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The Role of Quorum Sensing on *copA* and *copB* Activity in Virulence Plasmid Replication in *Yersinia pseudotuberculosis*

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Thesis submitted to the University of Nottingham for the degree of Master of Research in Microbiology

Declaration

I declare that the thesis is the result of my own work which has been undertaken during my period of registration for this degree at The University of Nottingham. Unless otherwise acknowledged, the work presented in this thesis is entirely my own. No part has been submitted for another degree in The University of Nottingham or any other institute of learning.

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Abstract

Replication of the type three secretion pYV virulence plasmid is tightly controlled in *Yersinia pseudotuberculosis* through the activity of its two repressors *copA* and *copB.* Although the functionality and action of these genes have been well studied, the systems governing their activity have yet to be established. Previous observations suggested that a *Y. pseudotuberculosis* quorum sensing deficient mutant lost its virulence plasmid more rapidly than its parental counterpart. To examine if there was a regulatory relationship between the quorum sensing system and plasmid replication two bioluminescent *lux*-based promoter fusions were constructed to measure the activity of *copA* and *copB.* These reporter plasmids were then cloned into three strains, a *Y. pseudotuberculosis* parent and two quorum sensing mutants, one deficient in the *N*-acyl homoserine lactone synthases *ytbI* and *ypsI* and the other in the transcriptional regulators *ytbR and ypsR*. Promoter activity was determined as a measure of bioluminescence and cell density and *copA* activity was found to be greatly reduced at 26^{o}C and 37^{o}C when compared to the parent and *ytbR/ypsR* mutant. It was also evident that activity of *copA* at host temperatures was highest during the early stages of growth to 3 hours and then fell steadily through the later stages of growth. Similar activity was seen at 26^{o}C although the latent activity remained higher when compared to 37^{o}C. In contrast, *copB* activity was independent of the quorum sensing system with activity being identical in all three strains. *copB* activity was however found to be dependent on temperature, with the activity of *copB* being at its highest during later stages of growth at host temperatures and was inactive at 26^{o}C. These data provide evidence that temperature and the quorum sensing system play a role in plasmid replication in *Y. pseudotuberculosis.*

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- Jordan

Abbreviations

%	Percent
3’	Three prime end
5’	Five prime end
AHL	*N*-acyl homoserine lactone
AMP	Ampicillin
bp	Base pairs
BSL	Biosafety Level
CM	Chloramphenicol
DNA	Deoxyribose nucleic acid
dNTP	Deoxyribonucleotide Triphosphates
EDTA	Ethylenediaminetetraacetic acid
ERD	Enteric red mouth disease
FACS	Fluorescence activated cell sorting
FESLF	Far east scarlet like fever
g/L	grams/litre
GDP	Guanosine diphosphate
GENT	Gentamycin
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
KAN	Kanamycin
kb	Kilobase pairs
kV	Kilovolts
LB	Luria broth
M	Moles
MAPK	Mitogen-activated protein kinase
mg/mL	Milligrams per millilitre
mL	Millilitre
mm	Millimetre
mM	Millimolar
mRNA	Messenger ribose nucleic acid
ms	Milliseconds
NAL	Nalidixic acid
ng/µL	Nanograms/microlitre
nM	Nanomolar
^{o}C	Degrees centigrade
OD_{600}	Optical density at 600nm
ORF	Open reading frame
ORI	Origin of replication
PCR	Polymerase chain reaction
pH	Power of hydrogen
QS	Quorum Sensing
RLU	Relative light units

RNA	Ribose nucleic acid
RNAse III	Ribonuclease III
Rpm	Rotations per minute
Spp.	Species
Syc	Specific Yop chaperone
T3SS	Type three secretion system
TAE	Tris acetate EDTA
V	Volt
v/v	Volume per volume
w/v	Weight per volume
x g	Times gravitational force
YLA	*Yersinia* luria agar
YLB	*Yersinia* luria broth
Yop	*Yersinia* outermembrane protein
Ypm	*Yersinia pseudotuberculoisis* derived mitogen
µF	Microfarads
µg/mL	Micrograms per millilitre
µL	Microlitre
µm	Micromitre
Ω	Ohm

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CHAPTER 1

Introduction

1.1 The genus *Yersinia*

The genus *Yersinia* consists of a group of 26 different species of Gram-negative bacillius shaped bacteria that are widely distributed across the world. The genus was named after Danish-French microbiologist Alexandre Yersin; for his role in the identification of the infectious agent responsible for plague, during the 1894 plague outbreak in Hong Kong. Yersin, along with Shibasaburo Kitasato would go onto propose that rodents and their fleas were vectors for plague transmission which was proven through the infection of rodents using bubo extract from deceased plague victims, which developed plague symptoms before death. (4)

Originally *Yersinia* spp. fell within the very large and diverse *Enterobacteriaceae* family, which at the time was the only family within the *Enterobacterales* order. In 2016 the family was sub-divided into the 7 current distinct families after phylogenetic studies revealed conserved signature insertions and deletions (indels) which allowed for more precise taxonomic classifications and groupings within the order (5). The new proposed family *Yersiniaceae* which contained the *Yersinia* genus and other closely related genera (such as *Serratia*) were characterised by their highly conserved indels within the family's TetR transcriptional regulators (5).

Yersinia spp. can tolerate temperatures, ranging from 0^{o}C to 45^{o}C but are optimal for growth between 25-28^{o}C and can be found in a diverse range of environments; existing as pathogens within warm blooded mammals as well as free living in soil and water (6). The environmental hardiness of the genus has been well documented, illustrated by the fact that *Yersinia intermedia* was selected as a candidate in astrobiological studies relating to habitability of Mars environment (7).

While the majority of members of genus *Yersinia* are environmental species and pose minimal risk to humans, three members, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis* and *Yersinia pestis* are clinically significant human pathogens. The other pathogenic species, *Yersinia ruckeri* causes enteric redmouth disease (ERD) a haemorrhagic disease in salmonid fish with associated high mortality rates (8). Financially *Y. ruckeri* affects global fish populations and continues to cause problems in small fisheries where control strategies necessary to stop its spread are absent and contributes to the $6 billion annual losses due to emerging aquatic diseases (9).

1.1.1 *Yersinia pseudotuberculosis*

Y. pseudotuberculosis infections traditionally present in humans as self-limiting gastroenteritis that persists for a few weeks, although recently it has been linked to Far east scarlet like fever (FESLF) which patients are usually diagnosed with severe inflammatory symptoms. Historically the presence of FESLF has been geographically limited to the far east of Russia (10) and in Japan where the disease is endemic, often causing annual outbreaks of the disease with younger age groups being more susceptible (11).

While *Y. pseudotuberculosis* can always cause gastroenteritis symptoms in humans it is not always able to manifest as FESLF; while the reasons for this are currently not clear, research into FESLF has revealed that strains of *Y. pseudotuberculosis* taken from FESLF patients usually belong to a group of serotypes that predominantly produce the superantigen ypm (12).

1.1.2 *Yersinia enterocolitica*

Y. enterocolitica is the causative agent for the disease yersiniosis in humans. The disease is accompanied with enteritis and diarrhoea; which is usually self-limiting without treatment and lasts for 1-2 weeks (13). Research into *Y. enterocolitica* outside of humans has revealed a complex and varied life cycle; where the bacteria can exist in aquatic environments (14) or in their large natural reservoirs which are made up of birds and mammals (13).

1.1.3 *Yersinia pestis*

Y. pestis is the causal agent of plague and remains a historically significant disease in the history of human societal development; being the cause of three major pandemics within the last 1500 years. Bubonic plague is caused by infection of the lymphatic system which presents as characteristic buboes which are painful swellings and inflammation of the lymph nodes. Pneumonic plague is a disease of the respiratory tract, which is associated with high mortality rates, and is spread through human populations through contaminated respiratory droplets. Septicaemic plague like pneumonic plague has high mortality rates when untreated and a low latent period which quickly lead to sepsis and the rapid deterioration of health of infected individuals.

