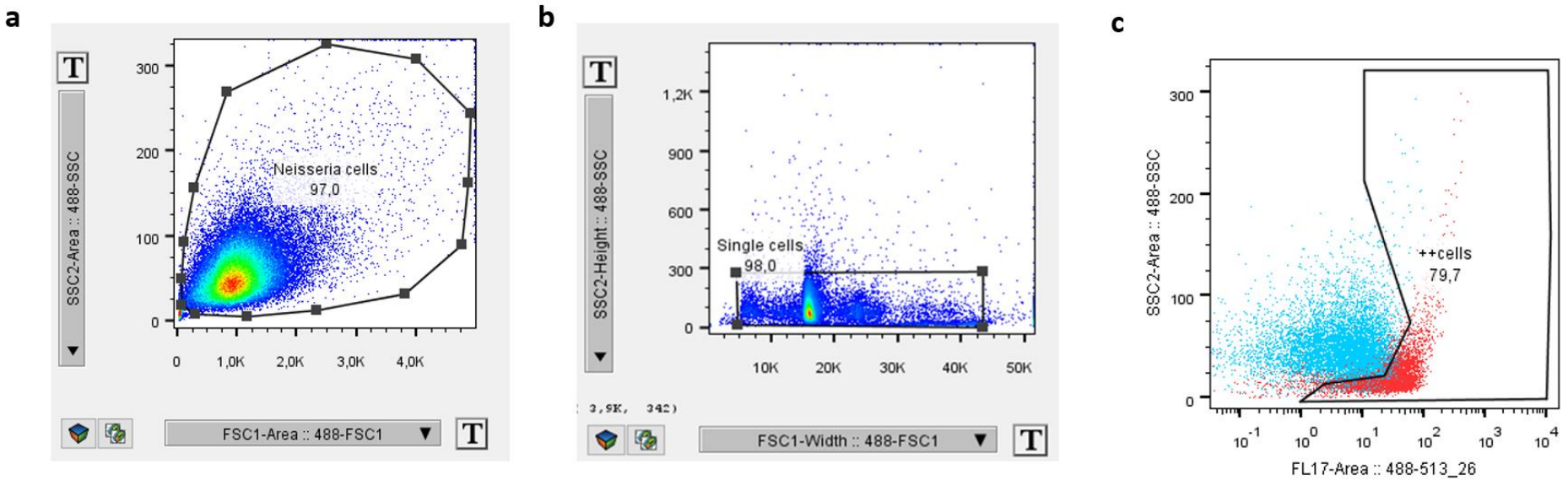

Figure- Gating strategy for identifying stained Neisserial cells. FlowJo v10 software was used to analyse FACS data. **a.** Data show Dot-plot (Side scatter vs Forward scatter) of total population gated to remove cellular debris. **b.** Dot-plot [SSC (height) x FSC1 (width)] of pulse processing to remove aggregates/doublets. **c.** Dot-plot discriminating between the cell cycle populations based on FITC staining plotted against cells stained with secondary antibody only (negative control). Each region was gated and the data shown are from a typical experiment where a minimum of 100,000 events were recorded and conducted in triplicate.

Bead	ABC	Channel
Blank		2.1
Bead #1	7311	35.4
Bead #2	44263	114.5
Bead #3	161876	405.36
Bead #4	269561	608.57

Regression Coefficient:	0.9956
Detection Threshold:	248

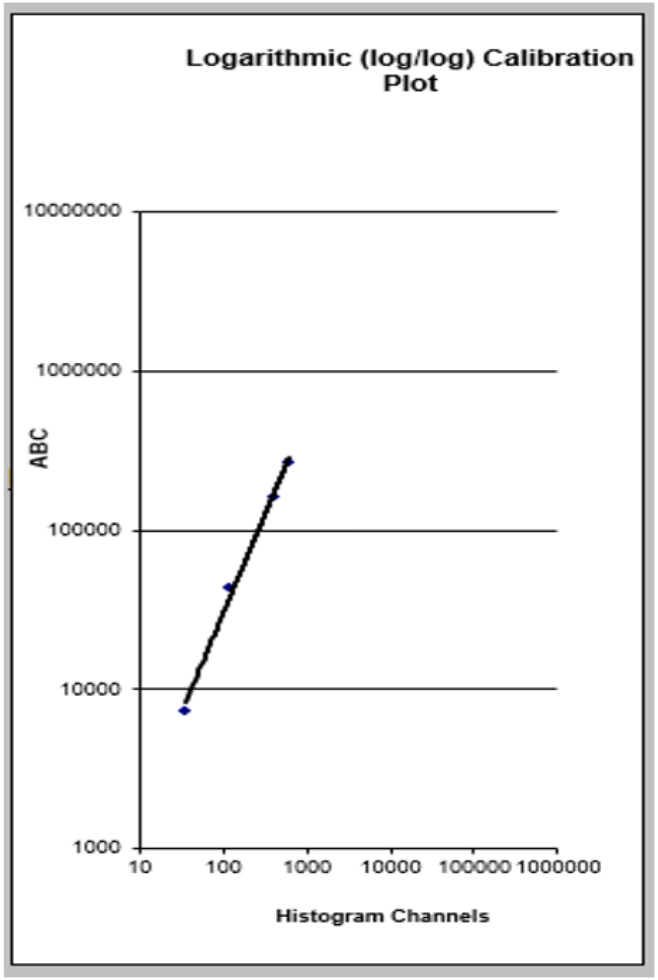

Figure- Standard curve (number of antibody vs fluorescence) generated by Quickcal v2.3 (Bangs Lab)

Appendix XI- SOE of *lolA* and *lolB*

Table. *lolA and lolB SOEing primers*

HA1_lolA_Fwd_EcoRI	GATAGAATTCCAAAACAGGCTTGTCCGGTC
Rev_HA1_lolA	GATGATGGTTGTATTTTTCCTTGTCGGGATG
Kan_f_lolA	CATCCCGACAAGGAAAAATACAACCATCATCGATG
Kan_r_LolA	GGACGGCATTCAGACGGCATGTAATGCTCTGCC
HA2_lolA_fwd	ATGCCGTCTGAATGCCGTCCGCCCCGATGCCG
HA2_lolA_BamHI_rev	CTTAGGATCCCTGATACGCGCCCGCTTTGATTTGC
HA1_lolB_Fwd_EcoRI	GATAGAATTCGGTCAGTTGTTTACGATCG
Rev_HA1_lolB	GATGATGGTTGCAGAATTTCCTTAACGGATGC
LolB_Kan_f	GCATCCGTTAAGGAAATTCTGCAACCATCATCGATG
LolB_Kan_r	CTGTCCGCCTTCAGACGGCATGTAATGCTCTGCC
HA2_lolB_fwd	ATGCCGTCTGAAGGCGGACAGATGAATATTGC
HA2_lolB_BamHI_rev	CTTAGGATCCTATGCTTGAGGCGGAATTTCG

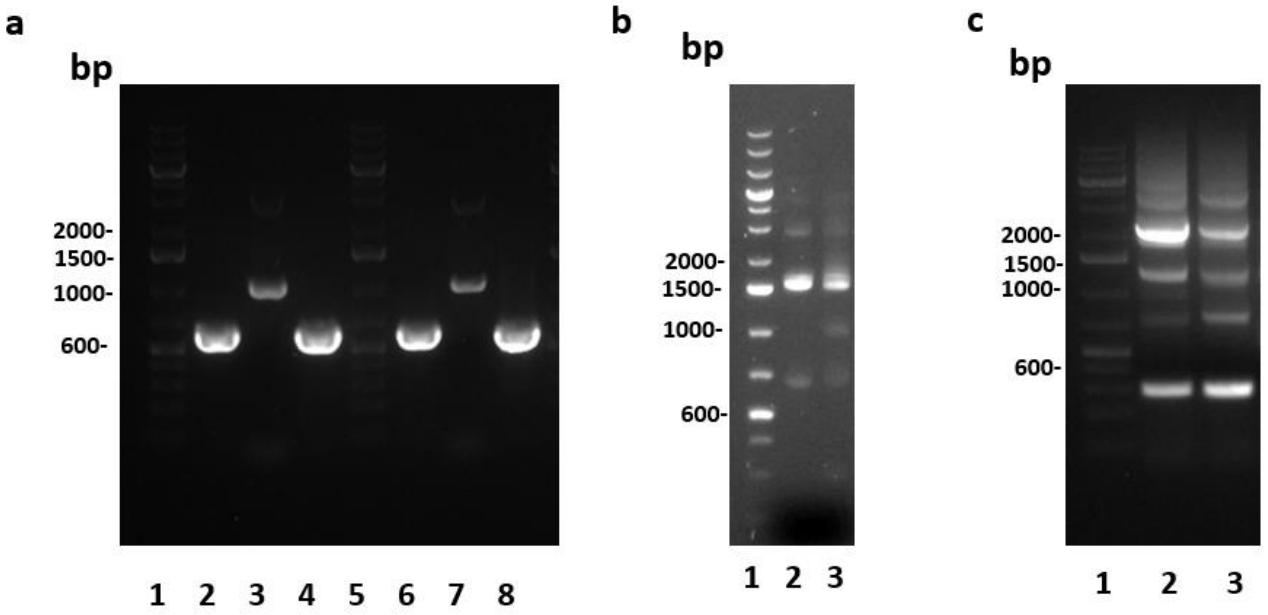

Figure- PCR amplification steps involved in SOE of *lolA and lolB*

a. First round of PCR amplification of *lolA* and *lolB* is shown. Lanes; 1 and 5, ladder; 2 and 6, HA1 upstream of *lolA* and *lolB,* 3 and 7; *kan* gene; 4 and 8, HA2 downstream of lolA *and* lolB. **b.** Second round of PCR amplification using HA1 and *kan* products as template for this round. Lanes; 1, ladder; 2, *lolA*_HA1_kan product; 3, *lolb*_HA1_kan product. **c.** Final round of PCR amplification. HA1_kan and HA2 were used as template to generate the last PCR product to be used for transformation. Lanes; 1, ladder; 2 and 3, HA2 to *lolA*_HA1_kan product and *lolb*_HA1_kan product, respectively.

Appendix XII- DE genes and KEGG list

GeneID	GeneName	log2FoldChange	padj	KEGG
NMB0577	hypothetical protein	-1.66	1.64E-05	K19339
NMB0792	transporter	-0.83	5.10E-09	K14445
NMB0437	hypothetical protein	-0.76	9.01E-04	K13771
NMB1719	efflux pump component MtrF	-1.24	1.77E-07	K12942
NMB1737	secretion protein	-0.81	1.88E-07	K12340
NMB1400	ABC transporter family protein	-0.72	5.46E-03	K11004
NMB1738	secretion protein	-1.13	3.60E-07	K11003
NMB0789	amino acid ABC transporter ATP-binding protein	-0.89	3.58E-02	K10010
NMB0070	polysialic acid capsule biosynthesis protein SynX	-0.84	2.33E-08	K08068
NMB0583	IS1016C2 transposase	-1.01	2.11E-09	K07488
NMB1411	IS1016C2 transposase	-0.91	3.66E-04	K07488
NMB1259	IS30 family transposase	-0.86	9.17E-05	K07482
NMB0248	hypothetical protein	-0.69	3.96E-03	K07461
NMB0578	copper ABC transporter substrate-binding protein	-1.00	8.55E-05	K07218
NMB1669	heme utilisation protein	-0.84	8.45E-03	K07215
NMB2037	hypothetical protein	-0.86	1.22E-05	K07172
NMB2038	hypothetical protein	-0.84	2.16E-03	K07171
NMB2003	hypothetical protein	-0.79	2.10E-07	K06518
NMB1493	carbon starvation protein A	-1.04	2.37E-03	K06200
NMB1496	23S rRNA pseudouridine synthase E	-1.05	4.31E-11	K06181
NMB0689	transcription elongation factor GreB	-1.08	4.15E-02	K04760
NMB1995	nitrogen regulatory protein P-II 1	-1.09	2.14E-02	K04751
NMB1398	superoxide dismutase	-0.71	4.55E-05	K04565
NMB0649	hypothetical protein	-0.69	2.14E-02	K04095

NMB0255	adenosine monophosphate-protein transferase NmFic	-0.69	3.36E-05	K04095
NMB0394	quinolinate synthetase	-1.35	4.38E-02	K03517
NMB0378	phosphate permease	-1.70	2.88E-04	K03306
NMB0018	pilin PilE	-1.25	4.80E-10	K02650
NMB0721	translation initiation factor IF-3	-0.74	4.49E-04	K02520
NMB0462	spermidine/putrescine ABC transporter substrate-binding protein	-1.14	5.47E-09	K02055
NMB0879	sulfate ABC transporter ATP-binding protein	-1.03	9.68E-04	K02045
NMB1996	phosphoribosylformylglycinamidine synthase	-0.82	4.67E-09	K01952
NMB0688	N-(5'-phosphoribosyl)anthranilate isomerase	-1.07	1.83E-02	K01817
NMB0284	adenylosuccinate lyase	-0.72	7.31E-03	K01756
NMB0763	cysteine synthase	-1.04	1.40E-05	K01738
NMB1572	aconitate hydratase B	-0.80	8.16E-03	K01682
NMB0726	type II restriction enzyme	-0.78	5.59E-03	K01155
NMB1997	hydroxyacylglutathione hydrolase	-0.94	3.73E-11	K01069
NMB1588	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase	-0.68	7.46E-04	K00995
NMB1154	sulfate adenylyltransferase subunit 2	-1.81	2.77E-05	K00957
NMB1153	sulfate adenylyltransferase subunit 1	-1.98	2.97E-09	K00956
NMB0337	branched-chain amino acid aminotransferase	-0.78	3.49E-02	K00826
NMB2051	cytochrome C1	-0.71	1.01E-06	K00413
NMB1151	sulfite reductase subunit beta	-1.98	1.44E-07	K00381
NMB1152	sulfite reductase flavoprotein subunit alpha	-1.48	3.21E-06	K00380
NMB1623	major anaerobically induced outer membrane protein	-1.47	1.59E-02	K00368
NMB0258	NADH dehydrogenase subunit M	-0.77	8.43E-04	K00342
NMB0251	NADH dehydrogenase subunit I	-0.74	1.00E-04	K00338
NMB0242	NADH dehydrogenase subunit B	-0.79	1.23E-02	K00331
NMB0241	NADH dehydrogenase subunit A	-1.09	1.75E-06	K00330
NMB0949	succinate dehydrogenase hydrophobic membrane anchor protein	-0.89	5.64E-03	K00242

NMB0948	succinate dehydrogenase cytochrome b556 subunit	-0.93	1.18E-04	K00241
NMB2096	malate:quinone oxidoreductase	-0.68	1.34E-02	K00116
NMB1162	hypothetical protein	-5.37	1.10E-03	-
NMB0120	hypothetical protein	-1.89	2.96E-14	-
NMB0993	rubredoxin	-1.78	2.05E-04	-
NMB1541	lactoferrin-binding protein	-1.69	5.56E-27	-
NMB1712	L-lactate permease-like protein	-1.59	1.67E-10	-
NMB0663	outer membrane protein	-1.44	2.20E-10	-
NMB0119	hypothetical protein	-1.33	1.83E-06	-
NMB1739	hypothetical protein	-1.31	1.63E-05	-
NMB1786	hypothetical protein	-1.28	1.67E-10	-
NMB1753	VapD-like protein	-1.28	3.14E-07	-
NMB1211	hypothetical protein	-1.27	2.56E-04	-
NMB0866	hypothetical protein	-1.23	6.52E-04	-
NMB1212	hypothetical protein	-1.20	1.51E-04	-
NMB1495	hypothetical protein	-1.18	6.56E-03	-
NMB1213	lipoprotein	-1.15	7.46E-04	-
NMB0985	hypothetical protein	-1.08	1.98E-03	-
NMB0297	hypothetical protein	-1.04	1.88E-03	-
NMB1780	hemolysin activation protein HecB	-1.03	4.38E-03	-
NMB1994	adhesin/invasin	-1.02	4.69E-03	-
NMB1124	hypothetical protein	-0.97	5.47E-05	-
NMB2113	hypothetical protein	-0.96	1.01E-06	-
NMB0256	hypothetical protein	-0.96	6.41E-10	-
NMB0915	hypothetical protein	-0.94	5.71E-10	-
NMB0373	hypothetical protein	-0.91	1.54E-03	-
NMB1053	class 5 outer membrane protein	-0.88	3.97E-04	-

NMB1214	hemagglutinin/hemolysin-like protein	-0.87	6.66E-04	-
NMB1126	hypothetical protein	-0.87	1.10E-03	-
NMB0024	fimbrial protein	-0.86	1.83E-02	-
NMB1856	LysR family transcriptional regulator	-0.86	1.91E-07	-
NMB2132	transferrin-binding protein-like protein	-0.86	2.81E-03	-
NMB0654	hypothetical protein	-0.85	1.13E-03	-
NMB0065	hypothetical protein	-0.84	2.20E-05	-
NMB1494	hypothetical protein	-0.83	6.70E-03	-
NMB1007	transcriptional regulator	-0.82	5.08E-03	-
NMB0648	hypothetical protein	-0.81	2.44E-06	-
NMB0986	hypothetical protein	-0.81	3.29E-02	-
NMB0122	hypothetical protein	-0.80	8.09E-04	-
NMB0916	hypothetical protein	-0.79	1.43E-05	-
NMB2097	hypothetical protein	-0.78	2.56E-02	-
NMB0371	hypothetical protein	-0.78	2.29E-02	-
NMB0252	hypothetical protein	-0.77	1.57E-08	-
NMB0818	hypothetical protein	-0.76	8.85E-09	-
NMB2112	hypothetical protein	-0.76	4.36E-03	-
NMB1843	MarR family transcriptional regulator	-0.75	1.54E-05	-
NMB0104	hypothetical protein	-0.75	8.33E-03	-
NMB0653	MafB-like protein	-0.73	1.89E-02	-
NMB1410	hypothetical protein	-0.73	3.33E-04	-
NMB0910	transcriptional regulator	-0.72	3.37E-05	-
NMB0655	hypothetical protein	-0.72	7.41E-03	-
NMB0656	hypothetical protein	-0.72	9.59E-04	-
NMB0467	hypothetical protein	-0.72	5.85E-09	-
NMB1088	hypothetical protein	-0.71	2.90E-03	-

NMB0731	hypothetical protein	-0.71	1.15E-02	-
NMB0605	histone deacetylase	-0.70	6.70E-03	-
NMB1117	hypothetical protein	-0.70	1.33E-02	-
NMB0817	hypothetical protein	-0.70	2.78E-05	-
NMB0882	hypothetical protein	-0.69	3.89E-02	-

GeneID	GeneName	log2FoldChange	padj	KEGG
NMB1426	hypothetical protein	1.64	2.90E-06	-
NMB1475	hypothetical protein	1.51	1.16E-02	-
NMB0020	fimbrial protein	1.42	5.18E-06	-
NMB1103	hypothetical protein	1.19	1.45E-03	-
NMB1102	hypothetical protein	1.13	9.60E-06	-
NMB1101	hypothetical protein	1.07	1.58E-04	-
NMB0661	bis(5'-nucleosyl)-tetraphosphatase	1.01	2.84E-06	-
NMB0346	hypothetical protein	1.00	1.64E-05	-
NMB0023	fimbrial protein	0.97	3.32E-03	-
NMB1104	phage sheath protein	0.97	2.15E-07	-
NMB0019	fimbrial protein	0.97	1.67E-10	-
NMB1253	hypothetical protein	0.95	4.01E-06	-
NMB2002	hypothetical protein	0.94	9.62E-07	-
NMB1565	hypothetical protein	0.94	3.93E-10	-
NMB0345	cell-binding factor	0.93	6.17E-05	-
NMB0747	hypothetical protein	0.88	1.99E-03	-
NMB0313	hypothetical protein	0.87	2.80E-03	-
NMB0087	hypothetical protein	0.84	2.90E-02	-
NMB1105	hypothetical protein	0.83	1.51E-04	-
NMB1106	hypothetical protein	0.82	1.74E-04	-
NMB0742	hypothetical protein	0.80	4.43E-08	-
NMB1100	hypothetical protein	0.78	2.56E-04	-
NMB1273	alginate o-acetyltransferase AlgI	0.77	6.02E-04	-
NMB1110	tail protein	0.76	1.98E-03	-
NMB2095	adhesin complex protein	0.73	3.47E-02	-
NMB1845	thioredoxin	0.73	5.35E-05	-
NMB0086	hypothetical protein	0.69	4.05E-03	-