The role of arthropods as vectors for *Y. pestis* has been well studied, with fleas belonging to the order Siphonaptera playing a major role in the spread of the disease. The cycle of transmission begins when the flea takes a bloodmeal from an infected mammal and the bacteria propagate in the flea gut and subsequently form a biofilm which blocks the fleas foregut (15). Through repeated feeding attempts the bacteria are transferred to a new susceptible mammalian host when contaminated flea gut contents are mixed with the hosts blood.

1.2 *Y. pseudotuberculosis* vs *Y. pestis*

Initial estimates suggest that *Y. pestis* diverged from *Y. pseudotuberculosis* around 1,500-20,000 years ago (16) but a recent study of 3,800 year old human remains from the late neolithic/bronze age has suggested that *Y. pestis* emerged earlier than previously thought (17). These phylogenetic studies examined the relationship between *Y. pseudotuberculosis* and *Y. pestis* using DNA-DNA hybridization techniques as a way to compare the genomes of the two species, revealing high conservation at the DNA level (98%). However, despite their genetic similarity the two pathogens are radically different in how they spread as well as their potential as a pathogen. The reason for this in *Y. pestis* appears to be related to a handful of key mutations within its genome as well as the acquisition of two unique virulence associated plasmids; pPla (PCP) and PMT1(pFra) (18). The presence of both the pPla and the PMT plasmids leads to an overall increase in the virulence of *Y. pestis* but plays no role in the ability to cause disease in mammals.

From a research standpoint the homology between these two species acts as a useful tool for any groups wanting to research gene function or protein expression within *Y. pestis;* by allowing *Y. pseudotuberculosis* to be used a model for research where both species contain functionally identical components. The main advantage of this is the lower biological safety rating of *Y. pseudotuberculosis* being only a BSL-2 organism, allowing it to be used in standard category-2 microbiology research labs. This is in direct contrast to the far more pathogenic *Y. pestis* which requires a much larger financial and time investment in terms of equipment, training and regulation of research (19).

1.3 *Yersinia* in the modern day

Plague is perhaps one of the most devastating diseases that has affected human populations in history with the black death (*Ca* 1346-1353 CE) killing - an estimated 25 million people which accounted for 30% of Europe's population during that time (20). In schools plague is often our first introduction to disease as children, usually as a way to put diseases into context and the consequences of diseases spreading; additionally, it is taught as a major part of European history (21) with its passing causing major social change for the old world. For these reasons it is understandable why the common belief in the developed world is that plague is exactly as it is taught: a historical disease.

However plague is still endemic in some parts of the world with many outbreaks in Asia, Africa and South America; with the World Health Organisation declaring Madagascar, The Democratic Republic of Congo and Peru as major endemic regions (22), Madagascar reports annual cases of bubonic plague during the epidemic season which lasts from October to April (23) where case numbers remain steady despite the country's introduction of a national plague program in 1996, to monitor urban cases and rodent populations . More recently, the program aimed to mitigate the spread of the disease using pesticides to eliminate the flea vectors (24).

Another issue that the World Health Organisation faces when determining the severity and pattern of these seasonal outbreaks is due to the confirmation and reporting of new cases. In most of the regions where plague is endemic the diagnostic infrastructure is insufficient for confirmation of patient samples, leading to the vast majority of plague cases going unreported (24).

As a zoonotic infection *Y. pestis* populations are maintained outside human populations and are thought to circulate in natural reservoirs with up to 200 species of rodents known to be affected (25).

One puzzling aspect of modern day plague is its global distribution between the latitudes of 50°N and 35°S despite the presence of suitable mammal hosts existing beyond these latitudes and its presence within these areas in past pandemics. If this is related to temperatures then global warming could allow for emergence of plague at higher latitudes as we have seen with other emerging vector diseases such as dengue in Europe (26).

1.4 Multidrug resistance in *Yersinia* spp.

One major concern regarding any emerging modern-day infectious disease is the acquisition of multidrug resistance mechanisms because of the inappropriate use of antibiotics in medicine and agriculture.

The risk of these broad spectrum resistance mechanisms becoming more common in *Y. pestis* is a worrying scenario to both the public health of the countries it is currently found in as well as the world. This could result in a situation where countries like the United States of America, where plague instances exist but are usually sporadic and easily treatable with the use of antibiotics become an untreatable and fatal disease. The emergence of antibiotic resistant *Yersinia* spp. has been increasing over several decades with some patients infected with strains carrying multiple resistance genes on the same transmissible plasmid. For *Y. pestis,* strain IP275 carried resistance to three frontline antibiotics, and in addition also contained resistance to ampicillin, kanamycin, spectinomycin, minocycline and sulfonamides (27).

The appearance of multidrug resistant strains are still rare but the emergence of another strain similar to IP275 in a high-density urban population would pose a massive threat to both the public health and infrastructure of the region, essentially creating a pre-antibiotic scenario where we would see plagues historical 60-100 % mortality rates, depending on the form of plague thats present.

1.5 Quorum sensing in *Yersinia* spp.

Quorum sensing is a bacterial cell-to-cell communication system which allows for the regulation of gene expression as a function of the bacterial population density. In Gram negative bacteria the system is often controlled by multiple two-component systems consisting of a *luxI*-type synthase gene which produces the quorum system auto inducer *N*-

acyl homoserine lactone (AHL), along with their *luxR*-type AHL receptor protein genes (28). The LuxR-type proteins often contain two active domains, one which acts as a binding site for AHLs which stabilises the protein and facilitates protein folding with the change in the tertiary structure of the protein allowing a second domain to bind to specific upstream DNA regions known as lux-boxes (29). The binding of the exposed second domain to the DNA results in gene expression of the target gene bearing the upstream lux-box DNA element.

AHLs are structurally diverse with many species synthesising several different classes of signal most being unique to the species' 'AHL identity' allowing individual species to measure their population density even within complex or highly diverse environments such as a biofilm. Additionally, most species contain multiple functional pairs of *luxIR* orthologues which are able to code for multiple AHLs which can bind to a variety of different lux-R type proteins (30). Figure 1.1 shows the general AHL structure and highlights two important components related to their structure; the lactone ring and the acyl side chain.

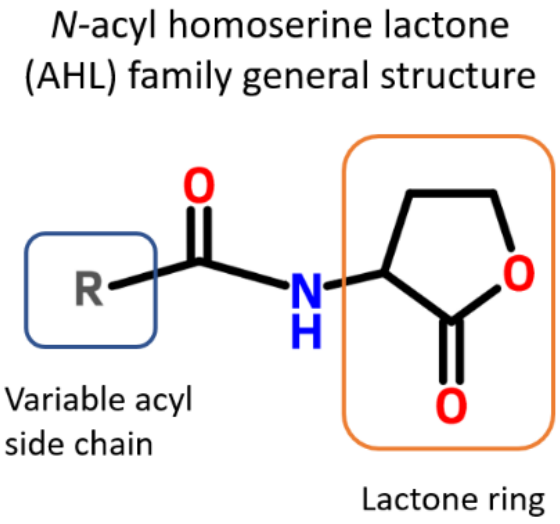


Figure 1.1: The general structure of quorum sensing *N*-acyl homoserine signal molecules. The variable acyl side chains increase resistance of the lactone ring to pH changes and prevents the pH dependant opening of the lactone ring ester bond. Opened lactone rings are rendered inactive and cannot bind to AHL binding domains on LuxR homologues.

It has been shown in both *Y. pseudotuberculosis* and *Pseudomonas aeruginosa* that AHL stability and activity is pH dependent with shifts towards higher pH ranges resulting in an inactivation of the AHL signal molecules through the opening of the lactone ring (31). Additionally, lactone rings on AHLs with longer acyl side chains have a greater resistance to pH change and remain active at higher pH ranges. This change is reversable and lowering the pH will cause the lactone ring to close, reverting the signal molecule back to its active state (31).