NMB1562	hypothetical protein	0.68	1.77E-05	-
NMB1377	L-lactate dehydrogenase	1.03	2.84E-02	K00101
NMB0111	methionyl-tRNA formyltransferase	0.81	1.06E-05	K00604
NMB1799	S-adenosylmethionine synthetase	0.76	5.65E-04	K00789
NMB1432	3-phosphoshikimate 1-carboxyvinyltransferase	0.85	1.09E-02	K00800
NMB1254	GTP cyclohydrolase II	0.93	1.57E-09	K01497
NMB0878	threonine dehydratase	0.78	5.80E-09	K01754
NMB0871	pantoate--beta-alanine ligase	0.73	1.15E-03	K01918
NMB0420	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase	0.76	4.81E-04	K01925
NMB0964	TonB-dependent receptor	1.32	4.85E-05	K02014
NMB0623	spermidine/putrescine ABC transporter substrate-binding protein	1.01	1.61E-04	K02055
NMB0752	bacterioferritin-associated ferredoxin	0.72	4.10E-02	K02192
NMB0702	competence protein	0.88	3.97E-08	K02238
NMB0017	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase	0.87	7.21E-04	K02535
NMB1862	50S ribosomal protein L11 methyltransferase	0.80	2.79E-07	K02687
NMB1315	uracil permease	0.78	1.13E-06	K02824
NMB0942	50S ribosomal protein L31 type B	1.34	2.62E-02	K02909
NMB0941	50S ribosomal protein L36	1.52	4.98E-03	K02919
NMB0743	ubiquinone/menaquinone biosynthesis methyltransferase	0.70	2.15E-07	K03183
NMB0268	RNA methyltransferase	1.00	5.74E-04	K03216
NMB1716	membrane fusion protein	0.82	1.41E-06	K03585
NMB0412	cell division protein FtsL	0.79	6.91E-04	K03586
NMB1846	ATP-binding protein	0.85	9.21E-09	K03593
NMB0687	GTP-binding protein Era	0.70	3.41E-07	K03595
NMB0683	transcription antitermination protein NusB	0.71	7.60E-04	K03625

NMB0267	hypothetical protein	1.02	5.36E-03	K03642
NMB0294	thiol:disulfide interchange protein DsbA	1.13	4.64E-03	K03673
NMB0686	ribonuclease III	0.82	2.57E-06	K03685
NMB0822	heat shock protein HtpX	0.83	8.03E-04	K03799
NMB1807	penicillin-binding protein 1	0.69	1.51E-04	K05366
NMB0344	BolA/YrbA family protein	0.88	1.56E-05	K05527
NMB0549	ABC transporter ATP-binding protein	1.13	8.98E-06	K05685
NMB0342	intracellular septation protein A	0.78	1.11E-02	K06190
NMB0419	hypothetical protein	0.74	5.35E-05	K07126
NMB0347	D-tyrosyl-tRNA(Tyr) deacylase	1.02	3.93E-10	K07560
NMB1949	soluble lytic murein transglycosylase	0.79	2.05E-03	K08309
NMB0343	hypothetical protein	0.85	1.95E-09	K09780
NMB1646	hemolysin	1.07	1.51E-04	K11068
NMB1566	phosphoribosylglycinamide transformylase	1.21	1.45E-18	K11175
NMB1200	ribonuclease R	1.03	3.88E-11	K12573
NMB0992	adhesin	0.96	3.82E-09	K12690
NMB0548	AcrA/AcrE family protein	1.12	1.35E-06	K13888
NMB1988	iron-regulated outer membrane protein FrpB	1.75	7.38E-04	K16087
NMB0036	hypothetical protein	0.78	1.06E-03	K16301

Appendix XIII- RNAseq genes

GeneID	GeneName	baseMean	log2FoldChange	padj
NMB1541	lactoferrin-binding protein	739.83	-1.69	0.000
NMB1566	phosphoribosylglycinamide transformylase	2719.17	1.21	0.000
NMB0120	hypothetical protein	196.74	-1.89	0.000
NMB1997	hydroxyacylglutathione hydrolase	4711.43	-0.94	0.000
NMB1200	ribonuclease R	4033.65	1.03	0.000

NMB1496	23S rRNA pseudouridine synthase E	644.60	-1.05	0.000
NMB1712	L-lactate permease-like protein	235.08	-1.59	0.000
NMB1786	hypothetical protein	460.25	-1.28	0.000
NMB0019	fimbrial protein	12337.36	0.97	0.000
NMB0663	outer membrane protein	8469.22	-1.44	0.000
NMB1565	hypothetical protein	2151.82	0.94	0.000
NMB0347	D-tyrosyl-tRNA(Tyr) deacylase	1754.16	1.02	0.000
NMB0018	pilin PilE	102991.54	-1.25	0.000
NMB0915	hypothetical protein	1105.84	-0.94	0.000
NMB0256	hypothetical protein	463.22	-0.96	0.000
NMB1254	GTP cyclohydrolase II	1413.43	0.93	0.000
NMB0343	hypothetical protein	6211.24	0.85	0.000
NMB0583	IS1016C2 transposase	1712.58	-1.01	0.000
NMB1153	sulfate adenylyltransferase subunit 1	77.86	-1.98	0.000
NMB0992	adhesin	2459.17	0.96	0.000
NMB1996	phosphoribosylformylglycinamidine synthase	35363.68	-0.82	0.000
NMB0792	transporter	987.53	-0.83	0.000
NMB0462	spermidine/putrescine ABC transporter substrate-binding protein	21416.56	-1.14	0.000
NMB0878	threonine dehydratase	3275.13	0.78	0.000
NMB0467	hypothetical protein	5925.72	-0.72	0.000
NMB0818	hypothetical protein	1185.98	-0.76	0.000
NMB1846	ATP-binding protein	4087.08	0.85	0.000
NMB0252	hypothetical protein	2822.27	-0.77	0.000
NMB0070	polysialic acid capsule biosynthesis protein SynX	5032.61	-0.84	0.000
NMB0702	competence protein	669.84	0.88	0.000
NMB0742	hypothetical protein	1000.34	0.80	0.000
NMB1151	sulfite reductase subunit beta	141.26	-1.98	0.000
NMB1719	efflux pump component MtrF	6994.04	-1.24	0.000

NMB1737	secretion protein	717.96	-0.81	0.000
NMB1856	LysR family transcriptional regulator	1836.36	-0.86	0.000
NMB2003	hypothetical protein	945.38	-0.79	0.000
NMB0743	ubiquinone/menaquinone biosynthesis methyltransferase	3193.05	0.70	0.000
NMB1104	phage sheath protein	768.01	0.97	0.000
NMB1862	50S ribosomal protein L11 methyltransferase	2640.98	0.80	0.000
NMB1753	VapD-like protein	2537.96	-1.28	0.000
NMB0687	GTP-binding protein Era	1094.57	0.70	0.000
NMB1738	secretion protein	743.65	-1.13	0.000
NMB2002	hypothetical protein	739.72	0.94	0.000
NMB2113	hypothetical protein	342.16	-0.96	0.000
NMB2051	cytochrome C1	27705.67	-0.71	0.000
NMB1315	uracil permease	4074.73	0.78	0.000
NMB0548	AcrA/AcrE family protein	4524.52	1.12	0.000
NMB1093	hypothetical protein	1134.99	-0.66	0.000
NMB1716	membrane fusion protein	15104.14	0.82	0.000
NMB0241	NADH dehydrogenase subunit A	1772.49	-1.09	0.000
NMB0709	hypothetical protein	4314.40	-0.67	0.000
NMB0119	hypothetical protein	386.58	-1.33	0.000
NMB1857	modulator of drug activity B	6057.55	0.62	0.000
NMB0648	hypothetical protein	2954.53	-0.81	0.000
NMB0686	ribonuclease III	1049.40	0.82	0.000
NMB0661	bis(5'-nucleosyl)-tetraphosphatase	1484.75	1.01	0.000
NMB1426	hypothetical protein	1459.93	1.64	0.000
NMB1152	sulfite reductase flavoprotein subunit alpha	86.66	-1.48	0.000
NMB1253	hypothetical protein	2193.19	0.95	0.000
NMB0386	phosphatidylglycerophosphatase A	1870.74	0.65	0.000
NMB0020	fimbrial protein	179.86	1.42	0.000

NMB2093	methionine aminopeptidase	2047.09	0.59	0.000
NMB0746	hypothetical protein	825.73	-0.63	0.000
NMB0549	ABC transporter ATP-binding protein	4219.43	1.13	0.000
NMB1102	hypothetical protein	179.02	1.13	0.000
NMB0111	methionyl-tRNA formyltransferase	3480.82	0.81	0.000
NMB2037	hypothetical protein	1752.56	-0.86	0.000
NMB0763	cysteine synthase	116710.46	-1.04	0.000
NMB0916	hypothetical protein	458.07	-0.79	0.000
NMB1843	MarR family transcriptional regulator	17038.21	-0.75	0.000
NMB0344	BolA/YrbA family protein	3327.05	0.88	0.000
NMB1739	hypothetical protein	156.64	-1.31	0.000
NMB0577	hypothetical protein	3332.57	-1.66	0.000
NMB0075	transcriptional accessory protein Tex	1153.43	0.65	0.000
NMB0346	hypothetical protein	10267.25	1.00	0.000
NMB1562	hypothetical protein	1168.44	0.68	0.000
NMB0065	hypothetical protein	4348.43	-0.84	0.000
NMB1154	sulfate adenylyltransferase subunit 2	42.08	-1.81	0.000
NMB0817	hypothetical protein	2468.76	-0.70	0.000
NMB0255	adenosine monophosphate-protein transferase NmFic	1703.54	-0.69	0.000
NMB0910	transcriptional regulator	3702.61	-0.72	0.000
NMB0479	hypothetical protein	7536.99	-0.64	0.000
NMB0559	ubiquinone biosynthesis protein UbiB	5530.63	0.58	0.000
NMB0296	hypothetical protein	4100.49	-0.63	0.000
NMB0676	hypothetical protein	1613.86	0.60	0.000
NMB1398	superoxide dismutase	4725.84	-0.71	0.000
NMB0964	TonB-dependent receptor	470.48	1.32	0.000
NMB1513	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	1771.44	0.53	0.000
NMB1845	thioredoxin	355.38	0.73	0.000

NMB0419	hypothetical protein	790.96	0.74	0.000
NMB1124	hypothetical protein	197.01	-0.97	0.000
NMB0345	cell-binding factor	28757.54	0.93	0.000
NMB1019	5-(carboxyamino)imidazole ribonucleotide synthase	5268.10	-0.58	0.000
NMB0675	3-deoxy-manno-octulosonate cytidylyltransferase	2400.77	0.60	0.000
NMB0578	copper ABC transporter substrate-binding protein	1331.52	-1.00	0.000
NMB0253	NADH dehydrogenase subunit J	2816.41	-0.66	0.000
NMB1259	IS30 family transposase	312.29	-0.86	0.000
NMB1325	cation transporter E1-E2 family ATPase	2400.55	0.60	0.000
NMB1717	transcriptional regulator MtrR	1641.76	0.64	0.000
NMB0251	NADH dehydrogenase subunit I	2553.63	-0.74	0.000
NMB0948	succinate dehydrogenase cytochrome b556 subunit	4585.28	-0.93	0.000
NMB0662	ribonuclease	3148.87	0.58	0.000
NMB1212	hypothetical protein	1613.23	-1.20	0.000
NMB1807	penicillin-binding protein 1	4551.59	0.69	0.000
NMB1105	hypothetical protein	234.25	0.83	0.000
NMB1646	hemolysin	2068.55	1.07	0.000
NMB0772	hypothetical protein	611.31	0.66	0.000
NMB1101	hypothetical protein	193.90	1.07	0.000
NMB0623	spermidine/putrescine ABC transporter substrate-binding protein	12875.56	1.01	0.000
NMB1106	hypothetical protein	314.98	0.82	0.000
NMB0993	rubredoxin	980.21	-1.78	0.000
NMB2022	23S rRNA (pseudouridine(1915)-N(3))-methyltransferase RlmH	1234.14	0.54	0.000
NMB1685	D-lactate dehydrogenase	6548.89	0.50	0.000
NMB0422	undecaprenyldiphospho-muramoylpentapeptide beta-N-acetylglucosaminyltransferase	4496.53	0.64	0.000
NMB1211	hypothetical protein	1096.61	-1.27	0.000
NMB1359	CDP-6-deoxy-delta-3%2C4-glucoseen reductase	744.83	0.65	0.000
NMB1100	hypothetical protein	295.95	0.78	0.000

NMB1893	hypothetical protein	5293.63	-0.65	0.000
NMB1655	N5-glutamine S-adenosyl-L-methionine-dependent methyltransferase	2292.21	-0.53	0.000
NMB0378	phosphate permease	51154.84	-1.70	0.000
NMB0960	succinyl-CoA synthetase subunit alpha	36840.49	-0.48	0.000
NMB0669	hypothetical protein	1439.16	0.48	0.000
NMB0928	hypothetical protein	12417.77	0.54	0.000
NMB1035	hypothetical protein	3473.88	-0.60	0.000
NMB1410	hypothetical protein	286.74	-0.73	0.000
NMB1987	tRNA modification GTPase TrmE	2176.48	0.64	0.000
NMB1411	IS1016C2 transposase	348.32	-0.91	0.000
NMB1053	class 5 outer membrane protein	123116.61	-0.88	0.000
NMB0961	hypothetical protein	4064.08	-0.51	0.000
NMB1711	GntR family transcriptional regulator	685.12	0.58	0.000
NMB0114	nitrogen regulation protein NtrY	5071.92	0.61	0.000
NMB0721	translation initiation factor IF-3	12851.22	-0.74	0.000
NMB0250	NADH dehydrogenase subunit H	3540.09	-0.62	0.000
NMB1789	preprotein translocase subunit SecB	42726.69	0.45	0.000
NMB1512	2-C-methyl-D-erythritol 2%2C4-cyclodiphosphate synthase	1160.83	0.56	0.000
NMB0420	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase	3326.29	0.76	0.000
NMB0082	capsule polysaccharide modification protein	1080.43	0.56	0.000
NMB0626	peptide chain release factor 3	2911.47	0.62	0.000
NMB1023	C32 tRNA thiolase	1926.56	0.54	0.000
NMB1799	S-adenosylmethionine synthetase	5866.49	0.76	0.001
NMB0268	RNA methyltransferase	1363.02	1.00	0.001
NMB1442	DNA mismatch repair protein	1480.52	0.58	0.001
NMB0825	ADP-heptose synthase	1708.85	0.53	0.001
NMB1225	hypothetical protein	1083.63	-0.61	0.001
NMB1515	transporter	3815.37	0.50	0.001

NMB1641	hypothetical protein	920.40	0.59	0.001
NMB1273	alginate o-acetyltransferase AlgI	937.87	0.77	0.001
NMB0866	hypothetical protein	222.09	-1.23	0.001
NMB1294	1-acyl-sn-glycerol-3-phosphate acyltransferase	1290.88	0.64	0.001
NMB1983	hypothetical protein	3042.16	-0.66	0.001
NMB1214	hemagglutinin/hemolysin-like protein	10186.30	-0.87	0.001
NMB1863	oligoribonuclease	1389.40	0.64	0.001
NMB0332	type IV prepilin peptidase	1785.81	0.52	0.001
NMB0412	cell division protein FtsL	981.71	0.79	0.001
NMB0017	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase	5562.24	0.87	0.001
NMB1988	iron-regulated outer membrane protein FrpB	1688.25	1.75	0.001
NMB1213	lipoprotein	2473.36	-1.15	0.001
NMB1588	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase	1277.27	-0.68	0.001
NMB0683	transcription antitermination protein NusB	2016.43	0.71	0.001
NMB0710	hypothetical protein	1903.89	-0.46	0.001
NMB1353	aldehyde dehydrogenase	2628.86	0.64	0.001
NMB0822	heat shock protein HtpX	12069.48	0.83	0.001
NMB0122	hypothetical protein	2021.04	-0.80	0.001
NMB1658	phosphopantothenoylcysteine decarboxylase/phosphopantothenate--cysteine ligase	3098.92	0.44	0.001
NMB0576	glutamyl-tRNA reductase	7043.42	-0.60	0.001
NMB0258	NADH dehydrogenase subunit M	6781.46	-0.77	0.001
NMB0437	hypothetical protein	2347.95	-0.76	0.001
NMB0411	S-adenosyl-methyltransferase MraW	7799.33	0.46	0.001
NMB0656	hypothetical protein	183.78	-0.72	0.001
NMB0879	sulfate ABC transporter ATP-binding protein	2067.71	-1.03	0.001
NMB0036	hypothetical protein	1370.33	0.78	0.001
NMB1441a	hypothetical protein	1542.08	-0.48	0.001
NMB0315	hypothetical protein	597.94	0.58	0.001

NMB1715	multiple transferable resistance system protein MtrD	32599.16	0.61	0.001
NMB1162	hypothetical protein	11.67	-5.37	0.001
NMB1126	hypothetical protein	162.18	-0.87	0.001
NMB0654	hypothetical protein	503.74	-0.85	0.001
NMB0798	cell division protein FtsH	23287.05	0.43	0.001
NMB0871	pantoate--beta-alanine ligase	1377.31	0.73	0.001
NMB1521	phytoene synthase	1223.08	0.45	0.001
NMB0385	thiamine monophosphate kinase	2796.63	0.46	0.001
NMB1976	diaminopimelate decarboxylase	4358.53	0.59	0.001
NMB1461	drug resistance translocase	6487.51	0.45	0.001
NMB0930	xanthine/uracil permease	2023.05	0.56	0.001
NMB1876	N-acetylglutamate synthase	2610.11	0.52	0.001
NMB1103	hypothetical protein	90.84	1.19	0.001
NMB0373	hypothetical protein	333.87	-0.91	0.002
NMB0123	4Fe-4S type ferredoxin	1427.56	-0.66	0.002
NMB0539	porphobilinogen deaminase	3996.86	0.49	0.002
NMB0413	penicillin-binding protein 2	11217.67	0.52	0.002
NMB1231	ATP-dependent protease La	32515.60	0.45	0.002
NMB1650	leucine-responsive regulatory protein	1200.45	-0.55	0.002
NMB1073	hypothetical protein	6864.34	0.44	0.002
NMB1514	DNA polymerase III subunit epsilon	2216.98	0.48	0.002
NMB1568	DNA polymerase III subunit chi	1678.46	0.59	0.002
NMB0333	pilus assembly protein PilG	16908.00	0.44	0.002
NMB0601	twin arginine translocase A	2552.43	-0.48	0.002
NMB0297	hypothetical protein	137.59	-1.04	0.002
NMB0870	3-methyl-2-oxobutanoate hydroxymethyltransferase	2731.08	0.53	0.002
NMB0421	cell division protein	3896.49	0.41	0.002
NMB0985	hypothetical protein	245.33	-1.08	0.002

NMB1110	tail protein	190.37	0.76	0.002
NMB0747	hypothetical protein	255.87	0.88	0.002
NMB2004	hypothetical protein	1900.84	-0.68	0.002
NMB1714	multidrug efflux pump channel protein	13955.14	0.62	0.002
NMB1341	pyruvate dehydrogenase subunit E1	165194.67	0.47	0.002
NMB2066	metalloprotease TldD	3487.96	0.43	0.002
NMB0247	hypothetical protein	1125.43	-0.57	0.002
NMB1949	soluble lytic murein transglycosylase	4714.36	0.79	0.002
NMB2038	hypothetical protein	3890.99	-0.84	0.002
NMB1890	hypothetical protein	400.36	-0.65	0.002
NMB1587	protease	1434.52	0.45	0.002
NMB2076	D-beta-D-heptose 1-phosphate adenylyltransferase	1545.20	0.45	0.002
NMB1493	carbon starvation protein A	30939.07	-1.04	0.002
NMB1567	macrophage infectivity potentiator	33402.28	0.63	0.002
NMB0952	hypothetical protein	2517.72	-0.51	0.002
NMB0461	transferrin-binding protein 1	3517.47	0.43	0.002
NMB0414	UDP-N-acetylmuramoylalanyl-D-glutamate--2%2C6-diaminopimelate ligase	5132.55	0.37	0.003
NMB0234	hypothetical protein	1190.27	-0.50	0.003
NMB0112	16S rRNA (cytosine(967)-C(5))-methyltransferase	4188.73	0.48	0.003
NMB0405	competence protein ComM	7776.34	0.40	0.003
NMB0724	phenylalanyl-tRNA synthetase subunit alpha	3462.78	-0.63	0.003
NMB0313	hypothetical protein	1562.10	0.87	0.003
NMB2132	transferrin-binding protein-like protein	6426.58	-0.86	0.003
NMB1907	inner membrane protein translocase component YidC	9075.71	0.38	0.003
NMB1088	hypothetical protein	10807.01	-0.71	0.003
NMB1816	transcriptional regulator NrdR	258.22	0.65	0.003
NMB0550	thiol:disulfide interchange protein DsbC	7525.02	0.39	0.003
NMB1339	prolyl-tRNA synthetase	8453.64	0.55	0.003

NMB1028	hypothetical protein	981.55	0.43	0.003
NMB1284	hypothetical protein	2848.30	0.52	0.003
NMB1030	hypothetical protein	4771.62	-0.55	0.003
NMB1441	O-methyltransferase	3038.13	-0.45	0.003
NMB1575	hypothetical protein	5273.52	-0.59	0.003
NMB2013	hypothetical protein	3435.53	-0.48	0.003
NMB0012	hypothetical protein	2137.59	0.46	0.003
NMB0263	ribosome-associated GTPase	489.99	0.60	0.003
NMB0023	fimbrial protein	312.48	0.97	0.003
NMB2127	protease	2683.22	0.37	0.003
NMB1233	exodeoxyribonuclease V subunit alpha	8061.83	0.41	0.004
NMB0206	leucyl/phenylalanyl-tRNA--protein transferase	2088.88	0.45	0.004
NMB0246	NADH dehydrogenase subunit F	6215.69	-0.66	0.004
NMB1571	hypothetical protein	1234.94	0.41	0.004
NMB0248	hypothetical protein	730.63	-0.69	0.004
NMB0600	hypothetical protein	3802.74	-0.51	0.004
NMB1331	excinuclease ABC subunit B	2293.16	0.38	0.004
NMB0086	hypothetical protein	4802.38	0.69	0.004
NMB0751	integrase/recombinase XerD	1377.85	0.56	0.004
NMB1760	hypothetical protein	640.50	-0.62	0.004
NMB1519	thiol:disulfide interchange protein	37864.60	0.54	0.004
NMB2112	hypothetical protein	239.16	-0.76	0.004
NMB1780	hemolysin activation protein HecB	62.60	-1.03	0.004
NMB1357	hypothetical protein	2795.59	0.37	0.004
NMB0294	thiol:disulfide interchange protein DsbA	18673.51	1.13	0.005
NMB1402	hypothetical protein	258.66	-0.59	0.005
NMB2075	bifunctional biotin--[acetyl-CoA-carboxylase] ligase/pantothenate kinase	5660.07	0.48	0.005
NMB1994	adhesin/invasin	1906.78	-1.02	0.005

NMB0277	virulence factor MviN	3512.05	0.46	0.005
NMB1986	hypothetical protein	7102.19	-0.50	0.005
NMB1416	aminopeptidase	5436.81	0.63	0.005
NMB0941	50S ribosomal protein L36	3509.38	1.52	0.005
NMB1945	aromatic acid decarboxylase	520.22	0.52	0.005
NMB1326	excinuclease ABC subunit C	970.83	0.58	0.005
NMB1007	transcriptional regulator	797.96	-0.82	0.005
NMB1536q	hypothetical protein	1280.83	-0.52	0.005
NMB1219	transporter	1203.87	0.46	0.005
NMB0267	hypothetical protein	1021.42	1.02	0.005
NMB1796	hypothetical protein	16217.66	-0.61	0.005
NMB1400	ABC transporter family protein	1233.52	-0.72	0.005
NMB0726	type II restriction enzyme	5471.79	-0.78	0.006
NMB0949	succinate dehydrogenase hydrophobic membrane anchor protein	2841.44	-0.89	0.006
NMB0925	acyl-CoA thioesterase	2408.91	0.37	0.006
NMB1592	lipoprotein	10927.25	-0.37	0.006
NMB0106	aspartate carbamoyltransferase	5231.34	0.38	0.006
NMB0478	hypothetical protein	13346.59	-0.40	0.006
NMB1203	protein-PII uridylyltransferase	1249.92	0.49	0.006
NMB1869	fructose-1%2C6-bisphosphate aldolase	25949.27	-0.35	0.006
NMB0840	hypothetical protein	2013.27	-0.35	0.006
NMB2135	hypothetical protein	8222.18	0.38	0.006
NMB0835	type I restriction enzyme R protein	700.33	0.50	0.006
NMB1495	hypothetical protein	36.84	-1.18	0.007
NMB0455	hypothetical protein	2484.28	0.47	0.007
NMB1374	tRNA pseudouridine synthase B	1313.78	0.47	0.007
NMB1494	hypothetical protein	1819.40	-0.83	0.007
NMB0605	histone deacetylase	1203.82	-0.70	0.007

NMB1699	hypothetical protein	1955.61	-0.57	0.007
NMB0284	adenylosuccinate lyase	10591.25	-0.72	0.007
NMB0655	hypothetical protein	271.80	-0.72	0.007
NMB1803	cytochrome c-type biogenesis protein	4098.59	-0.37	0.007
NMB1222	uracil-DNA glycosylase	522.53	0.57	0.008
NMB1327	hypothetical protein	349.50	0.51	0.008
NMB1574	ketol-acid reductoisomerase	37333.69	-0.57	0.008
NMB1718	hypothetical protein	340.60	-0.50	0.008
NMB0779	hypothetical protein	6352.89	0.36	0.008
NMB1815	hypothetical protein	1721.04	0.42	0.008
NMB0819	hypothetical protein	1449.02	-0.48	0.008
NMB1443	DNA polymerase III subunits gamma and tau	2000.11	0.43	0.008
NMB0672	tetraacyldisaccharide 4'-kinase	1374.30	0.42	0.008
NMB1572	aconitate hydratase B	51592.80	-0.80	0.008
NMB0104	hypothetical protein	1026.61	-0.75	0.008
NMB1669	heme utilisation protein	10953.06	-0.84	0.008
NMB0185	phosphatidate cytidylyltransferase	3303.40	0.60	0.008
NMB0631	phosphate acetyltransferase	41381.43	0.67	0.008
NMB0507	hypothetical protein	1525.77	-0.60	0.009
NMB1207	bacterioferritin A	5033.40	-0.45	0.009
NMB1107	hypothetical protein	1385.84	-0.46	0.009
NMB1787	N-acetyl-gamma-glutamyl-phosphate reductase	2672.88	0.38	0.009
NMB0178	acyl-[acyl-carrier-protein]--UDP-N-acetylglucosamine O-acyltransferase	3193.68	0.33	0.010
NMB1844	hypothetical protein	4250.26	-0.57	0.010
NMB1899	hypothetical protein	1741.03	-0.44	0.010
NMB1372	ATP-dependent protease ATP-binding subunit ClpX	6182.42	0.44	0.010
NMB1459	hypothetical protein	1063.85	0.41	0.010
NMB1808	Tfp pilus assembly ATPase PilM	29064.19	-0.53	0.010

NMB0920	isocitrate dehydrogenase	36692.67	-0.49	0.011
NMB0708	DNA polymerase III subunit delta	4043.94	0.45	0.011
NMB1941	hypothetical protein	2407.53	-0.45	0.011
NMB1354	hypothetical protein	1058.85	0.51	0.011
NMB1432	3-phosphoshikimate 1-carboxyvinyltransferase	2524.76	0.85	0.011
NMB0342	intracellular septation protein A	4457.32	0.78	0.011
NMB1419	Holliday junction resolvase	957.57	-0.37	0.011
NMB0107	aspartate carbamoyltransferase	2190.90	0.37	0.011
NMB0731	hypothetical protein	681.57	-0.71	0.011
NMB1224	hypothetical protein	586.10	-0.48	0.011
NMB1475	hypothetical protein	1306.17	1.51	0.012
NMB1810	Tfp pilus assembly protein PilO	17461.01	-0.49	0.012
NMB0935a	hypothetical protein	2592.08	0.39	0.012
NMB0242	NADH dehydrogenase subunit B	2921.72	-0.79	0.012
NMB0254	NADH dehydrogenase subunit K	1072.46	-0.59	0.012
NMB0361	hypothetical protein	361.25	0.52	0.013
NMB2029	homoserine kinase	5761.47	0.40	0.013
NMB1234	ABC transporter ATP-binding protein	2552.25	0.49	0.013
NMB0820	hypothetical protein	1228.54	-0.35	0.013
NMB1117	hypothetical protein	2352.94	-0.70	0.013
NMB2096	malate:quinone oxidoreductase	15832.17	-0.68	0.013
NMB0456	N-acetylmuramoyl-L-alanine amidase	4359.07	0.33	0.014
NMB1262	peptidyl-prolyl cis-trans isomerase	650.72	0.46	0.014
NMB0782	DNA repair protein RadA	2966.11	0.38	0.014
NMB0249	NADH dehydrogenase subunit G	13211.74	-0.54	0.014
NMB0445	bicyclomycin resistance protein	3106.17	0.35	0.014
NMB1721	hypothetical protein	612.09	0.66	0.014
NMB2052	cytochrome B	40053.62	-0.42	0.014

NMB1555	long-chain-fatty-acid--CoA-ligase	5944.09	0.39	0.014
NMB2020	hypothetical protein	20774.88	0.46	0.015
NMB2155	electron transfer flavoprotein subunit beta	13838.60	-0.42	0.015
NMB1623	major anaerobically induced outer membrane protein	59458.95	-1.47	0.016
NMB1861	acetyl-CoA carboxylase biotin carboxylase subunit	17420.04	0.59	0.016
NMB1660	DNA-directed RNA polymerase subunit omega	3163.05	-0.45	0.016
NMB0503	hypothetical protein	543.94	-0.47	0.016
NMB1060	fructose-1%2C6-bisphosphatase	5635.38	0.38	0.017
NMB1070	2-isopropylmalate synthase	5886.78	-0.45	0.017
NMB0688	N-(5'-phosphoribosyl)anthranilate isomerase	357.37	-1.07	0.018
NMB0024	fimbrial protein	99.98	-0.86	0.018
NMB1609	O-succinylhomoserine sulfhydrolase	18215.51	-0.50	0.018
NMB1033	modification methylase	2170.53	-0.55	0.018
NMB2071	thiazole synthase	391.25	0.49	0.018
NMB0653	MafB-like protein	310.64	-0.73	0.019
NMB1608a	hypothetical protein	1889.09	-0.37	0.019
NMB1276	long-chain-fatty-acid--CoA ligase	3504.75	0.56	0.019
NMB2068	glycine oxidase ThiO	458.46	0.53	0.020
NMB2106	hypothetical protein	507.73	-0.47	0.020
NMB0466	aspartyl-tRNA synthetase	17771.91	0.39	0.020
NMB0865	hypothetical protein	492.44	-0.62	0.020
NMB1652	hypothetical protein	14265.46	-0.39	0.020
NMB0524	ribonuclease BN/unknown domain fusion protein	1844.84	0.35	0.020
NMB0195	4-hydroxythreonine-4-phosphate dehydrogenase PdxA	1622.93	0.32	0.021
NMB1995	nitrogen regulatory protein P-II 1	2300.63	-1.09	0.021
NMB0649	hypothetical protein	1546.05	-0.69	0.021
NMB0091	hypothetical protein	1739.86	-0.52	0.022
NMB0348	tRNA-dihydrouridine synthase A	3528.92	0.45	0.022

NMB0085	sodium/glutamate symport carrier protein	11402.95	-0.54	0.022
NMB0027	peptidyl-prolyl cis-trans isomerase	2453.47	0.32	0.022
NMB2044	phosphoenolpyruvate-protein phosphotransferase	4306.65	0.53	0.022
NMB0061	dTDP-4-dehydrorhamnose 3%2C5-epimerase	475.20	0.39	0.022
NMB1240	ABC-F family ATPase	21665.08	0.43	0.022
NMB1304	alcohol dehydrogenase	4252.93	0.41	0.022
NMB1420	DNA-binding protein Fis	614.23	-0.42	0.023
NMB0371	hypothetical protein	174.52	-0.78	0.023
NMB1838	GTP-dependent nucleic acid-binding protein EngD	7159.79	0.42	0.023
NMB1317a	hypothetical protein	2390.06	0.30	0.024
NMB0978	pyridine nucleotide transhydrogenase	6897.87	0.35	0.025
NMB0997	D-lactate dehydrogenase	13906.87	-0.53	0.025
NMB1533	outer membrane protein	36406.78	-0.49	0.025
NMB1072	prolipoprotein diacylglyceryl transferase	2835.23	0.50	0.025
NMB0674	hypothetical protein	1084.70	0.45	0.025
NMB1964	hypothetical protein	2734.67	0.40	0.025
NMB1109	phage virion protein	203.32	0.52	0.025
NMB2097	hypothetical protein	628.17	-0.78	0.026
NMB0942	50S ribosomal protein L31 type B	5278.20	1.34	0.026
NMB0516	hypothetical protein	2361.01	-0.29	0.026
NMB1528	methylated-DNA--protein-cysteine methyltransferase	1130.89	0.43	0.026
NMB1837	hypothetical protein	347.80	0.66	0.026
NMB1806	ribosome biogenesis GTP-binding protein YsxC	1420.61	-0.45	0.027
NMB1293	hypothetical protein	417.84	-0.39	0.027
NMB1024	hypothetical protein	5049.89	0.52	0.027
NMB1467	exopolyphosphatase	4621.39	0.36	0.027
NMB1536	preprotein translocase subunit SecA	24148.59	0.27	0.028
NMB0589	50S ribosomal protein L19	15012.68	0.48	0.028

NMB1784	hypothetical protein	382.21	-0.48	0.028
NMB1377	L-lactate dehydrogenase	1155.86	1.03	0.028
NMB1403	hypothetical protein	217.34	-0.53	0.029
NMB2094	hypothetical protein	10384.14	-0.36	0.029
NMB0087	hypothetical protein	697.94	0.84	0.029
NMB0282	hypothetical protein	3603.08	0.37	0.029
NMB1759	hypothetical protein	2444.69	-0.62	0.030
NMB1982	DNA polymerase I	7830.90	0.33	0.030
NMB0439	hypothetical protein	2631.30	0.42	0.030
NMB0330	hypothetical protein	234.21	0.55	0.031
NMB1111	baseplate assembly protein V	100.52	0.65	0.032
NMB0186	undecaprenyl pyrophosphate synthetase	5413.35	0.40	0.032
NMB0986	hypothetical protein	133.14	-0.81	0.033
NMB0593	hypothetical protein	1256.40	0.45	0.033
NMB1614	potassium transporter inner membrane associated protein	4574.37	0.28	0.033
NMB1919	ABC transporter ATP-binding protein	4341.94	0.34	0.034
NMB0209	glutathione-regulated potassium-efflux system protein	1917.23	0.43	0.034
NMB0399	exodeoxyribonuclease III	2979.82	0.49	0.034
NMB0200	hypothetical protein	102.88	-0.62	0.034
NMB0883	hypothetical protein	4780.17	-0.36	0.034
NMB0841	hypothetical protein	3326.64	-0.32	0.035
NMB2095	adhesin complex protein	75787.85	0.73	0.035
NMB0370	hypothetical protein	380.54	-0.48	0.035
NMB0337	branched-chain amino acid aminotransferase	18914.90	-0.78	0.035
NMB0789	amino acid ABC transporter ATP-binding protein	4581.32	-0.89	0.036
NMB1811	Tfp pilus assembly protein PilP	15118.34	-0.44	0.036
NMB2159	glyceraldehyde 3-phosphate dehydrogenase C	63089.14	-0.66	0.036
NMB0401	bifunctional proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase	16899.15	-0.43	0.037

NMB0369	hypothetical protein	164.98	-0.49	0.037
NMB1696a	hypothetical protein	2907.27	-0.47	0.038
NMB1805	cytochrome c4	13493.11	-0.48	0.038
NMB0727	N-6 adenine-specific DNA methylase	2366.50	-0.62	0.039
NMB0882	hypothetical protein	1764.61	-0.69	0.039
NMB1812	Tfp pilus assembly protein PilQ	86486.80	-0.45	0.040
NMB2001	hypothetical protein	1944.68	0.51	0.040
NMB2053	ubiquinol-cytochrome c reductase iron-sulfur subunit	13264.13	-0.45	0.040
NMB1263	GTPase	2370.97	0.32	0.041
NMB0752	bacterioferritin-associated ferredoxin	935.09	0.72	0.041
NMB0470	C4-dicarboxylate transporter	9460.01	-0.61	0.041
NMB0271	hypothetical protein	600.75	0.35	0.041
NMB0259	NADH dehydrogenase subunit N	6451.91	-0.50	0.041
NMB1369	hypothetical protein	3308.94	0.57	0.041
NMB1250	LuxR family transcriptional regulator	1039.14	0.65	0.041
NMB0689	transcription elongation factor GreB	353.19	-1.08	0.041
NMB1578	hypothetical protein	18844.67	-0.46	0.041
NMB2098	hypothetical protein	489.14	-0.35	0.041
NMB1732	transporter	1759.01	0.41	0.041
NMB1679	tRNA (uracil-5-)-methyltransferase	5238.13	0.27	0.042
NMB2131	hypothetical protein	1122.73	-0.42	0.042
NMB0933	cytidine and deoxycytidylate deaminase	366.84	0.45	0.042
NMB0394	quinolinate synthetase	1008.52	-1.35	0.044
NMB2040	thiamine biosynthesis protein ThiC	529.10	-0.43	0.044
NMB1603	potassium-tellurite ethidium and proflavin transporter	443.37	0.39	0.044
NMB1696b	hypothetical protein	3162.51	-0.38	0.045
NMB1330	hypothetical protein	593.45	-0.68	0.045
NMB0632	iron(III) ABC transporter ATP-binding protein	1363.29	0.31	0.046

NMB1332	carboxy-terminal peptidase	10286.97	0.35	0.046
NMB1201	inosine 5'-monophosphate dehydrogenase	17424.81	-0.45	0.046
NMB0118	DNA topoisomerase I	7316.32	0.41	0.046
NMB1670	paraquat-inducible protein A	2433.63	0.35	0.047
NMB1855	carbamoyl phosphate synthase large subunit	38209.69	0.54	0.048
NMB1693	hypothetical protein	2102.91	0.35	0.048
NMB2059	hypothetical protein	2140.76	0.32	0.048
NMB1620	hypothetical protein	2484.41	0.44	0.048
NMB0766	GTP-binding protein LepA	9646.15	0.35	0.049
NMB1835	tyrosyl-tRNA synthetase	5761.80	0.46	0.051
NMB0909	hypothetical protein	1188.88	-0.52	0.052
NMB0638	UTP-glucose-1-phosphate uridylyltransferase	6648.41	-0.44	0.053
NMB0028	hypothetical protein	153.63	1.12	0.053
NMB0245	NADH dehydrogenase subunit E	3227.95	-0.47	0.053
NMB0740	DNA repair protein RecN	4393.44	-0.27	0.054
NMB0805	IS30 family transposase	88.60	-0.61	0.054
NMB1305	esterase D	2423.21	0.41	0.055
NMB1573	ornithine carbamoyltransferase	3531.53	-0.42	0.056
NMB0725	modification methylase	2731.21	-0.53	0.057
NMB1666	hypothetical protein	1819.55	-0.42	0.057
NMB1897	leucyl-tRNA synthetase	18482.52	0.30	0.057
NMB0809	hypothetical protein	866.65	0.34	0.057
NMB0381	transcriptional regulator CysB-like protein	2755.61	-0.28	0.057
NMB0848	hypothetical protein	370.77	0.41	0.057
NMB1324	thioredoxin reductase	10760.90	0.47	0.057
NMB0807	inorganic polyphosphate/ATP-NAD kinase	1425.92	-0.41	0.057
NMB1686	peptide chain release factor 1	1781.81	0.27	0.058
NMB0476	hypothetical protein	13284.32	-0.24	0.059

NMB1954	hypothetical protein	1924.48	0.37	0.059
NMB0030	methionyl-tRNA synthetase	7532.24	0.44	0.059
NMB0265	Holliday junction DNA helicase RuvA	1617.45	0.31	0.060
NMB1068	gamma-glutamyl phosphate reductase	1639.36	-0.74	0.061
NMB1474	4-oxalocrotonate tautomerase	692.02	-0.32	0.061
NMB1509	amino acid ABC transporter permease	3355.90	0.30	0.061
NMB0302	transposase	272.08	-0.46	0.061
NMB0703	competence lipoprotein	13478.80	0.29	0.061
NMB1891	helix-turn-helix family protein	1737.92	-0.52	0.061
NMB1534	hypothetical protein	1401.20	-0.49	0.061
NMB0214	oligopeptidase A	18260.26	0.39	0.062
NMB1591	AraC family transcriptional regulator	1620.91	-0.38	0.063
NMB1665	hypothetical protein	2201.24	-0.48	0.063
NMB1524	oxidoreductase	1172.51	0.49	0.064
NMB0469	agmatinase	6659.68	-0.33	0.065
NMB0873	outer membrane lipoprotein LolB	511.19	0.37	0.065
NMB1356	aspartyl/glutamyl-tRNA amidotransferase subunit A	7770.98	0.43	0.065
NMB1437	hypothetical protein	12143.26	0.29	0.066
NMB1833	isoleucyl-tRNA synthetase	24613.94	0.30	0.066
NMB1188	dihydroxy-acid dehydratase	23.28	-1.46	0.067
NMB0745	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine diphosphokinase	1263.01	-0.42	0.067
NMB0115	nitrogen assimilation regulatory protein NtrX	5138.99	0.40	0.067
NMB1653	hypothetical protein	2398.05	-0.39	0.067
NMB0442	opacity protein	2732.03	-0.44	0.067
NMB0906	hypothetical protein	308.02	-0.38	0.067
NMB0999	tRNA-dihydrouridine synthase	761.17	-0.59	0.067
NMB1761	hypothetical protein	1626.84	-0.38	0.067
NMB1675	hypothetical protein	1732.74	-0.40	0.070