This system allows for coordination and uniform expression within bacterial populations and is dependent on the local concentrations of AHLs and their ability to diffuse. At low population densities AHLs are produced but fail to meet the threshold required for gene

expression and diffuse out to their environments. At higher cell densities AHLs accumulate when rate of AHL production is greater than AHL removal from the local area, which surpasses the threshold required for *luxR* homologues to trigger gene expression throughout the population (32).

In the clinically relevant *Yersinia spp.* quorum sensing is controlled by two pairs of convergently transcribed *luxI/luxR* orthologues, *ytbI/ytbR and ypsI/ypsR (Y. pseudotuberculosis)* (33), *ypeI/ypeR* and *yepI/yepR* (*Y. pestis)* (18) and *yenI/yenR* in *Y. enterocolitica* (34). Previous studies of the quorum sensing system within *Yersinia spp.* have shown that the systems have major impacts on cell phenotype as well as cell behaviour.

In *Y. pseudotuberculosis,* the quorum sensing system has been shown to regulate several key components related to biofilm formation (35) and motility (33). It was shown that an AHL synthase deficient *ΔytbI/ypsI* mutant displayed a difference in activity in the regulation of the flagellar master operon *flhDC* and the transcriptional factor *fliA* when compared to the parent and that quorum sensing modulated motility through the expression of *flhDC* and *fliA* (33).

This activity has also been shown to be similar in *Y. enterocolitica*, which revealed that the luxI/R homologues affected both cell motility and host cell attachment using quorum system deficient mutants (34). Loss of quorum sensing components through mutations of key components in *ΔyenI* and *ΔyenR/ycoR* showed a significant reduction of adhesion to host cells when compared to the parent (34).

Additionally there exists AHL degrading enzymes called lactonases, such as AiiA which is encoded by *aiiA* and was originally discovered in *Bacillus spp.* (36). Lactonases are a class of metalloenzymes which target and inactivate AHLs, through hydrolysis of the ester bond found on the homoserine lactone ring. This activity is identical to how higher pH inactivates the signal molecule and is outlined in figure 1.2.

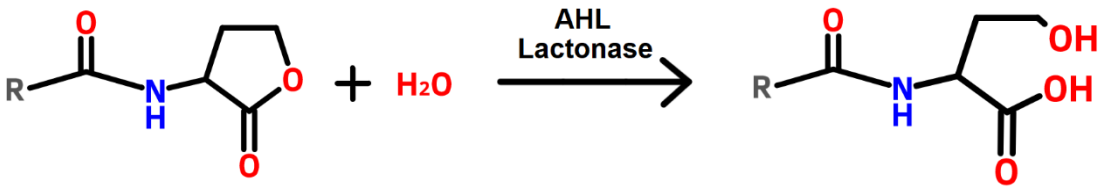


Figure 1.2: The activity of AHL lactone metalloenzymes on AHLs structure and function. Inactivation of AHLs occurs through hydrolysis of the ester bond on the homoserine lactone ring rending the AHL non-functional.

Due to their broad range of AHL targets and rapid activity, their significance has been debated regarding their 'quorum quenching' mechanisms and how they could be applied to human diseases that rely on quorum sensing associated regulation of virulence (36). One main critique of their use in the treatment of systemic diseases lies in the reversibility of the hydrolysis, since the mode of inactivity is identical to that of the pH inactivity a drop in pH

would reactivate the signal molecules (37). Additionally due to the difficulties relating to the mode of delivery to sites of infection, limits the usability of these enzymes in the treatment of human disease.

However, AiiA has found use as a valuable molecular microbiology research tool for the study of quorum sensing systems, often being utilized in the form of an inducible recombinant plasmid to check the effects of quorum signalling molecules on cell phenotypes and processes (33, 38, 39).

1.6 Virulence in *Yersinia pestis*

The type III secretion system (T3SS) is a complex series of virulence factors which allow bacteria to disrupt and bypass the physical membrane barrier of the host cells and inject multiple virulence associated effector proteins into the host cells. All components of the T3SS are coded for on a 68 kb plasmid which is essential for virulence in all three human pathogens (40). Central to this system is the synthesis and assembly of the injectisome, a complex multi-protein, transmembrane needle like structure which protrudes from the bacterial cell surface, the structure of which is outlined in figure 1.3. The structure of the injectisomes consist of four components: an inner membrane basal structure ring, a connector protein within the periplasm, an outer membrane ring and the structural needle (41). The injectisome needle is assembled from multiple Ysc proteins, with the basal section consisting of 5 proteins (YscR, YscS, YscT YscU and YscV), the outer membrane structural ring being primarily formed from YscC and the majority of the needle structure being formed from YscF (1). At its tip the needle is capped by YopB, YopD and LcrV, a protein complex pore known as the translocon that is required for successful attachment of the needle to the host (41).

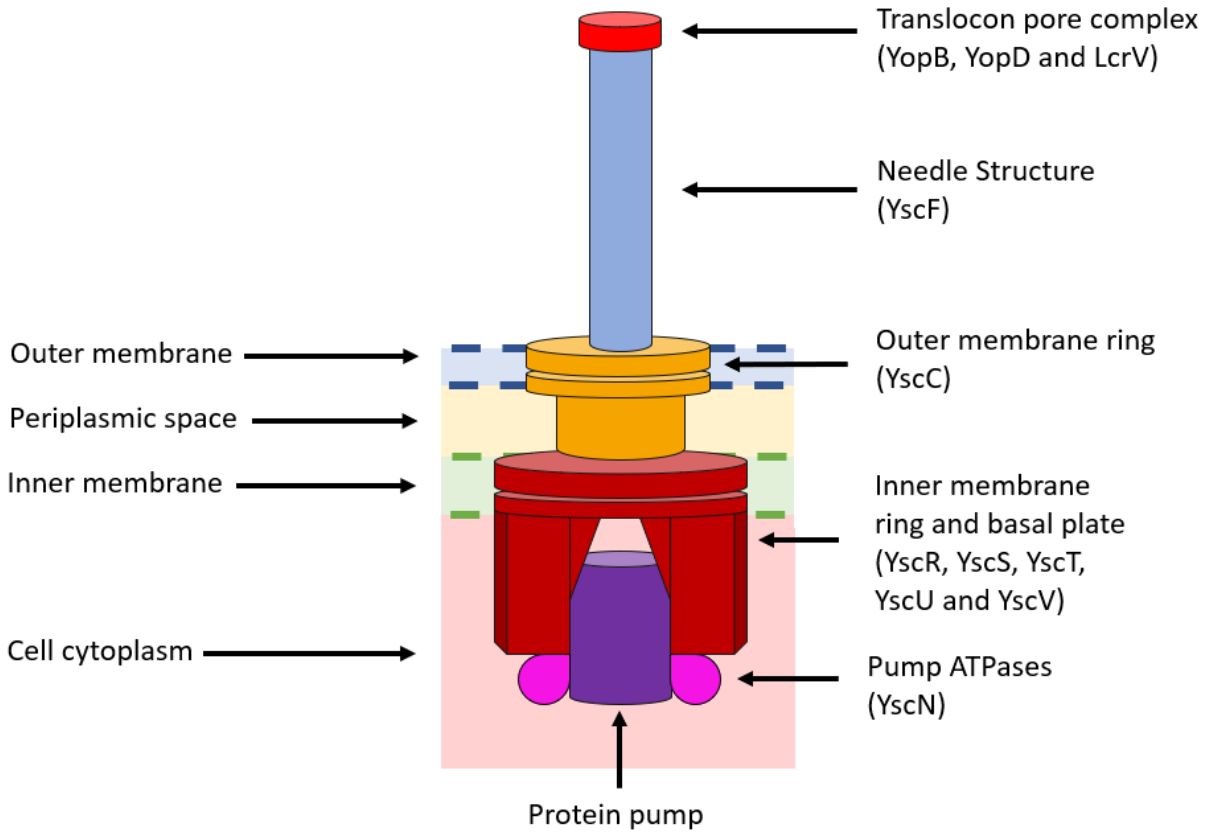


Figure 1.3: The assembled structure of an Injectisome in *Y. pseudotuberculosis*. The transmembrane structure comprises of 4 major components: the inner membrane ring, basal plate, the outer membrane ring, the needle structure and cap containing the translocator components. Adapted from (1)

1.6.1 *Yersinia* outer membrane proteins (Yops) and T3SS chaperones

The Yop effectors are a group of secreted proteins which work in conjunction with each other to disrupt key pathways within the target host cells and cause disruption of the host cytoskeleton (figure 1.4) (42). There are 6 Yops that are present across the three pathogenic *Yersinia* species; YopO, YopH, YopE, YopM, YopT and YopP/YopJ.

YopH is a potent multifunctional tyrosine phosphatase which has a major role in preventing host immune cells phagocytosis. The protein targets several key proteins within phagocytes, which is delivered via the injectisomes of the T3SS directly into the host cells. YopH dephosphorylates $p130^{Cas}$, a focal adhesion protein that is a major cytoskeleton component related to cell motility and is required for phagocytosis to occur (43). Dephosphorylation leads to disruption of the actin microfilaments which ultimately affects the cell motility and the whole process of phagocytosis (44). Interestingly there is also evidence to suggest that components of $p130^{Cas}$ are related to the regulation of cellular apoptosis (45) which if prevented would be beneficial to *Y. pestis* (44).

YopO/YpkA like YopH is a multifunctional protein which plays a role in both disruption of the actin cytoskeleton and affecting palette formation within local tissues (44). The protein

acts as an inhibitor to Gαq a G-protein with close ties to the RhoA signalling pathway and to Rac1, a protein related for the production of new actin fibres (46). One major result of the inhibition of Gαq is its effects on palette aggregation and bleeding within the host which has been witnessed in both plague infections and in Gαq knockout mice (47).

YopE is a broad Inhibitor of multiple G-proteins, which inhibits their function by reducing them from their active GTP state to their inactive GDP state through the activation of the protein's GTPase activity (48). This works in conjunction with YopO to disrupt the host cell cytoskeleton through the disruption of the RhoA pathway which leads to depolymerization of the actin fibres within the host cells.

YopM and YopJ play a major role in the disruption of the internal cell cycle and the process of apoptosis. YopM is a non-catalytic monomer scaffold protein which has two domains present at either end of the protein (46). Its function as a scaffold protein is to bind to the kinases Rsk1 and Prk2 to form a trimer, by doing so it activates the autophosphorylation mechanisms of Rsk1 which leads to the phosphorylation of Prk2 (46). When active Prk2 has been shown to phosphorylate transcription factors, cell cycle regulators and apoptotic factors with the cell and components of the mitogen-activated protein kinase (MAPK) pathway (49). YopJ plays a more active role as a catalytic protein by disrupting MAPK pathways within the cells leading to loss of MKK regulation and an increase in pro-apoptotic factors.

YopT is a cysteine protease that targets host Rho, Rac and CDC42 anchor sites which hold the proteins to the cell membranes (50). YopT cleaving activity leads to the disruption of RhoA dependent components of the actin cytoskeleton and a reduction in cytoskeletal control necessary for the process of phagocytosis (44).

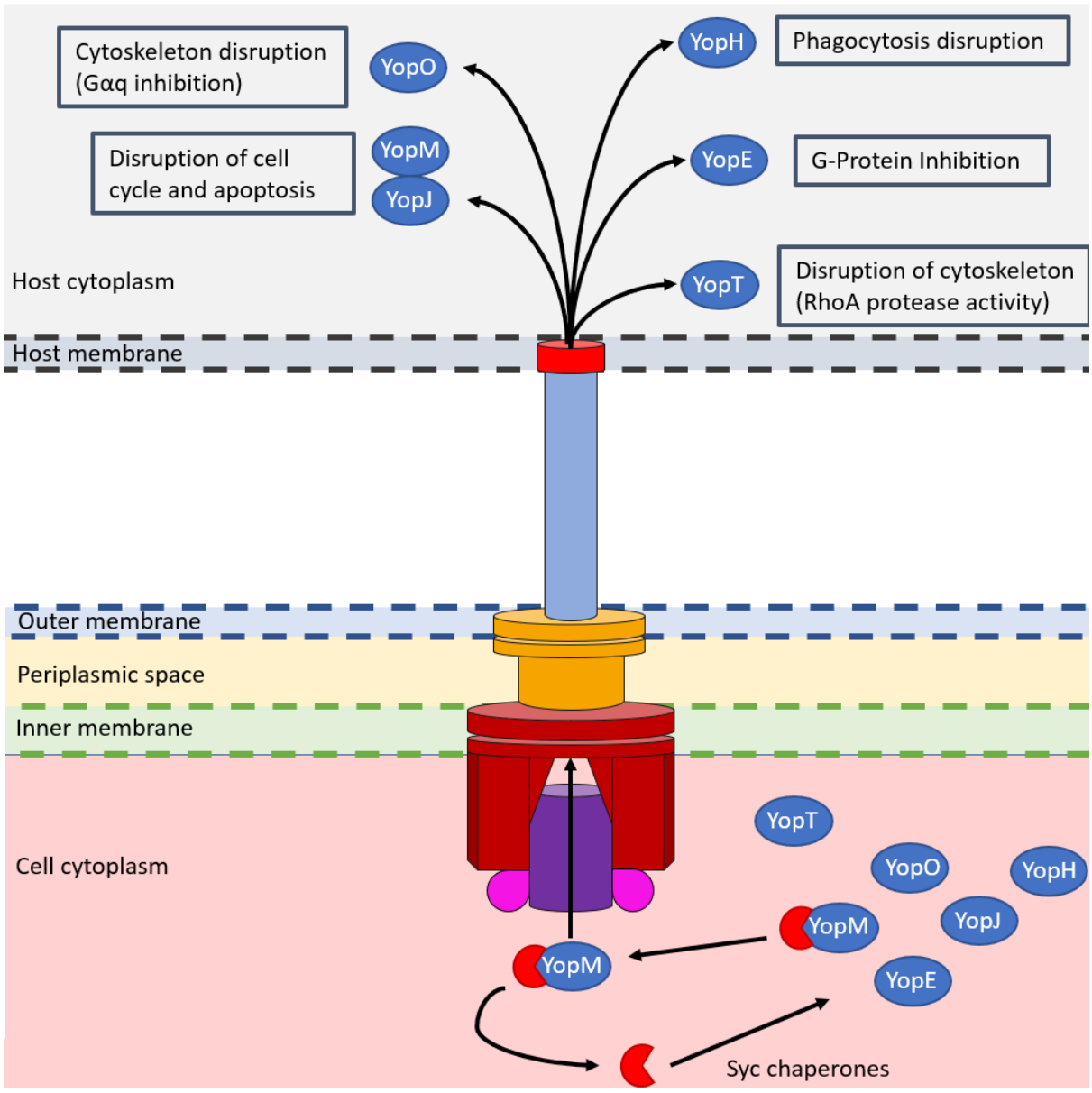


Figure 1.4: T3SS injectisome complex with host cell. Syc chaperones guide Yop proteins to the transmembrane injectisome channel and facilitates in the transfer into the host cell cytoplasm. host cytoplasm the Yop effectors begin disruption of key cell pathways which leads to disruption of cell cytoskeleton and a pro-apoptotic phenotype. Adapted from (1, 2)

One other integral component of the T3SS is the presence of effector chaperones (figure 1.4); small essential carrier proteins which bind to the effector proteins within the bacterial cytoplasm. The chaperones serve two major functions: they bind to the effectors to prevent host proteases from interbacterial degradation of the T3SS effectors and to guide the effectors towards the secretion channel near the basal section of the injectisome. In *Yersinia spp.* these specific yop chaperones (syc) are crucial for the delivery of many of the yop effectors to the host cytoplasm, with many being unable to be secreted in syc^- mutants (51).

1.6.2 Virulence associated plasmids in *Y. pestis*

In addition to the pYV plasmid *Y. pestis* has also acquired two highly conserved virulence associated plasmids named pPla and PMT1 which are unique to *Y. pestis*.