NMB1998	serine-type peptidase	12956.79	-0.45	0.070
NMB1083	bacteriophage DNA transposition protein B	206.29	0.61	0.070
NMB0554	molecular chaperone DnaK	60201.46	0.45	0.070
NMB0103	bacteriocin resistance protein	329.21	-0.40	0.070
NMB0563	thiamine biosynthesis lipoprotein ApbE	620.53	0.48	0.071
NMB1950	30S ribosomal protein S21	16805.93	0.56	0.072
NMB0409	hypothetical protein	352.74	-0.35	0.072
NMB1898	lipoprotein	3658.36	-0.47	0.072
NMB0953	hypothetical protein	919.08	-0.55	0.072
NMB1244	ribulose-phosphate 3-epimerase	2366.54	-0.49	0.072
NMB0947	dihydrolipoamide dehydrogenase	14489.88	-0.41	0.072
NMB0074	capsule polysaccharide export ATP-binding protein CtrD	1341.46	0.30	0.072
NMB1618	ribonuclease H	621.11	0.43	0.072
NMB0603	phosphoribosyl-ATP pyrophosphatase	2597.23	-0.64	0.073
NMB1630	hypothetical protein	24.74	-0.94	0.075
NMB1694	hypothetical protein	7223.53	0.36	0.075
NMB0116	DNA processing protein DprA	2718.69	0.24	0.076
NMB0506	hypothetical protein	432.09	-0.53	0.076
NMB1624	hypothetical protein	8895.59	-1.20	0.077
NMB1763	toxin-activating protein	279.69	-0.55	0.077
NMB0845	phosphate starvation-inducible protein PhoH	7243.08	-0.34	0.079
NMB0203	4-hydroxy-tetrahydrodipicolinate reductase	3883.65	0.45	0.079
NMB0275	indole-3-glycerol phosphate synthase	1599.15	0.35	0.079
NMB2128	CinA-like protein	1172.28	-0.33	0.080
NMB1062	hypothetical protein	2070.27	-0.29	0.081
NMB2110	hypothetical protein	296.18	-0.54	0.081
NMB1661	guanylate kinase	9045.96	-0.25	0.081
NMB1288	ribonucleotide-diphosphate reductase subunit beta	7219.32	-0.28	0.082

NMB1823	valine--pyruvate transaminase	3527.91	0.29	0.082
NMB1274	hypothetical protein	413.16	0.48	0.082
NMB0100	hypothetical protein	138.91	-0.81	0.082
NMB1032	type II restriction enzyme	1516.28	-0.27	0.082
NMB0495	replication protein	455.44	-0.36	0.083
NMB1283	2-dehydro-3-deoxyphosphooctonate aldolase	4261.72	0.26	0.084
NMB1992	hypothetical protein	366.38	0.35	0.084
NMB0650	hypothetical protein	422.18	-0.48	0.085
NMB1290	C-5 cytosine-specific DNA-methylase	4551.20	-0.47	0.085
NMB0598	Maf-like protein	816.58	0.30	0.085
NMB0956	dihydrolipoamide succinyltransferase	29733.35	-0.31	0.086
NMB0350	hypothetical protein	1943.27	-0.46	0.086
NMB0257	NADH dehydrogenase subunit L	6966.21	-0.48	0.087
NMB1239	hypothetical protein	1654.25	-0.47	0.088
NMB0540	aromatic amino acid aminotransferase	13624.57	-0.35	0.088
NMB0588	ABC transporter ATP-binding protein	1806.23	-0.54	0.088
NMB0187	ribosome recycling factor	7150.36	0.24	0.088
NMB1991	iron(III) ABC transporter permease	163.49	0.56	0.088
NMB0893	deoxyuridine 5'-triphosphate nucleotidohydrolase	2028.72	0.29	0.089
NMB0912	hypothetical protein	185.40	0.41	0.089
NMB0314	hypothetical protein	1091.85	0.26	0.090
NMB1682	DNA topoisomerase IV subunit B	2331.61	0.44	0.091
NMB0416	UDP-MurNAc-pentapeptide synthetase	3371.89	0.34	0.091
NMB0454	hypothetical protein	727.16	0.36	0.091
NMB1705	alpha 1%2C2 N-acetylglucosamine transferase	1144.85	-0.28	0.092
NMB1612	amino acid ABC transporter substrate-binding protein	19224.45	-0.39	0.092
NMB1696	acyl carrier protein	1202.26	-0.32	0.092
NMB0773	hypothetical protein	7923.22	0.56	0.092

NMB0872	hypothetical protein	2922.26	0.28	0.093
NMB1849	carbamoyl phosphate synthase small subunit	9372.25	0.36	0.094
NMB1583	imidazoleglycerol-phosphate dehydratase	15428.18	0.27	0.094
NMB1918	ACP S-malonyltransferase	7287.94	0.35	0.095
NMB1342	dihydrolipoamide acetyltransferase	61367.26	0.34	0.095
NMB1697	acyl carrier protein	1186.66	-0.32	0.096
NMB1034	isopropylmalate isomerase small subunit	1621.20	-0.42	0.096
NMB1953	stringent starvation protein A	11399.34	-0.46	0.096
NMB2026	ABC transporter permease	638.58	0.37	0.097
NMB0907	hypothetical protein	146.73	-0.51	0.097
NMB0561	heat shock protein GrpE	6300.14	0.39	0.097
NMB0521	hypothetical protein	950.21	-0.33	0.098
NMB0666	DNA ligase	2710.12	0.26	0.098
NMB1119	hypothetical protein	77.35	0.57	0.099
NMB1438	hypothetical protein	28960.30	0.27	0.099
NMB1422	ATP-dependent RNA helicase	4048.36	0.24	0.101
NMB0240	hypothetical protein	3287.43	-0.45	0.104
NMB0496	hemolysin activator-like protein	59.97	-0.95	0.104
NMB0774	uracil phosphoribosyltransferase	4767.93	0.27	0.106
NMB1621	glutathione peroxidase	3877.30	0.49	0.106
NMB0526	hypothetical protein	479.50	0.36	0.107
NMB0913	hypothetical protein	1269.97	-0.26	0.108
NMB0374	MafB-like protein	377.97	-0.48	0.108
NMB1243	Holliday junction DNA helicase RuvB	869.28	0.26	0.110
NMB1048	hypothetical protein	822.86	0.46	0.110
NMB0758	polynucleotide phosphorylase	31389.45	0.40	0.114
NMB0492	hypothetical protein	437.24	-0.47	0.114
NMB1801	lipid A biosynthesis lauroyl acyltransferase	947.10	-0.33	0.115

NMB1767	hypothetical protein	133.23	-0.44	0.117
NMB1768	hemagglutinin/hemolysin-like protein	5467.30	-0.27	0.117
NMB0226	hypothetical protein	1488.00	0.35	0.117
NMB0043	hypothetical protein	2485.15	0.35	0.117
NMB1797	penicillin-binding protein 3	2739.61	0.32	0.118
NMB0560	serine acetyltransferase	1823.33	0.27	0.118
NMB0035	hypothetical protein	5930.34	0.32	0.118
NMB1460	ssDNA-binding protein	10088.19	0.23	0.119
NMB0594	sensor histidine kinase	3122.84	0.37	0.119
NMB1920	GMP synthase	11225.42	0.27	0.119
NMB0843	poly(A) polymerase	3112.05	0.26	0.119
NMB1785	hypothetical protein	98.26	-0.65	0.119
NMB2115	hypothetical protein	205.63	-0.44	0.120
NMB0715	hypothetical protein	51.44	0.67	0.120
NMB1510	thermonuclease	905.24	0.34	0.120
NMB1896	type II restriction enzyme	323.34	0.41	0.120
NMB1301	30S ribosomal protein S1	103904.34	0.57	0.122
NMB1059	hypothetical protein	23102.82	-0.76	0.124
NMB0201	hypothetical protein	119.42	-0.63	0.124
NMB0824	orotidine 5'-phosphate decarboxylase	2396.82	0.23	0.124
NMB1854	hypothetical protein	2719.74	0.38	0.125
NMB0359	glutamine synthetase	22569.15	-0.50	0.125
NMB1347	extragenic suppressor protein SuhB	4625.53	0.30	0.126
NMB1118	hypothetical protein	994.72	-0.38	0.126
NMB0954	type II citrate synthase	30596.45	-0.62	0.127
NMB0728	phenylalanyl-tRNA synthetase subunit beta	17198.15	-0.35	0.129
NMB1412	outer membrane protein FrpC	1109.17	-0.34	0.130
NMB2086	GTPase ObgE	4778.31	0.25	0.131

NMB1074	acetylglutamate kinase	2926.10	-0.77	0.132
NMB0092	hypothetical protein	1076.00	-0.35	0.132
NMB1484	5'-nucleotidase SurE	1708.80	0.32	0.132
NMB0443	IS30 family transposase	315.56	-0.50	0.133
NMB0436	hypothetical protein	3114.44	-0.30	0.133
NMB0935	tRNA delta(2)-isopentenylpyrophosphate transferase	398.78	0.34	0.133
NMB1447	ATP-dependent DNA helicase	1345.94	0.42	0.133
NMB2047	hypoxanthine-guanine phosphoribosyltransferase	4423.57	-0.38	0.134
NMB0858	hypothetical protein	168.92	-0.37	0.134
NMB0693	bifunctional folylpolyglutamate synthase/dihydrofolate synthase	4147.35	0.26	0.134
NMB1886	hypothetical protein	334.94	0.32	0.135
NMB0188	hypothetical protein	727.89	-0.27	0.136
NMB0431	2-methylcitrate synthase	835.45	-0.81	0.136
NMB0651	hypothetical protein	709.90	-0.45	0.137
NMB0109	hypothetical protein	34260.08	-0.24	0.138
NMB0582	bacteriocin resistance protein	156.51	-0.39	0.139
NMB0204	lipoprotein	1921.31	0.29	0.139
NMB0180	UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase	3896.87	0.33	0.139
NMB0815	adenylosuccinate synthetase	17393.17	0.35	0.139
NMB0643	MafB-like protein	2402.75	0.38	0.139
NMB0093	hypothetical protein	760.40	-0.35	0.139
NMB1047	hypothetical protein	2825.78	0.28	0.139
NMB0851	recombination associated protein	2286.51	-0.29	0.139
NMB1345	hypothetical protein	11485.39	0.25	0.139
NMB1503	hypothetical protein	90.82	-0.57	0.139
NMB0435	acetate kinase	666.46	-0.50	0.139
NMB0292	hypothetical protein	3214.00	-0.25	0.139
NMB0377	anhydro-N-acetylmuramic acid kinase	1260.36	-0.65	0.140

NMB0308	dihydrofolate reductase	2371.53	-0.35	0.140
NMB0198	ribosomal large subunit pseudouridine synthase C	1853.03	-0.32	0.140
NMB0352	KpsF/GutQ family sugar isomerase	3434.54	-0.40	0.140
NMB1358	aspartyl/glutamyl-tRNA amidotransferase subunit B	8670.03	0.29	0.143
NMB1809	Tfp pilus assembly protein PilN	15849.70	-0.35	0.143
NMB0787	amino acid ABC transporter substrate-binding protein	24497.09	-0.65	0.144
NMB1360	pyridoxamine 5'-phosphate oxidase	1744.48	-0.57	0.144
NMB1523	hypothetical protein	13627.98	-0.48	0.144
NMB0366	hypothetical protein	950.52	-0.30	0.144
NMB1125	hypothetical protein	97.58	-0.70	0.144
NMB1951	hypothetical protein	2324.92	-0.23	0.145
NMB1452	hypothetical protein	13586.11	-0.61	0.146
NMB2007	ATP-dependent DNA helicase	2594.06	-0.38	0.146
NMB1318	CDP-diacylglycerol--serine O-phosphatidyltransferase	3677.76	-0.25	0.146
NMB2148	IS30 family transposase	71.81	-0.66	0.146
NMB0220	acyl carrier protein	30753.76	-0.44	0.146
NMB2069	thiamin-phosphate pyrophosphorylase	384.83	0.35	0.148
NMB0005	arsenate reductase	3851.52	-0.53	0.150
NMB1636	opacity protein	137739.91	-0.30	0.150
NMB0208	4Fe-4S type ferredoxin	327.66	0.45	0.150
NMB1334	hypothetical protein	749.35	-0.46	0.150
NMB2049	glyoxalase II family protein	446.46	0.28	0.150
NMB1668	hemoglobin receptor	3192.16	-0.62	0.152
NMB0547	type IV pilin protein	1790.84	-0.30	0.152
NMB0402	sodium/proline symporter	3876.80	-0.48	0.152
NMB0243	NADH dehydrogenase subunit C	3204.95	-0.38	0.155
NMB1827	DNA polymerase III subunit alpha	8851.70	0.26	0.155
NMB2143	hypothetical protein	1391.95	0.31	0.155

NMB1279	membrane-bound lytic murein transglycosylase B	5000.27	0.47	0.155
NMB1435	drug resistance translocase	1062.98	-0.26	0.156
NMB0670	thymidylate kinase	1162.58	0.24	0.156
NMB0744	hypothetical protein	1100.05	0.65	0.157
NMB1654	hypothetical protein	588.34	-0.25	0.157
NMB0781	uroporphyrinogen decarboxylase	5833.40	0.29	0.158
NMB0853	hypothetical protein	2798.83	-0.35	0.158
NMB1328	tRNA (guanine-N(7)-)-methyltransferase	1125.61	0.32	0.158
NMB1265	hypothetical protein	820.57	-0.27	0.158
NMB1485	hypothetical protein	2887.32	0.30	0.159
NMB1698	acyltransferase	608.85	-0.29	0.160
NMB2028	thermoresistant gluconokinase	848.71	0.25	0.161
NMB2074	hypothetical protein	3793.94	0.34	0.162
NMB0983	bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	14182.24	-0.84	0.162
NMB1281	transcription-repair coupling factor	7983.47	0.45	0.162
NMB0192	ribonuclease HII	395.40	-0.30	0.163
NMB0611	spermidine/putrescine ABC transporter permease	5669.08	0.31	0.163
NMB1202	hypothetical protein	228.16	-0.44	0.164
NMB1839	formate--tetrahydrofolate ligase	17702.01	-0.38	0.164
NMB1127	short chain dehydrogenase	119.68	-0.56	0.165
NMB0001	acetyltransferase	1481.11	0.23	0.166
NMB1723a	Cbb3-type cytochrome oxidase subunit 3	4928.87	-0.43	0.167
NMB0448	pyridoxine 5'-phosphate synthase	3146.17	0.20	0.167
NMB0753	hypothetical protein	202.57	0.59	0.167
NMB1770	IS30 family transposase	68.44	-0.64	0.167
NMB0771	hypothetical protein	5709.50	0.38	0.168
NMB1790	glutaredoxin 3	6813.71	0.19	0.168
NMB1967	AraC family transcriptional regulator	813.46	-0.32	0.169

NMB2140	hypothetical protein	3543.58	-0.48	0.170
NMB0998	oxidoreductase	6590.12	-0.48	0.170
NMB0326	octaprenyl-diphosphate synthase	4960.43	0.21	0.170
NMB1373	ribosome-binding factor A	1351.80	0.30	0.173
NMB1337	Holliday junction resolvase-like protein	1448.20	0.40	0.173
NMB1363	exodeoxyribonuclease VII large subunit	785.37	-0.90	0.174
NMB0021	fimbrial protein	1240.24	-0.35	0.174
NMB1206	bacterioferritin B	4048.71	-0.35	0.174
NMB0274	ATP-dependent DNA helicase	2519.78	0.26	0.174
NMB1344	dihydrolipoyl dehydrogenase	35231.94	0.47	0.174
NMB0430	2-methylisocitrate lyase	779.76	-0.83	0.174
NMB1723	cytochrome c oxidase subunit III	35458.40	-0.52	0.174
NMB0283	hypothetical protein	2324.59	-0.20	0.174
NMB0719	queuine tRNA-ribosyltransferase	3349.59	0.22	0.174
NMB0959	succinyl-CoA synthetase subunit beta	39734.41	-0.23	0.176
NMB0546	alcohol dehydrogenase	195664.91	-0.60	0.176
NMB1230	DNA-binding protein HU-beta	28138.87	0.22	0.177
NMB1423	hypothetical protein	328.96	-0.37	0.177
NMB1701	hypothetical protein	1262.82	-0.27	0.177
NMB0551	primosome assembly protein PriA	1408.57	-0.23	0.177
NMB1824	hypothetical protein	1158.97	0.37	0.177
NMB0837	ATP-dependent Clp protease adaptor protein ClpS	2054.64	-0.39	0.178
NMB1064	hypothetical protein	357.56	0.40	0.178
NMB0684	6%2C7-dimethyl-8-ribityllumazine synthase	2337.89	0.45	0.178
NMB1877	prolyl oligopeptidase	1313.37	0.21	0.180
NMB0270	pimeloyl-[acyl-carrier protein] methyl ester esterase	1013.22	0.24	0.182
NMB1840	hypothetical protein	1354.81	-0.70	0.183
NMB1965	hypothetical protein	2231.41	0.31	0.184

NMB2121	hypothetical protein	101.29	-0.57	0.184
NMB1394	keto-hydroxyglutarate-aldolase/keto-deoxy-phosphogluconate aldolase	8105.96	-0.50	0.184
NMB0418	phospho-N-acetylmuramoyl-pentapeptide-transferase	2931.10	0.24	0.184
NMB0830	hypothetical protein	533.23	0.27	0.185
NMB1663	hypothetical protein	5731.96	0.27	0.186
NMB1037	glutamate--cysteine ligase	5144.91	0.34	0.186
NMB0349	glutamyl-Q tRNA(Asp) synthetase	2056.51	-0.37	0.186
NMB0477	hypothetical protein	5818.28	-0.23	0.188
NMB2039	major outer membrane protein PIB	534579.92	0.35	0.189
NMB0880	sulfate ABC transporter permease	1051.93	-0.91	0.190
NMB0512	hypothetical protein	106.05	-0.50	0.190
NMB1832	lipoprotein signal peptidase	2172.33	-0.25	0.190
NMB0886	fimbrial protein FimT	5927.19	-0.18	0.190
NMB1671	paraquat-inducible protein B	2309.22	0.23	0.190
NMB1397	hypothetical protein	221.62	-0.30	0.190
NMB0796	hypothetical protein	288.47	0.42	0.190
NMB1506	arginyl-tRNA synthetase	8282.82	0.28	0.193
NMB1027	hypothetical protein	1021.44	0.22	0.193
NMB0736	PTS system nitrogen regulatory protein IIA	5519.61	-0.35	0.193
NMB1958	thioredoxin	1446.75	0.21	0.193
NMB1532	hypothetical protein	1422.92	-0.20	0.193
NMB0475	hypothetical protein	20999.48	-0.20	0.193
NMB0002	hypothetical protein	1714.23	0.26	0.197
NMB1917	hypothetical protein	1078.26	0.25	0.198
NMB2048	DNA ligase	869.11	0.43	0.198
NMB0358	shikimate dehydrogenase	1672.79	-0.21	0.199
NMB1540	lactoferrin-binding protein A	567.52	-0.30	0.199
NMB1336	hypothetical protein	2498.07	0.38	0.199