The 9.6 kb pPla plasmid contains genes for the production of Pla, a protease which plays a key role within the dissemination of *Y. pestis* during infection by dampening the hosts ability to activate coagulation factors (52). Through the multifunctional site of pla it is able to cleave a number of host components such as plasminogen (to its active fibrin degrading enzyme form plasmin), α2-Antiplasmin (a key inhibitor of plasmin) and plasminogen activator inhibitor-1 (plasminogen regulator and neutrophil recruiter) (52). Through pla clevage of the hosts regulatory mechanisms there is a loss of regulation and plasminogen is converted into plasmin which causes an increase in fibrin clot degradation and increase in *Y. pestis* dissemination within the host tissues. In pla negative strains of *Y. pestis* its ability to spread into neighbouring tissue is reduced in both pneumonic and bubonic infections

The 102 kb PMT1 plasmid contains several virulence genes related to production of the F1 capsule protein and the yersinia murine toxin. The F1 capsule is a bacterial surface polymer coded for by the genes *caf1A* and *caf1M* which play an active role in *Y. pestis* survival by preventing the process of phagocytosis from occurring within the host(53). When the F1 capsule proteins are co-expressed alongside YopH and YopE the bacteria become highly resistant to phagocytosis as shown in one study where 2 ΔF1 mutants (ΔF1 and ΔF1/ΔYopH/ΔYopE) showed significant reduction in phagocyte resistance when compared to the wild type parent (53).

The presence of the T3SS plasmid, and in *Y. pestis* the combination of these plasmids and their effectors results in a significantly unfit cell phenotype with major mobility disruption to the actin cytoskeleton, an increase in pro-apoptotic factors and decrease in immune cell recruitment. The death of host cells then provides the pathogen with access to key nutrients required for further growth and enables the bacteria to further disseminate within the host. The rapid cycle of infection and expansion in *Y. pestis* is highly efficient and causes the rapid deterioration characteristic of plague infections going from a mild illness to a life threatening condition over the course of several hours.

1.7 T3SS virulence plasmid replication

pYV replication when studied in *E. coli* is tightly regulated by the initiator protein RepA which is located 170 bp ahead of the origin of replication (Ori). The repression of RepA expression is outlined below in figure 1.5 and is regulated by CopA, a 90 bp gene located upstream of RepA and its other ancillary regulator CopB.

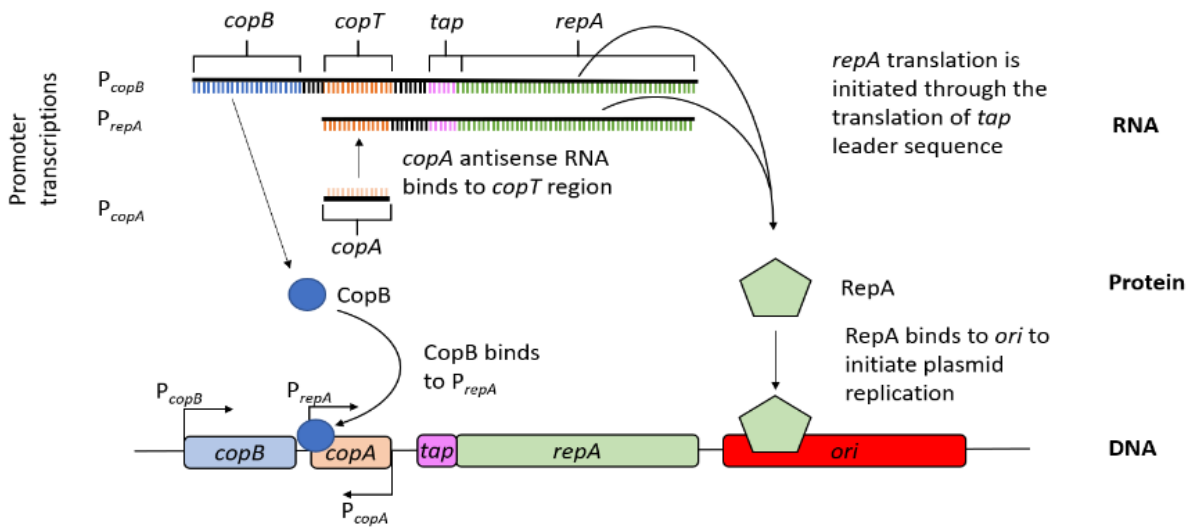


Figure 1.5: An overview of the plasmid replication system and regulation through *copA* and *copB.* Plasmid replication is initiated through the translation of *repA* sequences to produce the replication initiation protein RepA. RepA can be transcribed from both the P_{copB} and P_{repA} transcription sequences. *copA* produces a non-translated anti-sense mRNA sequence which forms an mRNA duplex with complementary site *copT,* this prevents the *repA* leader peptide sequence *tap* from being translated which prevents *repA* translation. *copB* is a repressor of the P_{repA} promoter which when bound prevents *repA* transcription.

Figure 1.6 outlines the activity and function of CopA as a post transcriptional inhibitor of RepA by producing the non-coding anti-sense RNA sequence CopA-RNA which targets the complementary site of the RepA-mRNA sequence CopT. Once CopA is bound to CopT on the RepA-mRNA strand it will remain bound; preventing further translation of the *repA*-mRNA sequence into the RepA protein (3). This arrest of translation is caused by the interaction of *copA*-*copT*, which prevents the peptide leader sequence *tap* from being translated, which is required for translation of *repA* (54). The process is finally complete when the mRNA strand is digested by RNAseIII which cleaves the double strand close to the CopA/CopT duplex which renders the RepA-mRNA strand unusable by the cell (55).

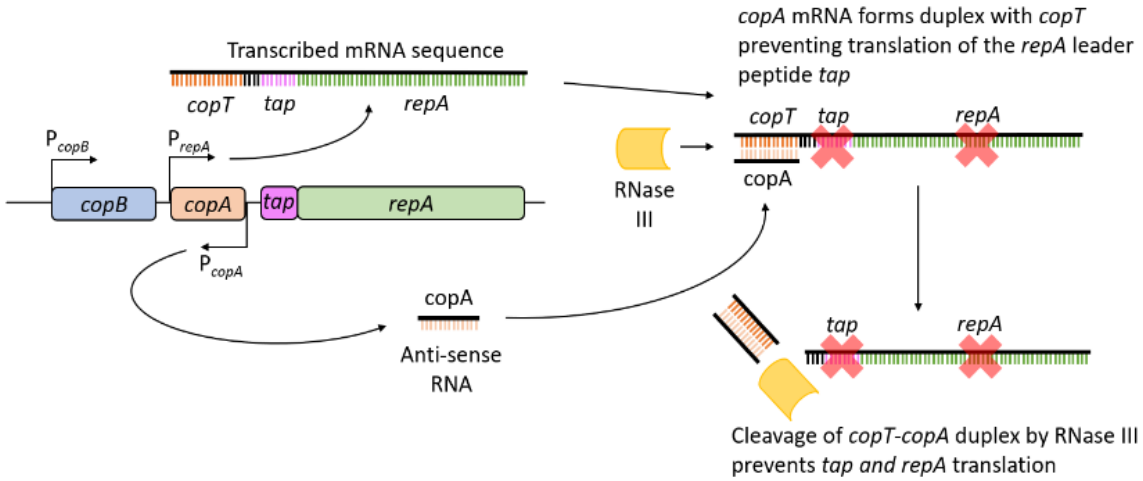


Figure 1.6: Overview of the post-transcriptional repressor *copA*. *copA* transcription produces a non-translatable antisense mRNA which binds to the complementary region *copT* located on the transcribed *repA* mRNA sequence. The resulting *copA-copT* duplex prevents translation of the leader peptide *tap* and RepA. RNAse III binds to the ds-RNA and cleaves the *copA-copT* duplex close to *tap* which prevents further translation.

Blomberg *et al.*, (1990) revealed that this form of RNAseIII mediated repression has been shown to be highly effective as a means of preventing RepA synthesis with almost complete cleavage of the mRNA strands by the RNAseIII activity while full-length RepA-mRNAs were rare whereas, the shorter cleaved RNA fragments were abundant in number (55).