NMB0059	molecular chaperone DnaJ	3263.87	0.33	0.200
NMB1677	cytochrome c5	21218.11	-0.47	0.200
NMB1039	hypothetical protein	3312.47	-0.19	0.200
NMB0464	phospholipase A1	4887.79	-0.20	0.203
NMB1500	hypothetical protein	8848.74	-0.47	0.203
NMB0338	hypothetical protein	2307.75	-0.20	0.203
NMB1543	hypothetical protein	40.70	-0.65	0.203
NMB0447	DNA repair protein RecO	4241.82	0.17	0.204
NMB0877	penicillin-binding protein	1109.41	-0.29	0.204
NMB1706	hypothetical protein	139.16	-0.42	0.205
NMB0937	elongation factor P	17508.54	0.45	0.207
NMB0690	amidophosphoribosyltransferase	7208.58	-0.85	0.208
NMB0010	phosphoglycerate kinase	33133.97	-0.57	0.208
NMB0404	hypothetical protein	3087.36	-0.26	0.208
NMB1782	hypothetical protein	165.69	-0.34	0.209
NMB0788	amino acid ABC transporter permease	5343.15	-0.59	0.209
NMB1656	hypothetical protein	48.70	-0.58	0.209
NMB1025	hypothetical protein	2279.58	0.32	0.209
NMB1659	guanosine-3'%2C5'-bis(diphosphate) 3'-pyrophosphohydrolase	13770.02	-0.25	0.210
NMB0311	hypothetical protein	8007.95	-0.35	0.210
NMB1831	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	4528.16	0.30	0.210
NMB1295	formamidopyrimidine-DNA glycosylase	1025.76	0.39	0.210
NMB1421	dihydrouridine synthase	1247.71	-0.23	0.210
NMB0110	peptide deformylase	7387.43	0.22	0.210
NMB1084	hypothetical protein	2953.08	-0.23	0.210
NMB0667	hypothetical protein	7386.53	0.27	0.212
NMB1462	transglycosylase	2758.32	0.33	0.212
NMB1041	GTP-binding protein	1647.39	-0.26	0.212

NMB1008	hypothetical protein	1011.95	-0.43	0.212
NMB0367	hypothetical protein	82.34	-0.43	0.213
NMB1853	hypothetical protein	636.78	0.24	0.215
NMB0510	hypothetical protein	282.52	-0.30	0.215
NMB1199	GTP-binding protein TypA	26284.08	0.17	0.217
NMB1131	chaperone protein HscA	37.81	0.89	0.217
NMB0829	type I restriction enzyme M protein	1073.22	0.31	0.218
NMB1684	seryl-tRNA synthetase	12978.61	-0.42	0.220
NMB0946	peroxiredoxin 2 family protein/glutaredoxin	81989.61	-0.31	0.220
NMB1300	cytidylate kinase	958.64	0.27	0.220
NMB1952	ClpXP protease specificity-enhancing factor	3216.64	-0.26	0.222
NMB0264	ABC transporter ATP-binding protein	2573.85	0.20	0.222
NMB0379	coproporphyrinogen III oxidase	2639.09	0.24	0.222
NMB0355	hypothetical protein	2589.75	0.22	0.223
NMB1989	iron ABC transporter substrate-binding protein	1469.20	0.19	0.224
NMB0052	twitching motility protein PilT	20348.53	-0.33	0.225
NMB1531	hypothetical protein	1093.82	-0.39	0.225
NMB1362	oxalate/formate antiporter	2369.35	-0.53	0.228
NMB1926	lacto-N-neotetraose biosynthesis glycosyl transferase LgtE	2011.61	-0.19	0.229
NMB0532	protease Do	6568.20	0.17	0.230
NMB0868	hypothetical protein	1335.35	0.19	0.231
NMB1720	exodeoxyribonuclease V subunit RecC	5447.05	0.25	0.231
NMB0126	transcription antitermination protein NusG	8977.35	-0.28	0.232
NMB1792	sensor histidine kinase	1691.65	0.19	0.233
NMB0434	AcnD-accessory protein PrpF	1309.26	-0.36	0.234
NMB2050	hypothetical protein	7476.23	0.21	0.234
NMB1687	hypothetical protein	936.71	0.22	0.234
NMB0875	ribose-phosphate pyrophosphokinase	15692.33	0.22	0.234

NMB0619	hypothetical protein	3385.64	0.34	0.234
NMB1725	cbb3-type cytochrome c oxidase subunit I	66574.24	-0.51	0.236
NMB0902	hypothetical protein	43.36	-0.56	0.238
NMB1145	UDP-N-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase	67.77	-0.49	0.238
NMB2133	serine/threonine transporter SstT	8189.35	-0.44	0.240
NMB2008	hypothetical protein	3058.98	-0.28	0.240
NMB0328	hypothetical protein	719.58	-0.39	0.243
NMB0854	histidyl-tRNA synthetase	4362.55	0.24	0.244
NMB0372	hypothetical protein	147.42	-0.41	0.246
NMB1577	acetolactate synthase isozyme III large subunit	30880.39	-0.35	0.246
NMB0759	hypothetical protein	803.34	-0.29	0.247
NMB1724	cbb3-type cytochrome c oxidase subunit II	23796.95	-0.45	0.247
NMB1576	acetolactate synthase small subunit	4961.00	-0.38	0.247
NMB0668	N-acetyl-anhydromuranmyl-L-alanine amidase	1500.72	-0.23	0.247
NMB0558	hypothetical protein	1871.46	0.21	0.247
NMB0509	hypothetical protein	193.57	-0.35	0.249
NMB1888	preprotein translocase subunit SecG	5755.29	-0.28	0.249
NMB0734	hypothetical protein	835.86	-0.25	0.250
NMB0679	acetyl-CoA carboxylase subunit beta	16504.15	0.28	0.250
NMB0290	transcriptional regulator	1412.75	-0.25	0.251
NMB1749	hypothetical protein	298.68	-0.29	0.252
NMB1842	4-hydroxyphenylacetate 3-hydroxylase small subunit	1703.06	-0.29	0.252
NMB1563	GntR family transcriptional regulator	4858.08	0.22	0.253
NMB1902	DNA polymerase III subunit beta	16707.02	-0.32	0.255
NMB1026	hypothetical protein	701.83	0.24	0.257
NMB0193	tRNA uridine 5-carboxymethylaminomethyl modification protein GidA	2499.72	0.18	0.257
NMB0707	rare lipoprotein B	2665.01	0.25	0.260
NMB1870	hypothetical protein	20967.76	-0.37	0.261

NMB0013	hypothetical protein	1030.94	0.20	0.262
NMB0446	chorismate mutase	9924.88	0.20	0.262
NMB0996	hypothetical protein	922.92	0.33	0.262
NMB0790	phosphoglucomutase	13027.98	0.42	0.262
NMB0432	hypothetical protein	683.04	-0.35	0.263
NMB1948	ABC transporter ATP-binding protein	826.85	-0.20	0.263
NMB1204	transcriptional regulator	330.92	0.30	0.263
NMB0279	hypothetical protein	6020.78	0.21	0.264
NMB0607	preprotein translocase subunit SecD	20229.49	0.22	0.266
NMB1505	nicotinate phosphoribosyltransferase	2105.90	0.19	0.268
NMB1582	histidinol-phosphate aminotransferase	10539.26	0.32	0.268
NMB0117	protein Smg	2398.29	-0.20	0.269
NMB1977	hypothetical protein	386.02	0.25	0.269
NMB1934	ATP synthase F0F1 subunit beta	59015.58	-0.37	0.270
NMB0786	hypothetical protein	5339.45	-0.30	0.271
NMB0610	spermidine/putrescine ABC transporter ATP-binding protein	8515.83	0.23	0.271
NMB0579	copper ABC transporter ATP-binding protein	680.85	0.30	0.273
NMB1590	hypothetical protein	17002.35	-0.47	0.275
NMB0244	NADH dehydrogenase subunit D	7924.45	-0.34	0.276
NMB0008	cell division protein FtsX	4475.46	0.18	0.276
NMB0444	hypothetical protein	2043.74	-0.23	0.278
NMB1909	Maf-like protein	1794.05	-0.49	0.279
NMB0050	hypothetical protein	2265.83	-0.49	0.279
NMB1601	IS1106 transposase	542.38	-0.21	0.279
NMB1581	histidinol dehydrogenase	15649.13	0.38	0.279
NMB2030	3-demethylubiquinone-9 3-methyltransferase	2474.13	0.38	0.279
NMB1302	integration host factor subunit beta	15207.52	0.55	0.281
NMB1011	hypothetical protein	1168.88	0.22	0.282

NMB1969	serotype-1-specific antigen	22463.74	-0.43	0.282
NMB0295	signal recognition particle protein	4899.12	0.34	0.282
NMB0783	hypothetical protein	4556.67	-0.34	0.284
NMB0473	hypothetical protein	815.50	0.21	0.284
NMB1933	ATP synthase F0F1 subunit epsilon	14701.57	-0.39	0.285
NMB1448	DNA polymerase IV	714.83	-0.55	0.289
NMB2099	5-formyltetrahydrofolate cyclo-ligase	968.50	-0.21	0.289
NMB0230	hypothetical protein	3408.18	-0.31	0.289
NMB0293	TonB-dependent receptor	132.37	-0.47	0.291
NMB1260	type III restriction-modification system enzyme subunit res	531.60	0.27	0.291
NMB0154	50S ribosomal protein L5	29168.64	0.36	0.292
NMB0535	glucose/galactose transporter	25231.14	-0.29	0.292
NMB1642	transcription elongation factor NusA	5953.59	0.26	0.293
NMB0590	tRNA (guanine-N(1)-)-methyltransferase	17055.21	0.24	0.294
NMB0356	ABC transporter ATP-binding protein	3019.78	0.23	0.295
NMB1096	hypothetical protein	136.17	0.36	0.296
NMB0895	hypothetical protein	3053.66	0.25	0.297
NMB1728	biopolymer transport protein	3865.56	0.44	0.300
NMB0025	fimbrial protein	351.45	0.38	0.304
NMB1921	3-ketoacyl-ACP reductase	12469.03	-0.25	0.305
NMB1765	hypothetical protein	159.25	-0.41	0.306
NMB0633	iron(III) ABC transporter permease	1630.33	-0.27	0.307
NMB0453	pyrophosphohydrolase MutT	1738.68	0.36	0.308
NMB0073	capsule polysaccharide export inner-membrane protein	1836.40	0.23	0.311
NMB0181	outer membrane protein OmpH	3691.07	0.17	0.312
NMB1692	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase	1740.96	0.21	0.312
NMB1881	hypothetical protein	13.65	1.00	0.312
NMB2010	hypothetical protein	1295.88	-0.17	0.313

NMB0387	ABC transporter ATP-binding protein	23466.15	-0.14	0.313
NMB1647	amino acid symporter	1378.09	-0.52	0.314
NMB0884	superoxide dismutase	16516.42	-0.22	0.314
NMB0053	hypothetical protein	2905.66	-0.28	0.315
NMB2156	lipopolysaccharide heptosyltransferase I	5534.68	-0.29	0.315
NMB1138	hypothetical protein	25.35	-0.73	0.317
NMB1069	gamma-glutamyl kinase	1014.32	-0.62	0.317
NMB2157	pyrazinamidase/nicotinamidase PncA	4373.81	-0.26	0.317
NMB1218	hypothetical protein	1341.86	-0.20	0.317
NMB0233	hypothetical protein	191.97	-0.47	0.318
NMB1451	DNA polymerase III subunit epsilon	950.29	-0.35	0.318
NMB0730	FxsA protein	587.57	-0.36	0.321
NMB0212	DNA gyrase subunit B	24903.76	-0.41	0.322
NMB0393	multidrug resistance protein	661.94	-0.55	0.325
NMB1875	hypothetical protein	1215.41	0.16	0.325
NMB2145	hypothetical protein	283.56	-0.26	0.327
NMB0981	phosphoserine phosphatase	1196.47	0.21	0.327
NMB1691	dihydropteroate synthase	1708.03	0.25	0.328
NMB0081	dTDP-4-keto-6-deoxy-D-glucose-3%2C6-epimerase	347.74	0.27	0.330
NMB1634	hypothetical protein	28.73	-0.59	0.333
NMB0768	twitching motility protein PilT	15193.99	-0.42	0.333
NMB0511	hypothetical protein	88.82	-0.37	0.333
NMB2154	electron transfer flavoprotein subunit alpha	14900.31	-0.23	0.333
NMB1291	ribonucleotide-diphosphate reductase subunit alpha	22168.59	-0.16	0.333
NMB1226	ABC transporter ATP-binding protein	3290.05	0.17	0.333
NMB2142	hypothetical protein	3716.65	0.19	0.333
NMB1361	23S rRNA pseudouridylate synthase B	2973.52	0.19	0.333
NMB0826	C-5 cytosine-specific DNA methylase	1649.75	0.25	0.333

NMB0869	spermidine synthase	1691.80	0.16	0.334
NMB1429	outer membrane protein PorA	274972.01	0.27	0.334
NMB0852	GTP-binding protein EngA	3088.88	-0.16	0.337
NMB2147	hypothetical protein	702.78	-0.32	0.337
NMB0108	hypothetical protein	2027.12	0.17	0.338
NMB0696	amino acid ABC transporter ATP-binding protein	6170.70	0.18	0.338
NMB0929	4-hydroxy-tetrahydrodipicolinate synthase	6349.32	0.22	0.338
NMB2062	molybdopterin/thiamine biosynthesis family protein	715.06	-0.24	0.342
NMB1963	hypothetical protein	8387.77	0.17	0.342
NMB1289	type II restriction enzyme	3998.41	-0.29	0.343
NMB1428	aminopeptidase	7978.69	0.34	0.343
NMB1489	hypothetical protein	602.43	-0.20	0.343
NMB2033	D%2CD-heptose 1%2C7-bisphosphate phosphatase	682.93	0.27	0.344
NMB1944	chromosome-partitioning protein ParB	6134.97	-0.33	0.346
NMB2139	hypothetical protein	3477.09	-0.17	0.346
NMB0228	LamB/YcsF family protein	1689.88	0.44	0.346
NMB1241	multifunctional tRNA nucleotidyl transferase/2'3'-cyclic phosphodiesterase/2'nucleotidase/phosphatase	3330.93	0.16	0.347
NMB1713	IS30 family transposase	40.80	0.51	0.347
NMB0660	hypothetical protein	171.75	0.47	0.350
NMB0216	catalase	2417.69	-0.41	0.352
NMB1051	ABC transporter ATP-binding protein	3009.27	0.14	0.352
NMB1382	hypothetical protein	526.82	-0.25	0.352
NMB0864	hypothetical protein	517.34	-0.29	0.354
NMB0572	hypothetical protein	1134.86	-0.22	0.355
NMB1966	ABC transporter ATP-binding protein	3720.20	0.18	0.356
NMB0508	hypothetical protein	139.76	-0.51	0.356
NMB2101	30S ribosomal protein S2	43329.84	0.39	0.356
NMB0979	hypothetical protein	1852.52	0.17	0.356

NMB1275	hypothetical protein	1364.12	0.27	0.358
NMB0266	hypothetical protein	1610.64	0.28	0.358
NMB0063	dTDP-D-glucose 4%2C6-dehydratase	44.43	-0.55	0.359
NMB0776	hypothetical protein	1963.39	0.20	0.360
NMB1454	4Fe-4S type ferredoxin	4331.37	-0.27	0.361
NMB0918	hypothetical protein	578.04	-0.18	0.361
NMB0480	hypothetical protein	57.19	0.58	0.361
NMB1246	hypothetical protein	1439.22	-0.21	0.362
NMB1990	iron ABC transporter permease	233.15	0.31	0.362
NMB1858	hypothetical protein	115.51	0.33	0.362
NMB0368	hypothetical protein	115.10	-0.32	0.362
NMB0452	4'-phosphopantetheinyl transferase	1533.57	0.31	0.363
NMB0914	hypothetical protein	1308.32	-0.16	0.364
NMB0064	UDP-glucose 4-epimerase	758.57	0.28	0.365
NMB1446	3-dehydroquinase	1084.18	0.15	0.367
NMB0045	signal recognition particle protein	3562.53	0.16	0.369
NMB0331	kinase	1156.23	0.15	0.369
NMB2116	hypothetical protein	265.93	-0.29	0.370
NMB1830	phosphoglycolate phosphatase	622.04	0.18	0.370
NMB0170	septum formation inhibitor	4623.19	-0.19	0.370
NMB1269	hypothetical protein	3887.05	0.20	0.372
NMB0944	5-methyltetrahydropteroyltriglutamate/homocysteine S-methyltransferase	66922.29	0.21	0.372
NMB0105	hypothetical protein	4803.21	-0.24	0.374
NMB0072	capsule polysaccharide export inner-membrane protein	3532.54	0.14	0.375
NMB0671	malate oxidoreductase	25281.12	0.19	0.376
NMB0458	glutamate racemase	2397.05	0.16	0.378
NMB0312	ATPase	556.02	-0.31	0.378
NMB2092	hypothetical protein	483.25	0.26	0.378

NMB1756	hypothetical protein	211.20	-0.25	0.379
NMB1710	glutamate dehydrogenase	171980.94	-1.17	0.379
NMB1235	hypothetical protein	3495.45	0.18	0.379
NMB0089	pyruvate kinase	31370.47	-0.42	0.380
NMB1554	CTP synthetase	15756.24	0.13	0.380
NMB2144	RNA polymerase sigma factor	723.86	-0.29	0.381
NMB0617	transcription termination factor Rho	5123.34	0.22	0.383
NMB0621	hypothetical protein	1087.88	-0.31	0.384
NMB0802	cystathionine gamma-synthase	6132.29	-0.26	0.385
NMB0718	ferrochelatase	1877.95	0.22	0.386
NMB0360	AmpG-like protein	1933.25	0.21	0.386
NMB0417	hypothetical protein	462.39	0.24	0.386
NMB1561	DeoR family transcriptional regulator	1671.96	0.18	0.387
NMB0130	50S ribosomal protein L10	70491.78	0.40	0.387
NMB1095	hypothetical protein	179.87	0.30	0.388
NMB0587	membrane protein	631.41	0.36	0.391
NMB2078	hypothetical protein	6680.21	0.19	0.392
NMB0584	outer membrane protein FrpC	232.66	-0.37	0.393
NMB1367	hypothetical protein	621.92	-0.20	0.394
NMB0003	glutamyl-tRNA synthetase	11659.11	0.20	0.394
NMB2091	hemolysin	4551.74	0.20	0.394
NMB1238	peptidyl-prolyl cis-trans isomerase-like protein	10475.34	0.14	0.394
NMB1306	hypothetical protein	8968.01	-0.21	0.395
NMB1439	5-(carboxyamino)imidazole ribonucleotide mutase	4511.61	0.21	0.395
NMB1867	1-deoxy-D-xylulose-5-phosphate synthase	9428.44	0.25	0.398
NMB1433	hypothetical protein	2643.45	-0.51	0.401
NMB1054	transposase	297.80	-0.27	0.401
NMB1868	integrase/recombinase XerC	1848.50	-0.25	0.401

NMB1314	cell division protein FtsK	4602.54	0.16	0.402
NMB0602	HIT family hydrolase	1710.90	-0.24	0.402
NMB1538	RNA polymerase sigma factor RpoD	11331.79	0.25	0.402
NMB0083	capsule polysaccharide modification protein	1659.52	-0.37	0.403
NMB1939	ATP synthase F0F1 subunit C	24732.51	-0.30	0.403
NMB1498	aspartate kinase	15616.35	-0.21	0.403
NMB0849	deoxycytidine triphosphate deaminase	1897.78	-0.23	0.403
NMB1804	cytochrome c-type biogenesis protein	5867.79	-0.20	0.405
NMB1613	fumarate hydratase	14305.20	-0.28	0.406
NMB0770	type IV pilus assembly protein PilZ	3791.57	0.28	0.406
NMB1560	glutaminyl-tRNA synthetase	13640.47	-0.18	0.407
NMB1050	IS30 family transposase	83.98	-0.31	0.408
NMB0923	cytochrome c	4559.50	-0.25	0.408
NMB0014	3-deoxy-D-manno-octulosonic acid transferase	1102.51	0.18	0.408
NMB1604	2%2C3-bisphosphoglycerate-dependent phosphoglycerate mutase	12734.18	-0.35	0.408
NMB1794	citrate transporter	1511.05	0.14	0.411
NMB0287	ATP-dependent DNA helicase DinG	2034.52	-0.16	0.413
NMB0939	hypothetical protein	1891.57	0.23	0.413
NMB2130	hypothetical protein	1281.42	-0.28	0.414
NMB1264	hypothetical protein	448.72	-0.22	0.414
NMB0184	1-deoxy-D-xylulose 5-phosphate reductoisomerase	4060.30	0.16	0.414
NMB2056	30S ribosomal protein S9	26203.92	0.27	0.414
NMB1904	50S ribosomal protein L34	5778.49	-0.19	0.417
NMB1586	hypothetical protein	215.97	0.23	0.417
NMB1476	glutamate dehydrogenase	3319.34	-0.13	0.419
NMB1055	serine hydroxymethyltransferase	14395.28	-0.29	0.419
NMB0353	hypothetical protein	3953.60	-0.17	0.419
NMB1959	hypothetical protein	922.57	-0.17	0.419

NMB0800	hypothetical protein	2778.56	0.13	0.419
NMB1313	trigger factor	31972.68	0.25	0.424
NMB0173	LysR family transcriptional regulator	14160.86	0.13	0.430
NMB1343	hypothetical protein	6575.30	0.38	0.431
NMB0596	hypothetical protein	2414.63	-0.16	0.432
NMB1884	hypothetical protein	452.63	-0.17	0.433
NMB0794	hypothetical protein	166.15	0.36	0.433
NMB0911	IS30 family transposase	53.02	-0.41	0.433
NMB0491	hypothetical protein	544.94	-0.27	0.433
NMB2072	hypothetical protein	20.87	0.57	0.433
NMB1228	homoserine dehydrogenase	4915.46	0.23	0.437
NMB1834	bifunctional riboflavin kinase/FMN adenylyltransferase	2807.04	0.16	0.437
NMB1852	hypothetical protein	903.33	0.16	0.437
NMB0712	RNA polymerase factor sigma-32	14714.48	0.17	0.437
NMB1237	recombination protein RecR	1694.07	0.17	0.437
NMB2089	hypothetical protein	1614.29	-0.15	0.438
NMB0799	cell division protein FtsJ	1492.26	0.18	0.438
NMB1384	DNA gyrase subunit A	18202.90	0.25	0.438
NMB0155	30S ribosomal protein S14	17410.48	0.30	0.439
NMB1020	hypothetical protein	1399.33	-0.23	0.439
NMB0900	hypothetical protein	2186.28	-0.20	0.439
NMB1885	protein-L-isoaspartate O-methyltransferase	2846.82	-0.16	0.439
NMB0033	membrane-bound lytic murein transglycosylase A	5269.94	0.13	0.439
NMB2045	sugar transport PTS system phosphocarrier protein HPr	1289.68	0.22	0.439
NMB0855	bacteriocin resistance protein	94.27	0.34	0.442
NMB1472	chaperone protein ClpB	19549.91	0.33	0.446
NMB1727	hypothetical protein	3521.55	0.16	0.448
NMB1488	succinate semialdehyde dehydrogenase	5261.86	0.19	0.450

NMB0767	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase	2494.54	0.15	0.450
NMB2035	hypothetical protein	239.89	0.19	0.450
NMB0129	hypothetical protein	13968.41	0.40	0.450
NMB1487	fimbrial assembly protein	3273.73	0.15	0.450
NMB0977	modulator of drug activity B	198.94	0.40	0.453
NMB1205	hypothetical protein	37.26	0.42	0.453
NMB1220	hypothetical protein	7397.40	0.25	0.454
NMB0309	hypothetical protein	1164.00	0.21	0.457
NMB0194	amino acid symporter	13465.93	-0.27	0.457
NMB1308	23S rRNA (adenine(2503)-C(2))-methyltransferase RlmN	6110.95	0.11	0.457
NMB0261	geranyltranstransferase	2236.08	0.15	0.457
NMB1457	transketolase	26004.77	0.18	0.461
NMB1466	hypothetical protein	64.97	0.36	0.462
NMB2122	MafB-like protein	34.39	-0.43	0.463
NMB0903	hypothetical protein	39.16	-0.43	0.463
NMB0810	TetR family transcriptional regulator	2041.26	0.23	0.463
NMB1946	outer membrane lipoprotein	23388.12	-0.14	0.463
NMB1270	hypothetical protein	4995.46	0.15	0.463
NMB1491	hypothetical protein	12.27	0.79	0.464
NMB1355	aspartyl/glutamyl-tRNA amidotransferase subunit C	1354.36	-0.21	0.466
NMB2054	hypothetical protein	1791.84	0.15	0.466
NMB1645	hypothetical protein	942.02	0.16	0.472
NMB0054	hypothetical protein	767.66	-0.22	0.475
NMB0665	coproporphyrinogen III oxidase	894.62	-0.20	0.475
NMB1733	hypothetical protein	693.69	-0.17	0.475
NMB0545	hypothetical protein	2344.06	0.12	0.475
NMB1383	chaperone protein HscB	674.90	0.29	0.475
NMB0121	hypothetical protein	417.80	-0.16	0.479

NMB1985	adhesion and penetration protein	34007.41	0.13	0.482
NMB1903	chromosomal replication initiation protein	9600.09	-0.13	0.484
NMB0056	DnaK suppressor protein	2153.05	0.12	0.485
NMB0803	GTP cyclohydrolase	3250.12	0.21	0.486
NMB1469	hypothetical protein	5471.54	0.36	0.486
NMB1617	tellurite resistance protein TehB	523.44	0.46	0.487
NMB1391	Sol/DevB family oxidoreductase	11790.75	-0.26	0.487
NMB1929	lacto-N-neotetraose biosynthesis glycosyl transferase	2769.06	-0.14	0.487
NMB1813	shikimate kinase	1464.62	0.16	0.491
NMB0756	dTDP-L-rhamnose synthase	3385.54	-0.22	0.492
NMB2153	hypothetical protein	2329.32	-0.20	0.492
NMB1908	hypothetical protein	2072.63	-0.27	0.493
NMB1900	polyphosphate kinase	4646.41	0.24	0.493
NMB0457	hypothetical protein	1369.46	-0.14	0.493
NMB0128	50S ribosomal protein L1	62343.72	0.29	0.493
NMB0357	monofunctional biosynthetic peptidoglycan transglycosylase	674.51	-0.16	0.496
NMB0382	outer membrane protein	198990.34	0.12	0.496
NMB1052	dedA protein	795.80	0.14	0.496
NMB0685	hypothetical protein	957.35	0.17	0.496
NMB1999	magnesium transporter	3528.96	0.19	0.496
NMB0569	Na(+)-translocating NADH-quinone reductase subunit A	13833.63	0.29	0.496
NMB1600	hypothetical protein	121.29	-0.25	0.497
NMB0232	DNA helicase II	2601.74	0.11	0.497
NMB2019	phosphopantetheine adenylyltransferase	1361.53	0.21	0.497
NMB1338	isomerase	812.43	0.20	0.499
NMB1962	hypothetical protein	1847.75	0.13	0.502
NMB1415	iron-regulated protein FrpC	3525.71	-0.14	0.506
NMB1836	acyltransferase	721.49	0.28	0.506

NMB0599	hypothetical protein	1310.63	-0.16	0.507
NMB0834	IS30 family transposase	97.33	-0.28	0.507
NMB0164	50S ribosomal protein L36	7997.74	-0.25	0.508
NMB0041	ABC transporter substrate-binding protein	10944.60	-0.29	0.509
NMB1018	hypothetical protein	372.04	-0.23	0.509
NMB1662	adenine phosphoribosyltransferase	2096.66	0.12	0.509
NMB1537	DNA primase	5363.37	0.15	0.509
NMB0764	hypothetical protein	689.27	0.17	0.509
NMB2017	competence protein ComEA	525.02	-0.22	0.509
NMB0323	hypothetical protein	10827.42	0.14	0.509
NMB0471	hypothetical protein	1782.27	-0.22	0.510
NMB0625	hypothetical protein	5603.52	-0.18	0.510
NMB1004	hypothetical protein	25.61	0.51	0.510
NMB1497	TonB-dependent receptor	22861.68	0.33	0.511
NMB1267	low molecular weight protein tyrosine-phosphatase	782.72	0.15	0.511
NMB1038	DNA repair protein RadC	459.52	0.17	0.511
NMB0801	delta-aminolevulinic acid dehydratase	5448.53	0.22	0.512
NMB0635	transposase IS30	55.62	-0.36	0.515
NMB0011	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	7394.11	0.10	0.516
NMB0222	hypothetical protein	371.20	0.18	0.516
NMB1310	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (flavodoxin)	5382.48	0.12	0.516
NMB1417	hypothetical protein	3634.20	-0.16	0.517
NMB1282	aspartate alpha-decarboxylase	1663.29	-0.13	0.517
NMB1851	hypothetical protein	910.88	0.12	0.517
NMB0780	hypothetical protein	2124.84	0.13	0.517
NMB0217	RNA polymerase sigma-54 factor RpoN	1584.41	-0.19	0.518
NMB1029	aspartate ammonia-lyase	5401.81	-0.18	0.518
NMB0497	hemagglutinin/hemolysin-like protein	336.64	0.21	0.520

NMB0976	hypothetical protein	80.44	0.37	0.520
NMB2160	DNA mismatch repair protein MutS	2762.94	0.15	0.522
NMB1075	hypothetical protein	2564.88	0.25	0.522
NMB0281	peptidyl-prolyl cis-trans isomerase	12000.27	0.17	0.525
NMB2123	hypothetical protein	139.10	-0.28	0.525
NMB1640	3-phosphoserine/phosphohydroxythreonine aminotransferase	4803.63	0.22	0.526
NMB2081	hypothetical protein	1228.25	-0.13	0.528
NMB0932	hypothetical protein	334.30	0.19	0.529
NMB1303	MerR family transcriptional regulator	1775.66	-0.19	0.530
NMB0055	pyrroline-5-carboxylate reductase	1780.22	0.17	0.530
NMB2034	1-acyl-SN-glycerol-3-phosphate acyltransferase	710.80	0.18	0.530
NMB1216	lipoyl synthase	2939.25	0.20	0.530
NMB0844	hypothetical protein	1320.38	0.17	0.531
NMB0785	exodeoxyribonuclease V subunit RecB	4339.44	0.19	0.531
NMB1961	VacJ-like protein	3869.74	0.13	0.531
NMB0227	hypothetical protein	3202.62	0.28	0.532
NMB1544	hypothetical protein	16.02	0.50	0.533
NMB0501	hypothetical protein	26.48	0.56	0.533
NMB0513	hypothetical protein	89.96	-0.25	0.533
NMB1960	hypothetical protein	6597.98	-0.13	0.534
NMB0423	UDP-N-acetylmuramate--L-alanine ligase	9090.33	0.16	0.534
NMB1887	triosephosphate isomerase	7158.19	-0.17	0.534
NMB0698	hypothetical protein	1610.38	0.12	0.534
NMB1044	ferredoxin--NADP reductase	8102.25	0.13	0.534
NMB1323	30S ribosomal protein S6	15929.59	0.13	0.536
NMB1924	inositol monophosphatase	755.99	0.22	0.536
NMB0022	hypothetical protein	96.02	-0.24	0.539
NMB1278	site-specific recombinase	3321.46	-0.22	0.539

NMB0597	hypothetical protein	265.88	0.19	0.539
NMB1468	hypothetical protein	21908.97	0.23	0.539
NMB0407	thiol:disulfide interchange protein DsbA	4812.82	-0.13	0.540
NMB1312	ATP-dependent Clp protease proteolytic subunit	6620.99	-0.17	0.541
NMB0124	elongation factor Tu	99742.37	-0.16	0.541
NMB1638	hypothetical protein	7723.79	0.13	0.543
NMB0797	hypothetical protein	1222.61	0.20	0.543
NMB0009	BolA/YrbA family protein	1877.46	0.17	0.547
NMB0062	glucose-1-phosphate thymidylyltransferase	19.68	-0.44	0.548
NMB0595	DNA-binding response regulator	19547.73	0.18	0.548
NMB1979	hypothetical protein	1845.13	0.23	0.549
NMB0931	23S rRNA (guanosine(2251)-2'-O)-methyltransferase RlmB	2488.56	0.13	0.550
NMB0060	hypothetical protein	9049.48	-0.19	0.551
NMB2136	peptide transporter	1365.81	-0.18	0.551
NMB0088	outer membrane protein P1	8160.07	-0.18	0.551
NMB0069	polysialic acid capsule biosynthesis protein SiaB	2337.93	-0.17	0.551
NMB0606	hypothetical protein	12147.50	-0.15	0.551
NMB0424	D-alanine--D-alanine ligase	6581.42	0.10	0.551
NMB0131	50S ribosomal protein L7/L12	60598.55	0.30	0.551
NMB1371	acetylornithine aminotransferase	5164.66	-0.20	0.552
NMB1063	dihydroneopterin aldolase	298.97	0.21	0.553
NMB1444	hypothetical protein	2673.73	-0.14	0.554
NMB1322	primosomal replication protein	9044.98	0.16	0.554
NMB0566	Na(+)-translocating NADH-quinone reductase subunit D	6523.10	-0.24	0.554
NMB1266	zinc uptake regulation protein	765.93	-0.15	0.554
NMB0341	T-cell stimulating protein TspA	22036.33	-0.26	0.555
NMB2100	hypothetical protein	310.07	-0.20	0.555
NMB1547	hypothetical protein	45.03	-0.33	0.556

NMB0172	cell division topological specificity factor MinE	4515.42	0.11	0.556
NMB0335	2%2C3%2C4%2C5-tetrahydropyridine-2%2C6-dicarboxylate N-succinyltransferase	19386.64	0.16	0.556
NMB1730	TonB protein	3926.44	0.17	0.556
NMB0051	twitching motility protein	18983.03	-0.23	0.558
NMB0862	hypothetical protein	348.22	-0.14	0.559
NMB0604	alcohol dehydrogenase	114024.42	-0.51	0.562
NMB1688	L-asparaginase	2181.54	0.11	0.564
NMB1473	aminotransferase	8860.56	0.13	0.565
NMB1597	hypothetical protein	506.82	-0.22	0.568
NMB1622	nitric oxide reductase	27266.40	-1.13	0.568
NMB0219	3-oxoacyl-ACP synthase	31553.99	-0.21	0.569
NMB0618	phosphoenolpyruvate synthase	87440.25	-0.20	0.569
NMB1657	DNA-binding protein	212.30	-0.15	0.569
NMB0778	uroporphyrin-III C-methyltransferase HemX	8025.45	0.12	0.569
NMB1703	3-oxoacyl-ACP synthase	4020.98	-0.16	0.570
NMB1637	hypothetical protein	2194.30	0.16	0.571
NMB0542	hypothetical protein	2732.19	-0.16	0.572
NMB2083	cysteinyl-tRNA synthetase	8594.71	-0.09	0.572
NMB0026	hypothetical protein	1486.05	0.10	0.572
NMB0398	ArsR family transcriptional regulator	1144.64	0.23	0.572
NMB0047	hypothetical protein	733.12	-0.20	0.572
NMB0811	UDP-N-acetylenolpyruvoylglucosamine reductase	1754.48	0.10	0.580
NMB0182	outer membrane protein OMP85	21660.28	0.12	0.580
NMB2141	hypothetical protein	34197.28	0.11	0.581
NMB1747	tspB protein	178.12	-0.17	0.585
NMB0015	6-phosphogluconate dehydrogenase	8919.79	0.10	0.587
NMB0580	protein disulfide isomerase NosL	1988.85	0.14	0.587
NMB1297	membrane-bound lytic murein transglycosylase D	7622.52	0.18	0.587

NMB0468	arginine decarboxylase	11369.15	0.14	0.588
NMB1518	acetate kinase	39451.22	0.15	0.588
NMB0967	anthranilate phosphoribosyltransferase	2297.85	0.11	0.589
NMB1817	riboflavin biosynthesis protein RibD	1058.92	0.11	0.591
NMB1874	orotate phosphoribosyltransferase	3614.91	-0.15	0.591
NMB0336	enoyl-(acyl carrier protein) reductase	11571.59	0.16	0.591
NMB1905	ribonuclease P	1974.12	-0.11	0.593
NMB0839	metalloprotease PmbA	2640.58	0.10	0.595
NMB2090	phosphoheptose isomerase	5254.00	0.12	0.595
NMB1386	transposase	419.27	-0.12	0.596
NMB1508	hypothetical protein	2201.76	0.15	0.598
NMB1425	lysyl-tRNA synthetase	8276.56	0.15	0.598
NMB0362	hypothetical protein	112.75	0.24	0.598
NMB1236	hypothetical protein	1844.84	0.12	0.598
NMB0842	single-stranded-DNA-specific exonuclease RecJ	2836.03	-0.11	0.606
NMB0525	7-cyano-7-deazaguanine synthase QueC	627.74	0.18	0.606
NMB1776	hypothetical protein	28.27	-0.40	0.606
NMB1061	hypothetical protein	1105.62	-0.13	0.607
NMB1012	hypothetical protein	11.28	0.52	0.607
NMB0760	diaminopimelate epimerase	2324.36	0.09	0.609
NMB1735	GTP pyrophosphokinase	5604.48	0.16	0.609
NMB1501	hypothetical protein	1467.89	0.11	0.610
NMB0493	hemagglutinin/hemolysin-like protein	6537.41	0.15	0.610
NMB0927	proline iminopeptidase	260.01	0.21	0.610
NMB0533	endonuclease III	948.30	-0.11	0.611
NMB1913	glycerol-3-phosphate acyltransferase PlsX	2849.70	0.18	0.613
NMB2087	hypothetical protein	541.65	0.17	0.613
NMB1748	hypothetical protein	115.08	-0.18	0.614

NMB1978	frataxin-like protein	888.07	-0.14	0.614
NMB2129	argininosuccinate synthase	22508.73	-0.13	0.614
NMB1734	glutaredoxin	3880.56	0.10	0.614
NMB0536	Na+/H+ antiporter	3205.16	0.17	0.614
NMB2018	hypothetical protein	830.10	0.19	0.614
NMB0211	L-serine dehydratase	1311.97	0.20	0.614
NMB0838	cold-shock protein CspA	47600.78	0.25	0.614
NMB1217	lipoate-protein ligase B	1927.19	-0.13	0.619
NMB1672	hypothetical protein	1196.94	-0.10	0.619
NMB2082	exodeoxyribonuclease	2491.34	-0.09	0.619
NMB0630	imidazole glycerol phosphate synthase subunit HisH	2506.93	0.09	0.619
NMB1729	biopolymer transport protein	5788.76	0.23	0.619
NMB1915	hypothetical protein	270.98	-0.16	0.623
NMB1031	3-isopropylmalate dehydrogenase	9882.37	-0.16	0.625
NMB1947	ABC transporter permease	561.62	-0.12	0.625
NMB1821	pilin glycosylation protein	3014.57	-0.09	0.627
NMB2120	hypothetical protein	327.34	-0.18	0.631
NMB1598	hypothetical protein	205.38	-0.26	0.633
NMB1938	ATP synthase F0F1 subunit B	32337.47	-0.18	0.633
NMB0541	hypothetical protein	324.30	0.14	0.633
NMB1352	hypothetical protein	448.79	-0.18	0.634
NMB1595	alanyl-tRNA synthetase	15464.80	0.12	0.634
NMB0919	IS1106 transposase	260.39	-0.15	0.634
NMB1766	hypothetical protein	353.82	-0.14	0.634
NMB0678	tryptophan synthase subunit alpha	8160.11	-0.12	0.634
NMB1370	hypothetical protein	171.25	0.31	0.634
NMB0408	undecaprenyl pyrophosphate phosphatase	3716.29	-0.13	0.637
NMB2025	hypothetical protein	2425.77	-0.09	0.637

NMB0286	hypothetical protein	2779.91	-0.09	0.637
NMB1910	hypothetical protein	14509.26	0.17	0.637
NMB0137	30S ribosomal protein S7	34570.67	0.18	0.637
NMB1492	hypothetical protein	14.76	0.42	0.637
NMB2105	mafB protein	2273.11	-0.10	0.637
NMB1393	phosphogluconate dehydratase	20461.74	-0.18	0.640
NMB0950	succinate dehydrogenase flavoprotein subunit	20205.94	-0.24	0.640
NMB1779	hemagglutinin/hemolysin-like protein	837.19	-0.15	0.640
NMB0153	50S ribosomal protein L24	17530.17	0.17	0.640
NMB1750	pilin gene inverting protein PivNM-2	262.89	-0.22	0.641
NMB2125	hypothetical protein	345.82	-0.13	0.641
NMB0538	hypothetical protein	1304.05	-0.10	0.646
NMB1829	TonB-dependent receptor	602.05	0.10	0.646
NMB0441	nitrilase	1423.78	0.17	0.646
NMB1388	glucose-6-phosphate isomerase	14058.60	-0.30	0.646
NMB1057	gamma-glutamyltranspeptidase	24079.83	-0.16	0.646
NMB0940	homoserine O-acetyltransferase	4007.07	0.18	0.646
NMB1177	acetyl-CoA carboxylase carboxyl transferase subunit alpha	16.84	-0.90	0.648
NMB2151	phosphoribosylamine--glycine ligase	3318.96	-0.11	0.648
NMB0706	hypothetical protein	1575.23	-0.10	0.652
NMB1159	ADP-dependent (S)-NAD(P)H-hydrate dehydratase	13.97	-0.43	0.653
NMB0700	IgA-specific serine endopeptidase	89015.65	-0.12	0.654
NMB1522	FKBP-type peptidylprolyl isomerase	17696.13	0.07	0.657
NMB0704	ribosomal large subunit pseudouridine synthase D	3384.91	0.08	0.658
NMB0555	hypothetical protein	392.99	-0.15	0.658
NMB0150	50S ribosomal protein L29	14699.38	0.26	0.658
NMB1702	3-ketoacyl-ACP reductase	1964.92	-0.14	0.659
NMB0565	Na(+)-translocating NADH-quinone reductase subunit E	5551.46	-0.22	0.661