The Ancillary repressor *copB* and its promoter P_{copB} is a 255 bp sequence located upstream from the RepA promoter P_{repA} also found on the pYV plasmid. The activity of CopB is shown in figure 1.7 as repressor of the P_{repA} promoter; which arrests all activity of P_{repA} while bound (56).

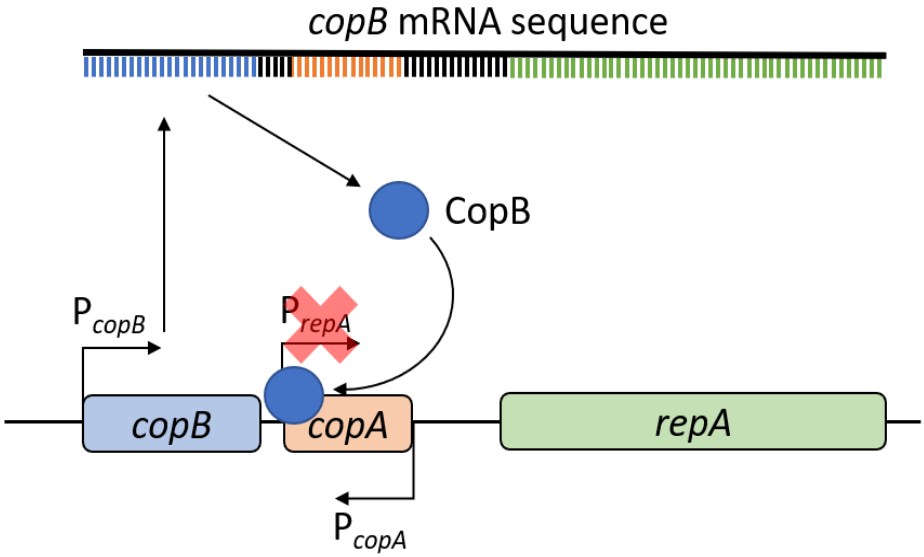


Figure 1.7: Overview of the activity of the pre-transcriptional activity of the repressor *copB.* Translated *CopB* is a repressor of P_{repA} which strongly binds to the promoter, this prevents transcription of the P_{repA} mRNA sequence and prevents RepA production and plasmid replication.

Interestingly there are two RepA promoters, namely P_{copB} and P_{copA} with P_{copB} producing the longer transcript. The majority of RepA translation occurs from the *repA* sequence transcribed on the P_{copB} mRNA strands with translation from P_{repA} mRNA sequences being largely inhibited by the pre-transcription binding of CopB or the post-transcriptional effects of *copA* (3).

This was further explored with a set of experiments outlined in figure 1.8 to see the effects of two separate mutations on the function of *copB* and its promoter on production and regulation of plasmid replication (57). A deletion of the promoter region and ORF of *copB* provided a mutant with no ability to regulate RepA using the copB system and led to an eight-fold increase in plasmid replication. However a frame shift mutation of the *copB* ORF alone retained its promoter functionality and led to a 40 fold increase in plasmid number (57).

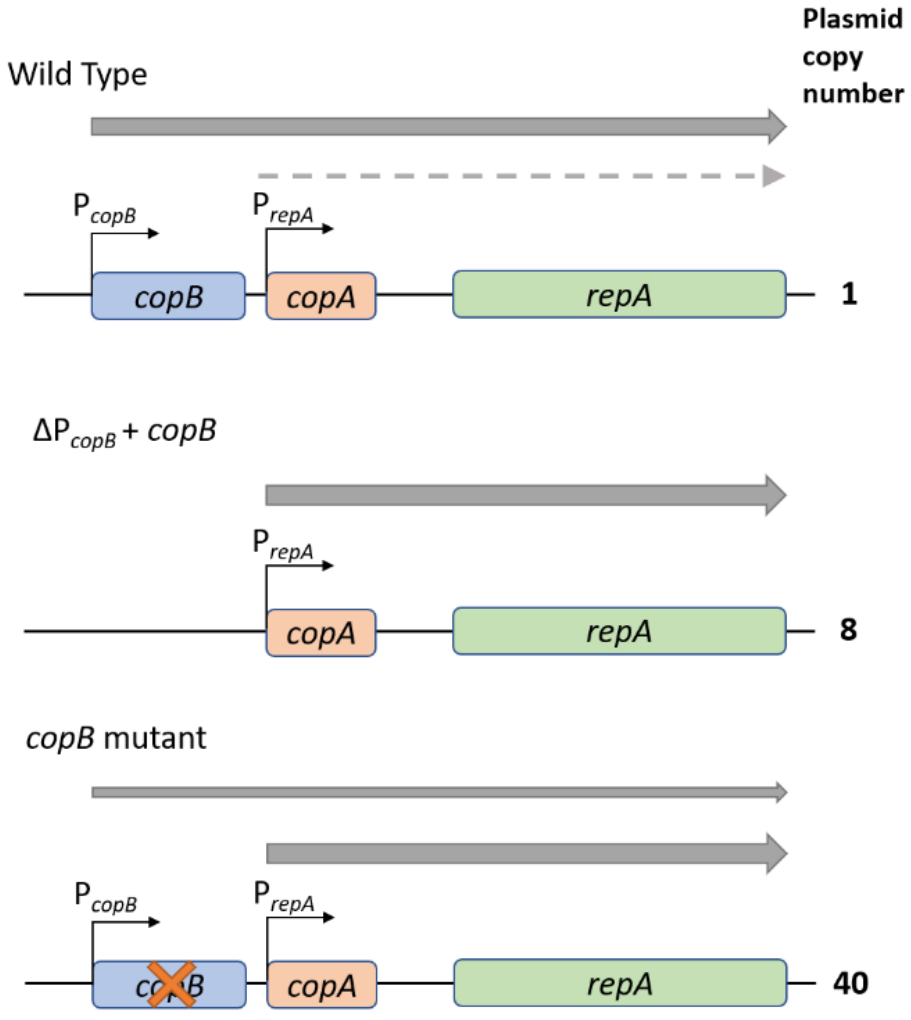


Figure 1.8: The effects of *copB* on relative plasmid copy number. Removal of both P_{copB} and *copB* repressor leads to an increase in repA transcription and an increase in copy number when compared to the wild type. Inactivation of copB lead to a large increase in repA translation from both the transcribed P_{repA} and P_{copB} mRNA and a large increase in plasmid replication. Thicker grey arrows indicated an increase in the transcription of that specific mRNA sequence. Figure adapted from (2, 3)

This would indicate that CopB plays a role as an inhibitor of P_{repA} and as an inducer of RepA translation when P_{copB} is transcribed, in direct contrast the *copA* antisense mRNA which only acts as a post transcriptional inhibitor of the RepA gene (58).

1.8 Virulence plasmid loss in QS mutants

While the activity and functionality of both copA and copB have been thoroughly studied in relation to *repA* expression, how the system is itself regulated is not well described. Previous work revealed that the copy number of pYV declined more rapidly in a *Y. enterocolitica* AHL synthase mutant *yenI* when compared to the parent strain (59). While the role of quorum sensing as a regulator of several diverse systems in *Y. pseudotuberculosis* are well documented (33, 35, 60) a link between the quorum sensing system and plasmid replication have never been investigated.

Unpublished observations by V. Garcia (2014) (figure 1.9) showed in a series of extended 15-day growth experiments that quorum sensing mutants in *Y. pseudotuberculosis* displayed different rates of virulence plasmid loss in their populations. The GFP tagged pYV virulence plasmids were used to measure plasmid presence within the total population (% GFP) and of the three strains that were tested, *ΔytbI/ΔypsI* (dIG) showed both a greater and earlier loss of pYV within its population, when compared to the parent (WG) and *ΔytbR/ΔypsR* (dRG).