NMB1859	S-adenosylmethionine--tRNA ribosyltransferase-isomerase	4461.34	-0.09	0.663
NMB0488	hypothetical protein	1659.12	0.09	0.666
NMB0694	protein FolI	1860.55	0.12	0.669
NMB1860	acetyl-CoA carboxylase biotin carboxyl carrier protein subunit	8039.67	0.17	0.669
NMB1098	hypothetical protein	24.89	0.37	0.670
NMB0148	30S ribosomal protein S3	67047.36	0.24	0.674
NMB1530	succinyl-diaminopimelate desuccinylase	3410.59	-0.12	0.675
NMB0567	Na(+)-translocating NADH-quinone reductase subunit C	7881.83	-0.19	0.676
NMB1285	phosphopyruvate hydratase	74522.97	0.19	0.676
NMB0804	NAD(P)H nitroreductase	5394.76	-0.08	0.676
NMB0499	hypothetical protein	49.97	-0.23	0.676
NMB1120	hypothetical protein	49.76	0.24	0.676
NMB0609	30S ribosomal protein S15	19964.68	-0.16	0.677
NMB0562	hypothetical protein	397.96	0.16	0.677
NMB0850	hypothetical protein	837.88	-0.16	0.678
NMB0723	50S ribosomal protein L20	24331.09	0.11	0.678
NMB0080	glucose-1-phosphate thymidylyltransferase	19.74	-0.32	0.679
NMB1937	ATP synthase F0F1 subunit delta	31316.77	-0.15	0.680
NMB1040	hypothetical protein	2219.26	-0.08	0.681
NMB0429	hypothetical protein	284.55	-0.66	0.682
NMB1639	hypothetical protein	737.81	0.16	0.682
NMB2118	hypothetical protein	195.51	-0.17	0.682
NMB1242	hypothetical protein	395.73	-0.13	0.682
NMB0904	hypothetical protein	39.10	-0.28	0.684
NMB0614	oxidoreductase	3437.40	-0.10	0.684
NMB1894	leucyl-tRNA synthetase	63.68	0.24	0.684
NMB1130	phytoene synthase	28.92	0.37	0.686
NMB1017	sulfate ABC transporter substrate-binding protein	1802.85	-0.36	0.687

NMB0179	(3R)-hydroxymyristoyl-ACP dehydratase	2122.38	0.08	0.689
NMB0146	30S ribosomal protein S19	19718.44	0.22	0.690
NMB1221	hypothetical protein	1468.29	0.10	0.690
NMB1380	scaffold protein	3732.30	0.23	0.692
NMB1477	hypothetical protein	56.48	0.24	0.692
NMB1427	hypothetical protein	29.49	0.30	0.692
NMB0955	2-oxoglutarate dehydrogenase subunit E1	48694.38	-0.10	0.694
NMB1940	ATP synthase F0F1 subunit A	13315.52	-0.14	0.695
NMB0278	thiol:disulfide interchange protein DsbA	5862.54	-0.13	0.695
NMB1880	ABC transporter substrate-binding protein	40.58	-0.26	0.695
NMB0737	HPr kinase/phosphorylase	5941.08	-0.14	0.695
NMB1511	ribose-5-phosphate isomerase A	3047.92	0.10	0.695
NMB1689	dedA protein	4850.79	0.11	0.695
NMB1925	16S rRNA (uracil(1498)-N(3))-methyltransferase	512.35	0.13	0.695
NMB0168	DNA-directed RNA polymerase subunit alpha	76071.61	0.17	0.695
NMB0149	50S ribosomal protein L16	43810.78	0.23	0.695
NMB0032	hypothetical protein	174.13	0.20	0.696
NMB0761	hypothetical protein	1013.35	-0.10	0.698
NMB1089	hypothetical protein	13.98	-0.35	0.702
NMB1414	outer membrane protein FrpC	140.27	-0.21	0.702
NMB0291	hypothetical protein	849.66	-0.16	0.702
NMB2085	hypothetical protein	7568.84	-0.10	0.702
NMB0427	cell division protein FtsZ	56972.18	-0.08	0.702
NMB0318	fatty acid efflux system protein	1962.09	0.19	0.702
NMB0174	valyl-tRNA synthetase	21552.58	-0.10	0.702
NMB1651	alanine racemase	6741.97	-0.12	0.703
NMB0889	hypothetical protein	4179.65	0.10	0.704
NMB1158	nickel-dependent hydrogenase b-type cytochrome subunit	49.68	-0.26	0.705

NMB0637	argininosuccinate lyase	13435.64	-0.06	0.705
NMB1333	hypothetical protein	13399.02	0.08	0.705
NMB1674	GDSL lipase	1140.42	0.09	0.705
NMB0739	hypothetical protein	2164.49	0.09	0.705
NMB2067	hydroxymethylpyrimidine transporter CytX	1114.54	0.14	0.705
NMB0570	hypothetical protein	4396.51	0.15	0.705
NMB0151	30S ribosomal protein S17	28842.74	0.24	0.705
NMB1133	hypothetical protein	12.74	0.47	0.705
NMB1135a	amino acid permease	29.42	-0.26	0.706
NMB0963	phosphatidylserine decarboxylase	7407.76	0.09	0.708
NMB2012	transcriptional regulator	1981.81	-0.12	0.709
NMB0917	death-on-curing protein	1386.72	-0.10	0.709
NMB1247	riboflavin synthase subunit alpha	1649.68	-0.10	0.709
NMB1430	transcription elongation factor GreA	3737.69	0.08	0.709
NMB2006	chloride channel protein	2683.00	0.08	0.710
NMB0498	hypothetical protein	550.44	0.09	0.711
NMB0894	succinyldiaminopimelate transaminase	6587.83	0.08	0.713
NMB0777	uroporphyrinogen-III synthase	1742.42	0.08	0.718
NMB1539	IS1106 transposase	290.59	-0.11	0.719
NMB1584	3-hydroxyacid dehydrogenase	16737.52	-0.17	0.720
NMB0808	hypothetical protein	1344.05	-0.07	0.720
NMB1321	30S ribosomal protein S18	9283.89	0.14	0.720
NMB0176	D-amino acid dehydrogenase small subunit	1186.64	-0.15	0.720
NMB0828	ADP-L-glycero-D-manno-heptose-6-epimerase	3673.22	-0.14	0.720
NMB0380	Crp/FNR family transcriptional regulator	3308.97	-0.06	0.720
NMB0191	ParA family protein	2521.23	0.07	0.720
NMB0571	hypothetical protein	389.46	0.09	0.720
NMB1516	hypothetical protein	232.34	0.12	0.720

NMB2042	ABC transporter ATP-binding protein	1402.71	0.13	0.720
NMB1892	hypothetical protein	367.83	0.13	0.720
NMB1268	hypothetical protein	2211.43	-0.07	0.720
NMB1296	hypothetical protein	1003.52	-0.12	0.723
NMB0167	30S ribosomal protein S4	52388.01	0.13	0.723
NMB0640	hypothetical protein	1661.55	-0.10	0.725
NMB1364	NH(3)-dependent NAD synthetase	1488.85	0.14	0.725
NMB1608	hypothetical protein	634.50	0.18	0.725
NMB0995	hypothetical protein	4682.10	-0.51	0.725
NMB0472	8-amino-7-oxononanoate synthase	1892.82	-0.09	0.726
NMB2088	hypothetical protein	2675.05	-0.12	0.726
NMB1864	glutamate-1-semialdehyde aminotransferase	11732.77	-0.12	0.726
NMB0215	hypothetical protein	149.64	0.15	0.726
NMB0319	fatty acid efflux system protein	2100.05	0.20	0.726
NMB2041	thiamin pyrophosphokinase-like protein	2399.71	0.11	0.727
NMB0924	short chain dehydrogenase/reductase oxidoreductase	3231.39	0.14	0.729
NMB0905	hypothetical protein	29.37	-0.24	0.729
NMB1564	hypothetical protein	4061.81	-0.10	0.729
NMB1585	MarR family transcriptional regulator	134.11	0.13	0.729
NMB0147	50S ribosomal protein L22	28748.64	0.21	0.729
NMB1557	hypothetical protein	20279.66	-0.21	0.730
NMB1464	hypothetical protein	1083.90	-0.09	0.730
NMB1436	hypothetical protein	16484.00	-0.07	0.730
NMB0042	hypothetical protein	5252.69	0.11	0.730
NMB0310	hypothetical protein	1569.80	0.08	0.731
NMB1936	ATP synthase F0F1 subunit alpha	72428.41	0.13	0.731
NMB1455	hypothetical protein	51.21	-0.31	0.731
NMB1139	acetyl-CoA carboxylase carboxyl transferase subunit alpha	134.75	-0.14	0.731

NMB1681	hypothetical protein	5382.97	0.12	0.731
NMB1882	TonB-dependent receptor	42.94	0.21	0.732
NMB1309	fimbrial biogenesis and twitching motility protein	4097.38	0.07	0.732
NMB0644	hypothetical protein	1531.74	-0.09	0.733
NMB0171	septum site-determining protein	17130.59	-0.07	0.735
NMB0885	replicative DNA helicase	6393.24	-0.15	0.737
NMB1602	transposase	75.50	0.19	0.739
NMB1619	hypothetical protein	1106.49	0.09	0.739
NMB0276	hypothetical protein	1202.25	0.07	0.743
NMB0564	Na(+)-translocating NADH-quinone reductase subunit F	13322.35	-0.16	0.744
NMB1320	50S ribosomal protein L9	18780.67	0.12	0.749
NMB0177	sodium/alanine symporter	3049.62	-0.17	0.751
NMB1643	translation initiation factor IF-2	20918.37	0.13	0.751
NMB1368	ATP-dependent RNA helicase	7178.66	0.45	0.751
NMB1971	hypothetical protein	195.67	-0.14	0.751
NMB2070	thiamine biosynthesis protein ThiS	121.08	0.13	0.751
NMB0750	bacterioferritin comigratory protein	2154.23	0.12	0.752
NMB1579	ATP phosphoribosyltransferase	10155.51	-0.14	0.754
NMB0897	hypothetical protein	2644.26	-0.08	0.754
NMB0806	hypothetical protein	1720.96	0.06	0.754
NMB1252	phosphoribosylaminoimidazole synthetase	14103.89	0.10	0.754
NMB1866	(dimethylallyl)adenosine tRNA methylthiotransferase	1406.99	0.07	0.754
NMB0142	50S ribosomal protein L3	49085.06	0.12	0.754
NMB0991	IS1106 transposase	36.60	0.22	0.754
NMB0340	lactoylglutathione lyase	32616.96	0.08	0.756
NMB2027	gluconate permease	2526.07	-0.07	0.759
NMB0958	hypothetical protein	2894.77	-0.06	0.759
NMB1616	phosphomethylpyrimidine kinase	5053.32	-0.14	0.759

NMB1229	hypothetical protein	1042.33	0.14	0.759
NMB1529	hypothetical protein	255.65	0.10	0.760
NMB1486	hypothetical protein	301.26	0.14	0.760
NMB1381	iron-sulfur cluster assembly protein IscA	3554.82	0.17	0.761
NMB1781	hypothetical protein	77.01	-0.18	0.762
NMB1366	thioredoxin I	7949.90	-0.14	0.764
NMB0317	7-cyano-7-deazaguanine reductase	553.53	-0.09	0.764
NMB2134	hypothetical protein	8003.04	0.08	0.764
NMB1046	threonine synthase	10497.84	0.06	0.764
NMB1828	hypothetical protein	109.08	-0.15	0.764
NMB2015	hypothetical protein	102.97	-0.20	0.765
NMB1754	cryptic plasmid protein A-like protein	60.86	-0.16	0.765
NMB0133	DNA-directed RNA polymerase subunit beta'	96777.84	-0.10	0.765
NMB0994	acyl-CoA dehydrogenase	2732.61	-0.49	0.766
NMB0132	DNA-directed RNA polymerase subunit beta	86983.15	-0.09	0.766
NMB1256	3%2C4-dihydroxy-2-butanone-4-phosphate synthase	1658.48	0.10	0.766
NMB1683	dinucleoside polyphosphate hydrolase	5089.99	-0.11	0.766
NMB0832	anticodon nuclease	571.74	-0.09	0.770
NMB0881	sulfate ABC transporter permease	1076.05	0.49	0.770
NMB1141	RNA methyltransferase	15.83	-0.26	0.770
NMB0175	zinc transporter ZupT	137.38	-0.30	0.771
NMB0483	hypothetical protein	12.24	-0.36	0.772
NMB1873	DNA polymerase	1828.51	-0.08	0.772
NMB0451	hypothetical protein	1290.36	-0.08	0.772
NMB0641	inorganic pyrophosphatase	20586.87	0.09	0.773
NMB0145	50S ribosomal protein L2	63551.54	0.16	0.774
NMB0544	UDP-2%2C3-diacylglucosamine hydrolase	482.59	0.07	0.778
NMB0951	succinate dehydrogenase iron-sulfur subunit	6646.89	-0.11	0.778

NMB1404	hypothetical protein	255.48	-0.10	0.778
NMB0463	30S ribosomal protein S20	20521.08	-0.10	0.778
NMB0428	hypothetical protein	12705.05	0.06	0.778
NMB2023	hypothetical protein	6062.50	-0.06	0.778
NMB1147	hypothetical protein	28.19	-0.25	0.778
NMB0557	iron-sulfur cluster insertion protein ErpA	8365.74	-0.08	0.778
NMB1981	S-ribosylhomocysteinase	5535.21	0.06	0.778
NMB0681	hypothetical protein	1015.17	0.08	0.778
NMB1286	hypothetical protein	14196.17	0.13	0.780
NMB0938	hypothetical protein	2075.07	-0.09	0.784
NMB0639	deoxyribonucleotide triphosphate pyrophosphatase	3304.55	-0.09	0.787
NMB0425	cell division protein	2765.41	-0.07	0.787
NMB1825	hypothetical protein	723.08	0.06	0.787
NMB0127	50S ribosomal protein L11	38630.08	0.10	0.787
NMB1526	SsrA-binding protein	3334.45	-0.05	0.788
NMB1471	tryptophanyl-tRNA synthetase	3658.92	0.06	0.789
NMB0812	multidrug efflux protein	1122.49	0.06	0.791
NMB2031	amino-acid transport protein	539.30	-0.11	0.793
NMB1003	hypothetical protein	20.08	0.27	0.793
NMB1731	hypothetical protein	2415.72	0.07	0.794
NMB0397	hypothetical protein	29.15	0.20	0.794
NMB0364	outer membrane protein FrpC	447.44	-0.09	0.795
NMB0528	hypothetical protein	509.79	-0.07	0.795
NMB0474	biotin synthesis protein BioC	763.33	-0.07	0.795
NMB1556	tRNA-specific 2-thiouridylase MnmA	4062.09	-0.07	0.795
NMB1930	glycyl-tRNA synthetase subunit beta	11996.54	0.06	0.795
NMB0680	hypothetical protein	671.29	0.08	0.795
NMB1115	tail fiber protein	13.75	0.25	0.795

NMB0659	hypothetical protein	19.78	0.27	0.795
NMB1664	protease	4065.93	0.05	0.797
NMB0395	hypothetical protein	2073.28	-0.10	0.797
NMB0608	preprotein translocase subunit SecF	9418.05	0.07	0.798
NMB1350	hypothetical protein	586.00	-0.10	0.798
NMB0748	RNA-binding protein Hfq	11769.29	0.05	0.798
NMB0136	30S ribosomal protein S12	27808.53	0.08	0.798
NMB2146	hypothetical protein	29.95	0.18	0.801
NMB0500	hypothetical protein	12.92	0.26	0.801
NMB0196	ribonuclease E	11701.40	0.08	0.803
NMB0962	excinuclease ABC subunit A	3447.68	0.08	0.804
NMB0856	hypothetical protein	208.28	0.11	0.804
NMB1150	dihydroxy-acid dehydratase	457.43	-0.12	0.805
NMB1418	lipid A biosynthesis lauroyl acyltransferase	586.64	-0.09	0.805
NMB2021	hypothetical protein	6586.76	-0.09	0.806
NMB0006	thioredoxin-like protein	8136.54	-0.10	0.806
NMB1049	transcriptional regulator	891.44	-0.09	0.806
NMB0125	preprotein translocase subunit SecE	3823.91	-0.07	0.806
NMB0749	penicillin-binding protein 4	1470.34	-0.07	0.806
NMB0775	hypothetical protein	4935.45	0.07	0.806
NMB1287	ferredoxin	35.53	0.17	0.806
NMB0363	hypothetical protein	49.15	-0.19	0.806
NMB1065	camphor resistance protein CrcB	268.96	-0.09	0.806
NMB0575	glycine cleavage system protein H	13067.64	0.08	0.806
NMB1036	3-isopropylmalate dehydratase large subunit	3537.79	0.06	0.808
NMB0143	50S ribosomal protein L4	47212.68	0.11	0.808
NMB1970	para-aminobenzoate synthetase component I/4-amino-4-deoxychorismate lyase	2701.24	-0.10	0.813
NMB2036	tRNA pseudouridine synthase A	763.24	-0.07	0.813

NMB0585	iron-regulated protein FrpA	1398.53	0.07	0.813
NMB0892	AzlC-like protein	345.79	0.08	0.813
NMB0162	preprotein translocase subunit SecY	30750.85	0.12	0.813
NMB0044	trifunctional thioredoxin/methionine sulfoxide reductase A/B protein	2594.10	0.11	0.814
NMB0433	aconitate hydratase	2117.44	-0.07	0.814
NMB0888	hypothetical protein	8107.67	0.05	0.814
NMB0695	hypothetical protein	2834.04	-0.06	0.814
NMB1227	hypothetical protein	803.27	0.05	0.816
NMB1606	sensor histidine kinase	1005.10	0.09	0.816
NMB1128	hypothetical protein	45.93	-0.19	0.817
NMB1871	hypothetical protein	5006.76	-0.06	0.817
NMB0351	transaldolase	9239.51	0.04	0.817
NMB1736	hypothetical protein	244.91	0.08	0.817
NMB1709	thymidylate synthase	1996.74	-0.08	0.821
NMB0205	ferric uptake regulation protein	4397.38	-0.06	0.821
NMB1210	toxin-activating protein	932.08	-0.06	0.821
NMB2064	hypothetical protein	9138.85	0.08	0.821
NMB0620	phosphoglycolate phosphatase	755.45	0.10	0.821
NMB0460	transferrin-binding protein B	1058.54	-0.11	0.821
NMB1872	ribosomal-protein-alanine acetyltransferase	938.90	0.08	0.821
NMB0324	50S ribosomal protein L27	20682.70	0.08	0.821
NMB0982	chloride channel protein	712.38	0.09	0.821
NMB0144	50S ribosomal protein L23	17271.05	0.12	0.821
NMB0720	threonyl-tRNA synthetase	14125.08	-0.04	0.827
NMB1819	hypothetical protein	79.29	0.15	0.830
NMB0957	dihydrolipoamide dehydrogenase	21019.82	-0.06	0.830
NMB0592	30S ribosomal protein S16	11501.26	0.05	0.830
NMB1916	3-oxoacyl-ACP synthase III	9379.45	-0.08	0.832