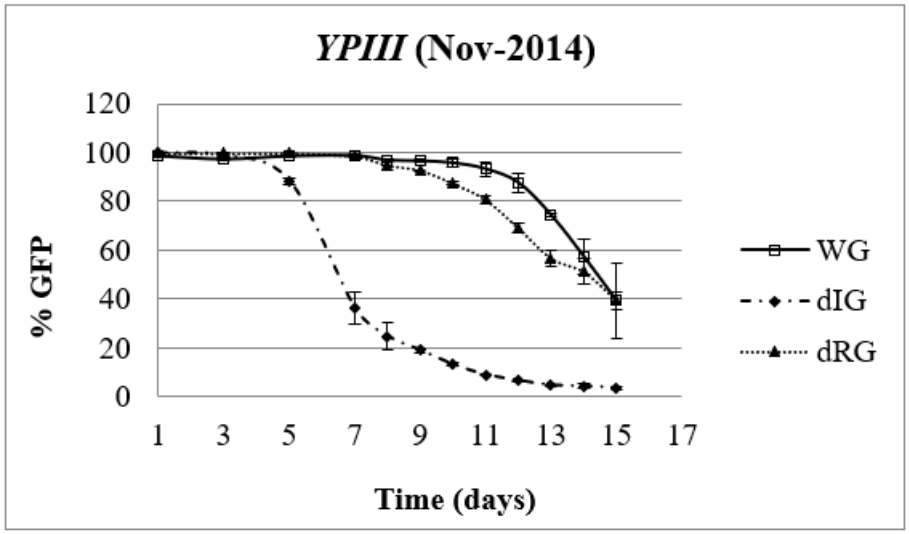


Figure 1.9: Unpublished GFP tagged virulence plasmid FACS data showing virulence plasmid presence within several *Y. pseudotuberculosis* strains; Parent (WG), *ΔytbI/ΔypsI* (dIG) and *ΔytbR/ΔypsR* (dRG). % GFP is a representation of virulence plasmid presence within the population, with a higher value representing a greater proportion of population containing the virulence plasmid. Plasmid loss occurs in all three strains, but occurs earlier in dIG at day 4 with the population effectively losing the GFP tagged virulence plasmid by day 13.

This observed difference in plasmid loss seen in the *ΔytbI/ΔypsI* raised the question if QS played a role in plasmid replication and partitioning in *Y. pseudotuberculosis* and if so what components does it interact with. These observations formed the hypothesis for this study

that pYV virulence plasmid replication is initiated/controlled by the QS system. The main objectives for this study were to investigate the role that QS plays in relation to the virulence plasmid initiation gene *repA* and if there were any changes in activity in either of its regulator genes *copA* or *copB*. This study would utilize the same two strains as used in the GFP flow cytometry data; *ΔytbI/ΔypsI* and *ΔytbR/ΔypsR* to investigate the effects of *luxI* and *luxR* deficient homologues on the pYV replication system.

To examine *copA* and *copB* promoter activity, reporter gene technologies were employed which have previously been used to measure gene expression as bioluminescence through fusions of the promoter of interest to the *luxCDABE* operon (31).

CHAPTER 2

Materials and Methods

2.1 Growth media and growth conditions

Unless otherwise stated *Y. pseudotuberculosis* strains were grown in *Yersinia* lysogeny broth (YLB) liquid cultures overnight at 30°C. YLB is a variant of Lysogeny broth with a reduced NaCl content: 10 g/L Tryptone, 5 g/L NaCl and 5 g/L yeast extract with the final pH being adjusted to pH7 using NaOH and HCl (61). The overnight cultures were then shaken at 200 rpm for a minimum length of 16 hours and a maximum length of 20 hours unless stated otherwise. For solid media such as agar plates, *Yersinia* lysogeny agar (YLA) was used which comprises of YLB with an addition of 15 g/L of agar (1.5 %).

Escherichia coli strains were grown overnight at 37°C in lysogeny broth (LB) which comprises of; 10 g/L tryptone, 10 g/L NaCl and 5 g/L yeast extract (62). Overnight cultures were shaken at 200 rpm for a minimum of 16 hours and a maximum length of 20 hours. Solid lysogeny agar was made by adding 15 g/L agar (1.5 %) to LB.

Antibiotic stocks for liquid and solid media were prepared as follows (unless stated in their preparation): Antibiotics were dissolved in the appropriate quantity of liquid, filter sterilised though a 0.2 µm filter and 1 mL aliquots of the sterile filtered antibiotic were transferred into sterile 1.5 µL microcentrifuge tubes and transferred to a -20°C freezer for storage. Freeze thaw cycles were recorded on the sides of the tubes and the tubes were discarded after five thaws. Details of each antibiotic as well as their solvents and concentrations can be found in table 2.1.

Antibiotic	**Abbreviation**	**Solvent**	**Stock Concentration (mg/mL)**	**Working Concentration (µg/mL)**
Ampicillin	AMP	H_2O	100	100
Chloramphenicol	CM	Ethanol (70%)	25	25
Gentamycin	GENT	H_2O	10	10
Kanamycin	KAN	H_2O	50	50
Nalidixic acid	NAL	H_2O – pH 11	30	15

Table 2.1: Antibiotics used in this study. Full names of each antibiotic as well as their abbreviations, solvent, stock and working concentrations.

2.2 Bacterial strains

Bacterial strains that were used in this study are listed in Table 2.2. All strains were stocked at -80^{o}C in a 20% glycerol mix.

Bacterial strain	Description	Source
Y. pseudotuberculosis		
YpIII pLB1	Parent strain of *Y. pseudotuberculosis* contains pYV (Nal^R). *Serotype O:3*	(63)
YpIII *ΔytbI/ΔypsI*	YpIII with double knockout of synthase YtbI and YpsI (Nal^R) *Serotype O:3*	(61)
YpIII ΔytbR/ΔypsR	YpIII with double knockout of AHL receptor YtbR and YpsR (Nal^R) *Serotype O:3*	(61)
Escherichia coli		
JM109	Cloning strain for transformation. LacZ for blue white screening. *traD36 proA$^+$B$^+$ lacI^q Δ(lacZ)M15/Δ(lac-proAB) glnV44 e14$^-$ gyrA96 recA1 relA1 endA1 thi hsdR17*	NEB
S17-1 λpir	Pir$^+$ strain used for conjugation in conjunction with Pir dependent suicide vectors. *thi pro hsd*R–*hsd*M+ *rec*A RP4 2-Tc::Mu-Km::Tn7	(64)
DH5α pBlue*luxCDABE*	DH5α strain containing the *luxCDABE* cassette (Amp^R). Fϕ80*lacZ*ΔM15Δ (*lacZYA-argF*) U169 *recAI endAI hsdR17* ($r_K^-m_K^+$) *supE44 thiI gyrI relAI*	(33)
Promoter fusions		
JM109 pGEM-T $P_{copA::lux}$	JM109 *E. coli* cloning strain containing lux fusion of the *copA* promoter of pYV on pGEM-T easy vector (Amp^R)	This study
JM109 pGEM-T $P_{copB::lux}$	JM109 *E. coli* cloning strain containing lux fusion of the *copB* promoter of pYV on pGEM-T easy vector (Amp^R)	This study
JM109 pHG327 $P_{copA::lux}$	JM109 *E. coli* cloning strain containing lux fusion of the *copA* promoter of pYV on PHG327 (Amp^R)	This study