NMB0321	50S ribosomal protein L28	25577.60	0.08	0.832
NMB1156	siroheme synthase	35.66	-0.25	0.833
NMB1762	hemolysin activation protein HecB	897.56	-0.05	0.833
NMB1678	aromatic amino acid aminotransferase	8096.16	-0.05	0.833
NMB0711	hypothetical protein	9306.15	0.07	0.833
NMB1453	hypothetical protein	1755.71	-0.06	0.834
NMB0699	tryptophan synthase subunit beta	10174.80	-0.06	0.834
NMB0968	hypothetical protein	152.34	0.13	0.834
NMB1481	hypothetical protein	562.66	-0.10	0.834
NMB2102	elongation factor Ts	50862.54	-0.09	0.835
NMB0713	apolipoprotein N-acyltransferase	366.00	-0.06	0.835
NMB0980	NAD(P) transhydrogenase subunit alpha	12062.26	-0.05	0.835
NMB0280	organic solvent tolerance protein	13441.27	0.08	0.837
NMB0459	hypothetical protein	84.59	-0.09	0.837
NMB1517	hypothetical protein	846.87	0.06	0.837
NMB1993	iron(III) ABC transporter ATP-binding protein	93.19	0.09	0.839
NMB1396	A/G-specific adenine glycosylase	237.13	0.09	0.839
NMB1559	glutathione synthetase	14158.28	0.09	0.839
NMB0034	hypothetical protein	1065.42	-0.07	0.840
NMB1387	hypothetical protein	5291.78	-0.09	0.841
NMB0876	50S ribosomal protein L25/general stress protein Ctc	27642.26	0.09	0.841
NMB1607	sigma-54 dependent response regulator	721.37	-0.10	0.843
NMB0573	AsnC family transcriptional regulator	4323.29	-0.05	0.843
NMB1973	co-chaperonin GroES	13545.30	0.05	0.843
NMB0795	peptidyl-tRNA hydrolase	1283.68	0.06	0.843
NMB0634	iron ABC transporter substrate-binding protein	16202.85	0.07	0.843
NMB0239	hypothetical protein	933.28	-0.08	0.845
NMB1818	lipopolysaccharide biosynthesis protein	1182.24	0.04	0.847

NMB0722	50S ribosomal protein L35	24065.59	0.06	0.847
NMB2058	hypothetical protein	16473.63	-0.08	0.847
NMB1527	ADP-heptose--LPS heptosyltransferase	2844.12	0.04	0.847
NMB0814	ATP phosphoribosyltransferase	12201.29	0.06	0.847
NMB0207	glyceraldehyde-3-phosphate dehydrogenase	2563.01	0.06	0.847
NMB0169	50S ribosomal protein L17	29573.71	0.10	0.847
NMB1134	2Fe-2S ferredoxin	20.82	0.20	0.847
NMB1928	lacto-N-neotetraose biosynthesis glycosyl transferase LgtB	2736.98	0.04	0.848
NMB0396	nicotinate-nucleotide pyrophosphorylase	2297.71	0.12	0.848
NMB1906	hypothetical protein	709.19	0.05	0.849
NMB1081	bacteriophage transposase	204.32	-0.08	0.852
NMB1092	hypothetical protein	16.87	0.22	0.852
NMB0857	hypothetical protein	45.47	0.17	0.854
NMB0531	hypothetical protein	4480.90	0.06	0.857
NMB0757	phosphoribosylaminoimidazole-succinocarboxamide synthase	5697.87	-0.07	0.857
NMB0836	ATP-dependent Clp protease ATP-binding subunit ClpA	8289.36	-0.10	0.860
NMB0440	prephenate dehydrogenase	763.21	0.04	0.860
NMB1649	disulfide bond formation protein B	2685.03	0.04	0.861
NMB1009	hypothetical protein	196.02	0.07	0.862
NMB0754	hypothetical protein	16.08	-0.17	0.862
NMB1911	50S ribosomal protein L32	8949.11	-0.06	0.862
NMB1440	hypothetical protein	1268.50	-0.04	0.862
NMB2103	uridylate kinase	4985.08	0.05	0.862
NMB0527	6-pyruvoyl tetrahydrobiopterin synthase	484.69	0.05	0.862
NMB1914	hypothetical protein	504.38	0.05	0.862
NMB0741	hypothetical protein	347.49	0.06	0.862
NMB1379	cysteine desulfurase	6728.55	0.10	0.862
NMB2011	hypothetical protein	1870.20	0.04	0.863

NMB2016	type IV pilin-like protein	1690.61	-0.04	0.863
NMB0887	type IV pilus assembly protein PilV	4199.25	-0.03	0.866
NMB0166	30S ribosomal protein S11	31525.12	0.05	0.866
NMB0874	4-diphosphocytidyl-2C-methyl-D-erythritol kinase	4646.47	0.06	0.866
NMB0568	Na(+)-translocating NADH-quinone reductase subunit B	10692.00	0.08	0.866
NMB0140	30S ribosomal protein S10	32050.28	0.09	0.866
NMB1802	DNA-binding/iron metalloprotein/AP endonuclease	3853.66	0.04	0.866
NMB0613	hypothetical protein	88.20	0.08	0.866
NMB1277	BCCT family transporter	4438.18	0.06	0.868
NMB0438	hypothetical protein	643.59	0.06	0.869
NMB1280	hypothetical protein	11401.50	-0.08	0.869
NMB1499	ribonuclease PH	5183.90	-0.08	0.869
NMB1378	hypothetical protein	909.56	0.11	0.869
NMB1955	cadmium resistance protein	2906.30	-0.07	0.869
NMB0139	elongation factor Tu	45900.14	0.10	0.872
NMB0732	adenosylmethionine--8-amino-7-oxononanoate aminotransferase BioA	1944.42	0.04	0.872
NMB0922	alpha-2%2C3-sialyltransferase	1369.32	0.06	0.872
NMB0389	aldose 1-epimerase	11944.38	0.08	0.872
NMB1146	biotin synthetase	15.56	-0.17	0.875
NMB2060	NAD(P)H-dependent glycerol-3-phosphate dehydrogenase	9815.70	0.03	0.875
NMB0622	outer membrane lipoprotein carrier protein	1418.94	0.06	0.875
NMB1390	glucokinase	26288.72	-0.11	0.876
NMB1580	hypothetical protein	11100.56	-0.09	0.876
NMB0735	4-hydroxybenzoate octaprenyltransferase	1677.14	0.05	0.876
NMB1673	DNA-3-methyladenine glycosylase I	645.01	0.05	0.876
NMB0534	hypothetical protein	82.88	0.07	0.876
NMB1667	hypothetical protein	12.62	-0.18	0.877
NMB0586	adhesin	11186.71	-0.13	0.877

NMB1002	hypothetical protein	51.12	-0.10	0.877
NMB0329	type IV pilus assembly protein	18227.15	0.04	0.877
NMB0529	hypothetical protein	668.10	0.04	0.877
NMB0038	UDP-N-acetylglucosamine pyrophosphorylase	3910.36	0.05	0.877
NMB2005	bifunctional ornithine acetyltransferase/N-acetylglutamate synthase	5451.83	0.06	0.877
NMB1458	fumarate hydratase	1491.02	-0.03	0.877
NMB0237	hypothetical protein	115.18	-0.10	0.878
NMB0793	hypothetical protein	16.81	0.18	0.878
NMB1389	DNA-binding transcriptional regulator	13559.20	-0.09	0.879
NMB1570	hypothetical protein	1590.82	-0.03	0.880
NMB2138	peptide chain release factor 2	6538.42	-0.03	0.881
NMB2063	hypothetical protein	1753.09	0.04	0.881
NMB0218	glycosyltransferase	1545.67	-0.04	0.884
NMB1005	hypothetical protein	26.75	0.12	0.884
NMB0697	dimethyladenosine transferase	7097.71	-0.03	0.884
NMB1149	hypothetical protein	38.09	-0.13	0.885
NMB0213	hypothetical protein	797.39	0.05	0.885
NMB0673	hypothetical protein	1451.73	-0.04	0.885
NMB0729	integration host factor subunit alpha	9976.98	-0.04	0.885
NMB0908	hypothetical protein	22.99	-0.15	0.886
NMB1395	alcohol dehydrogenase	9853.68	-0.12	0.887
NMB0615	ammonium transporter AmtB	1626.52	-0.35	0.889
NMB1365	hypothetical protein	5106.07	-0.19	0.889
NMB0733	dithiobiotin synthetase	970.81	-0.04	0.889
NMB1593	hypothetical protein	1931.93	0.03	0.890
NMB0612	spermidine/putrescine ABC transporter permease	4400.22	0.05	0.890
NMB0152	50S ribosomal protein L14	26044.00	0.05	0.891
NMB0007	ABC transporter ATP-binding protein	4924.19	-0.04	0.893

NMB1392	glucose-6-phosphate 1-dehydrogenase	27797.78	0.07	0.894
NMB0161	50S ribosomal protein L15	22039.99	-0.08	0.894
NMB1076	DnaA regulatory inactivator Hda	1806.68	-0.04	0.894
NMB1707	sodium- and chloride-dependent transporter	1573.77	0.05	0.894
NMB0784	phage shock protein E	1409.45	-0.04	0.896
NMB1094	hypothetical protein	91.32	-0.05	0.898
NMB0717	cytochrome	3391.10	0.07	0.899
NMB1042	cation transporter E1-E2 family ATPase	2111.17	-0.03	0.899
NMB1975	sodium- and chloride-dependent transporter	3315.72	-0.07	0.900
NMB0165	30S ribosomal protein S13	36872.79	-0.05	0.900
NMB1271	mercury transport periplasmic protein	1885.81	-0.03	0.901
NMB1504	segregation and condensation protein A	964.58	-0.03	0.901
NMB0574	glycine cleavage system aminomethyltransferase T	10825.04	-0.06	0.902
NMB0543	L-lactate permease	13464.97	-0.08	0.904
NMB1482	acyl-CoA thioesterase	2480.55	-0.03	0.904
NMB1972	molecular chaperone GroEL	102694.12	-0.02	0.904
NMB1772	hypothetical protein	143.08	0.05	0.904
NMB0334	glucose-6-phosphate isomerase	2038.07	0.07	0.904
NMB1016	hypothetical protein	926.81	-0.04	0.905
NMB1348	RNA methylase	1656.84	0.03	0.908
NMB0029	glycerate dehydrogenase	5380.66	-0.04	0.908
NMB0537	hypothetical protein	3684.16	-0.02	0.911
NMB2000	Hsp33-like chaperonin	6029.74	-0.05	0.911
NMB1317	hypothetical protein	14.45	0.12	0.912
NMB0628	imidazole glycerol phosphate synthase subunit HisF	2305.72	-0.03	0.913
NMB0642	dATP pyrophosphohydrolase	510.07	0.03	0.913
NMB0159	30S ribosomal protein S5	30313.70	-0.06	0.916
NMB1629	hypothetical protein	184.29	-0.06	0.916

NMB0591	16S rRNA-processing protein RimM	18392.31	0.02	0.916
NMB1335	hypothetical protein	2678.84	0.05	0.916
NMB0943	5%2C10-methylenetetrahydrofolate reductase	3737.01	0.03	0.917
NMB0926	opacity protein	1178.31	0.04	0.917
NMB2150	hypothetical protein	375.00	0.05	0.917
NMB1140	tRNA(Ile)-lysidine synthetase	11.44	0.11	0.917
NMB1071	hypothetical protein	759.54	0.04	0.919
NMB1611	hypothetical protein	1597.34	0.03	0.921
NMB2061	phosphoenolpyruvate carboxylase	15557.76	0.04	0.921
NMB0823	adenylate kinase	8023.29	0.03	0.921
NMB0426	cell division protein	11831.96	-0.02	0.921
NMB1788	ATP-dependent DNA helicase RecG	3564.89	0.02	0.921
NMB1822	pilin glycosylation protein	1852.04	-0.02	0.923
NMB1010	hypothetical protein	558.83	0.04	0.923
NMB1935	ATP synthase F0F1 subunit gamma	34251.45	-0.05	0.923
NMB0354	hypothetical protein	2564.60	0.03	0.923
NMB0163	translation initiation factor IF-1	6186.54	0.05	0.923
NMB1450	ferredoxin--NADP reductase	1548.53	-0.04	0.925
NMB0791	peptidyl-prolyl cis-trans isomerase	19210.71	0.04	0.925
NMB1445	recombinase A	14784.33	-0.02	0.925
NMB1791	cytoplasmic axial filament protein	5890.16	-0.04	0.926
NMB0138	elongation factor G	184079.84	0.04	0.927
NMB0755	hypothetical protein	24.89	-0.10	0.928
NMB0079	dTDP-D-glucose 4%2C6-dehydratase	28.77	-0.08	0.928
NMB0102	hypothetical protein	3270.78	-0.04	0.928
NMB0221	dihydroorotate dehydrogenase 2	4490.32	-0.03	0.928
NMB0183	hypothetical protein	4895.41	-0.02	0.928
NMB1351	hypothetical protein	4006.60	0.02	0.928

NMB0322	50S ribosomal protein L33	6547.72	0.04	0.928
NMB0966	anthranilate synthase component II	1820.35	-0.02	0.928
NMB1771	hypothetical protein	100.38	-0.05	0.930
NMB1956	50S ribosomal protein L31	13159.22	-0.03	0.930
NMB0339	hypothetical protein	5948.23	0.02	0.930
NMB0113	hypothetical protein	1616.60	0.02	0.930
NMB2152	hypothetical protein	536.21	0.02	0.930
NMB1648	hypothetical protein	6328.45	0.03	0.930
NMB0031	glucosamine--fructose-6-phosphate aminotransferase	7239.42	0.04	0.930
NMB1605	DNA topoisomerase IV subunit A	4127.02	-0.04	0.932
NMB0629	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide isomerase	3502.15	0.02	0.932
NMB0004	hypothetical protein	3707.03	-0.05	0.934
NMB1067	cell division protein FtsK	14919.34	0.03	0.934
NMB1251	IS30 family transposase	108.16	0.04	0.935
NMB0225	IS30 family transposase	137.04	-0.05	0.939
NMB0861	hypothetical protein	1738.32	0.02	0.939
NMB1820	pilin glycosylation protein PglB	953.90	0.02	0.939
NMB1307	nucleoside diphosphate kinase	4913.73	0.01	0.940
NMB1502	hypothetical protein	306.92	-0.05	0.941
NMB0307	phospho-2-dehydro-3-deoxyheptonate aldolase	3923.93	-0.02	0.942
NMB1066	hypothetical protein	770.67	-0.02	0.942
NMB1521a	hypothetical protein	348.51	-0.02	0.942
NMB0068	polysialic acid capsule biosynthesis protein SiaC	11172.93	0.02	0.943
NMB1077	ABC transporter ATP-binding protein	29.77	-0.05	0.943
NMB2057	50S ribosomal protein L13	30500.79	-0.02	0.943
NMB0037	phosphonate metabolism protein PhnA	613.32	0.02	0.943
NMB1399	IS1106 transposase	55.13	-0.04	0.946
NMB0067	polysialic acid capsule biosynthesis protein SiaD	1485.65	-0.02	0.946

NMB0392	L-aspartate oxidase	1074.92	0.05	0.946
NMB1137	hypothetical protein	35.27	-0.07	0.946
NMB0765	signal peptidase I	3075.01	-0.02	0.946
NMB0867	pseudouridine synthase	525.49	-0.02	0.946
NMB1249	nitrate/nitrite sensory protein NarX	2929.40	0.02	0.946
NMB1548	T-cell stimulating protein TspB	679.26	0.02	0.946
NMB1628	tspB protein	253.99	0.03	0.946
NMB0410	cell division protein MraZ	4796.15	0.02	0.946
NMB1841	mannose-1-phosphate guanylyltransferase	2766.47	-0.02	0.950
NMB1298	16S rRNA pseudouridine(516) synthase	1339.52	-0.01	0.952
NMB0502	hypothetical protein	287.76	-0.03	0.954
NMB1349	hypothetical protein	1406.99	-0.01	0.954
NMB0769	DNA polymerase III subunit delta'	7715.59	-0.03	0.954
NMB2065	release factor glutamine methyltransferase	2298.13	-0.01	0.956
NMB1633	hypothetical protein	32.94	0.04	0.956
NMB0158	50S ribosomal protein L18	22028.71	-0.03	0.956
NMB1644	hypothetical protein	1547.37	0.02	0.962
NMB0556	repressor protein	767.92	-0.01	0.963
NMB0406	hypothetical protein	5412.82	0.01	0.963
NMB2079	aspartate-semialdehyde dehydrogenase	19466.11	0.02	0.963
NMB1258	recombination factor protein RarA	2710.33	-0.02	0.964
NMB1774	hypothetical protein	13.68	-0.05	0.966
NMB1814	3-dehydroquinate synthase	4506.51	-0.01	0.966
NMB0390	maltose phosphorylase	48839.36	-0.04	0.966
NMB1912	hypothetical protein	2621.57	-0.02	0.968
NMB1932	glycyl-tRNA synthetase subunit alpha	6163.82	-0.01	0.968
NMB1434	phopholipase D-family protein	472.42	-0.01	0.968
NMB0682	dihydroorotase	2862.04	0.01	0.970

NMB0224	[glutamate--ammonia-ligase] adenylyltransferase	3477.55	0.01	0.970
NMB1099	IS30 family transposase	83.59	-0.02	0.970
NMB0273	hypothetical protein	1333.97	-0.01	0.970
NMB1826	hypothetical protein	764.86	-0.01	0.971
NMB0692	cell division protein	4827.37	0.02	0.971
NMB0581	electron transfer flavoprotein-ubiquinone oxidoreductase	3702.73	0.02	0.971
NMB2149	hypothetical protein	84.41	-0.03	0.974
NMB0157	50S ribosomal protein L6	38816.68	-0.02	0.974
NMB0262	exodeoxyribonuclease VII small subunit	1316.64	-0.01	0.974
NMB0627	phosphoribosyl-AMP cyclohydrolase	835.85	-0.01	0.974
NMB1726	hypothetical protein	3040.03	0.02	0.974
NMB2046	PTS system transporter subunit IIAB	1685.05	-0.01	0.976
NMB0465	hypothetical protein	3350.00	-0.01	0.977
NMB0388	sugar transporter	26045.47	-0.02	0.977
NMB1424	hypothetical protein	4447.64	-0.02	0.977
NMB1610	hypothetical protein	3505.35	-0.01	0.978
NMB1470	hypothetical protein	1166.62	-0.01	0.978
NMB0716	hypothetical protein	30.06	-0.02	0.979
NMB1108	hypothetical protein	61.75	-0.02	0.979
NMB2124	hypothetical protein	41.83	0.02	0.979
NMB1980	hypothetical protein	852.29	0.02	0.981
NMB1479	recombination regulator RecX	930.09	-0.01	0.981
NMB1021	anthranilate synthase component I	2625.81	0.01	0.982
NMB1690	phosphoglucosamine mutase	6353.39	0.01	0.982
NMB1889	hypothetical protein	55.85	-0.02	0.983
NMB0190	16S rRNA methyltransferase GidB	1579.87	-0.01	0.983
NMB0691	colicin V production protein	1760.17	-0.01	0.983
NMB1483	lipoprotein NlpD	16256.51	0.00	0.983

NMB0071	capsule polysaccharide export outer membrane protein	4406.59	0.00	0.985
NMB0738	hypothetical protein	3382.79	0.00	0.986
NMB2080	hypothetical protein	2181.19	0.00	0.986
NMB1968	aldehyde dehydrogenase	13945.23	0.02	0.986
NMB0552	hypothetical protein	316.30	0.01	0.988
NMB2024	hypothetical protein	1528.96	0.00	0.988
NMB0391	beta-phosphoglucomutase	11027.84	-0.01	0.991
NMB0890	type IV pilin-like protein	2921.29	0.00	0.991
NMB1405	FrpA/C-like protein	1733.72	0.00	0.991
NMB0325	50S ribosomal protein L21	22789.58	0.00	0.991
NMB0299	competence protein ComEA	292.21	0.00	0.991
NMB1775	hypothetical protein	58.97	-0.01	0.992
NMB1883	hypothetical protein	173.30	0.00	0.992
NMB2055	LysR family transcriptional regulator	591.90	0.00	0.992
NMB1045	hypothetical protein	3684.96	0.00	0.992
NMB1465	hypothetical protein	7255.40	0.00	0.992
NMB0705	transporter	924.78	0.00	0.992
NMB0156	30S ribosomal protein S8	20468.47	0.00	0.993
NMB1558	diacylglycerol kinase	3810.73	0.00	0.993
NMB1704	beta-1%2C4-glucosyltransferase	751.88	0.00	0.994
NMB0530	beta-hexosaminidase	3328.01	0.00	0.995
NMB0316	hypothetical protein	334.85	0.00	0.995
NMB0160	50S ribosomal protein L30	6212.71	0.00	0.996
NMB0833	type I restriction enzyme-like protein	365.33	0.00	0.996
NMB1680	chorismate synthase	3211.39	0.00	0.996
NMB1777	hypothetical protein	637.87	0.00	0.999
NMB0269	competence protein	487.91	0.00	0.999
NMB0199	lipid-A-disaccharide synthase	1267.47	0.00	0.999

NMB2158	hypothetical protein	560.05	0.00	0.999
NMB0646	ribonuclease inhibitor barstar	704.19	0.00	0.999
NMB1594	spermidine/putrescine ABC transporter substrate-binding protein	13445.27	0.00	0.999

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