S17-1 λpir pDM4.2 $P_{copB::lux}$	JM109 *E. coli pir*$^{+}$ conjugation strain containing lux fusion of the *copB* promoter of pYV on pDM4.2 (Gent^{R})	This study
YpIII $P_{copA::lux}$	YpIII parent containing lux fusion of the *copA* promoter of pYV on pHG327 (Amp^{R}) (Nal^{R})	This study
YpIII $P_{copB::lux}$	YpIII parent containing lux fusion of the *copB* promoter of pYV on PDM4.2 suicide vector (Gent^{R}) (Nal^{R})	This study
YpIII *ΔytbI/ΔypsI* $P_{copA::lux}$	YpIII *ΔytbI/ΔypsI* containing lux fusion of the *copA* promoter of pYV on PHG327 (Amp^{R})(Nal^{R})	This study
YpIII *ΔytbI/ΔypsI* $P_{copB::lux}$	YpIII *ΔytbI/ΔypsI* containing lux fusion of the *copB* promoter of pYV on PDM4.2 suicide vector (Gent^{R}) (Nal^{R})	This study
YpIII *ΔytbR/ΔypsR* $P_{copA::lux}$	YpIII *ΔytbR/ΔypsR* containing lux fusion of the *copA* promoter of pYV on PHG327 (Amp^{R}) (Nal^{R})	This study
YpIII *ΔytbR/ΔypsR* $P_{copB::lux}$	YpIII *ΔytbR/ΔypsR* containing lux fusion of the *copB* promoter of pYV on PDM4.2 suicide vector (Gent^{R}) (Nal^{R})	This study

Table 2.2 Bacterial strains that were used in this study

2.3 Molecular cloning methodologies

2.3.1 Plasmid extraction and digestion

Bacterial plasmids were extracted using plasmid GenElute miniprep kits (Sigma) as per the kits protocol as described by Schagat (65). Genomic DNA extractions were done using GenElute bacterial genomic DNA kits. Unless otherwise stated the plasmids were eluted from the DNA binding columns using ultrapure DNase/RNAse free distilled water and stored at -20^{o}C.

New England Bioscience (NEB) restriction enzymes were exclusively used for this project to prevent incompatibility from buffers. All digests were done according to the manufacturer's instructions with the exception of extending the digest time to 1 hour rather than the stated 15 minutes, this was to allow complete digestion to occur.

2.3.2 Gel electrophoresis and gel extraction

Gel electrophoresis was carried out at two different concentrations depending on the purpose of the gel. For routine DNA digest checks a 1% w/v agarose, 1x Tris-acetate-EDTA (TAE) buffer were used, containing 1x SYBR safe DNA gel stain (Thermofisher). DNA digests which were due to be extracted and cleaned up were run on 0.8 % agarose gels w/v agarose, 1x TAE gels containing 1x SYBR safe DNA gelstain.

Unless stated all gels were ran at 70V for 60 mins in 1x TAE buffer, using a 1 kb DNA ladder (NEB).

Purification and clean-up of PCR and DNA fragments was done using Monarch DNA Gel Extraction Kits (NEB) using the protocol outlined by the manufacturer. To increase the disassociation of the DNA from the column and increase concentration, the purified DNA was incubated at 37°C for 10 minutes before being eluted using half the volume which the instructions suggested.

2.3.3 Ligation reactions

Ligation reactions were carried out using T4 DNA Ligase (NEB) using the protocol outlined by the manufacturer. Ligations were carried out overnight at room temperature after an initial -20°C 5 min cooling step. Except where stated all digested DNA were first ran on an electrophoresis gel and cleaned up. Ligation ratios were set up for each new ligation using the equation:

$$\text{Mass of Insert (ng)} = \text{Desired Molar Ratio} \times \text{Mass of Vector (ng)} \times \left[\frac{\text{Insert size (bp)}}{\text{Vector size (bp)}}\right]$$

Ligations were tested at the ratios 1:1, 2:1, 3:1, 5:1 and 10:1 (Insert:Vector).

2.3.4 DNA Polymerase I, large Klenow fragment blunting

Digest blunting for double blunt end ligations was done using the DNA polymerase I, large Klenow fragment (NEB). The manufacturers protocol (NEB) for the blunting reactions and the dNTPs used were freshly prepared from a dNTP kit (Fisher),

The only change to the given protocol was the use of T4 ligase buffer (NEB) instead of the supplied Klenow buffer. The large Klenow fragment was confirmed by the manufacturer to be compatible with the ligase buffer. This was done to prevent DNA loss through an additional clean-up step and concentration decrease due to dilutions.

2.3.5 Preparation and transformation of electrocompetent cells

Electrocompetent cells were prepared using an overnight culture to seed 100 ml of YLB to an OD_{600} of 0.05-0.07. Liquid cultures were then grown in a shaking incubator until the OD_{600} reached the exponential phase of growth between 0.5-0.6. The cultures were then split into

two 50 ml centrifugation and harvested via centrifugation at 10,000 x g for 5 minutes. Three washes of 40 ml, 20 ml and 5ml respectively were performed using a 10 % (v/v) ice cold glycerol (10,000 x g for 5 min, at 4°C), removing the supernatant after each centrifuge. The final pellet was then resuspended with 40 µl of 10 % glycerol solution and aliquoted into 20 µl volumes which were stored at -80°C.

Transformation of the cells was done via electroporation using the BioRad pulser set to the Gram-negative setting (2.5 kV, 200 Ω and 25 µF) using a 2 mm electroporation cuvette. A maximum DNA volume of 2 µl was added to the cells to prevent the chance of arcing during electroporation. 1 ml of prewarmed media was then added to the recovered cells and incubated at the appropriate temperature for 1 hour. Bacterial cultures were then plated on plates containing the appropriate antibiotics to the resistance marker.

2.3.6 Preparation and transformation of chemically cells

Chemically competent cells were prepared by initially preparing three washes; 500 ml 0.1 M $MgCl_2$, 500 ml 0.05 M $CaCl_2$, 200 ml 0.1 M Cacl2 with 15 % (v/v) glycerol. Each solution was on the day and filter sterilised into sterile bottles, once sterilised the solutions were kept on ice until needed.

An overnight culture was added to 100 ml liquid media until the OD_{600} was 0.05-0.07. Liquid cultures were then grown in a shaking incubator until the OD_{600} reached the exponential phase of growth 0.5-0.6. The 100 ml culture was split centrifuged (4,000 x g for 10 minutes, 4°C). The supernatant was removed, and each pellet was resuspended with 12.5 ml 0.1 M $MgCl_2$ before incubating on ice for 10 minutes. A second centrifugation step was done, and the pellet was then resuspended with 12.5 ml 0.05M $CaCl_2$ before incubating on ice for 20 mins. A final centrifuge step was done, and the pellets were each resuspended with 5 ml 0.1 M Cacl2 with 15% (v/v) glycerol. The competent cells were then aliquoted into 100 µL volumes and stored at -80°C until needed.

Transformation of chemically competent cells was performed by adding the plasmid to the competent cells, heat shocking the bacteria at 42°C for 2 minutes and incubating on ice for 10 minutes. Bacteria were recovered in 1ml of liquid media incubated at the appropriate temperature for 1 hour. The bacteria were then plated on appropriate antibiotic containing media and incubated overnight.

Use of chemically competent cells was prioritized in some situations over electrocompetent cells. This is because chemically competent cells allow for greater volumes of DNA samples to be added to the cells unlike electrocompetent cells which are limited due to the risk of arcing. This was primarily done to increase the transformation rates of competent cells when working with low copy number plasmids. Chemically competent cells for JM109 *E. coli,* S-17 *E. coli*, the parent WT *Y. pseudotuberculosis,* ΔytbI/ΔypsI *Y. pseudotuberculosis* and ΔytbR/ΔypsR *Y. pseudotuberculosis* were generated.

2.3.7 Plasmids used in this study

All plasmids used in this study are listed in *table 2.3.*

Plasmid	Description	Source
pYV	*Y. pseudotuberculosis* virulence plasmid	This study
pBlue*lux*	pBluescript II KS+ Vector containing *luxCDABE* (AmpR)	(33)
pGEM-T easy	3' T overhang Cloning vector (Amp^R)	Promega
pDM4.2	Suicide vector *pir* dependent for replication (Gm^R). Modification of pDM4 (66) with Gm^R cassette.	Joanne Purves (unpublished)
pHG327	Low copy number vector (Amp^R)	(67)
pSU18	Medium copy number Empty vector containing a Cm^R cassette	(35)
pSUaiiA	Derivative of pSU18 carrying the AHL degrading *aiiA*	(35)
pGEM-T P_{copA}	pGEM-T cloning vector containing the promoter region P_{copA} (Amp^R)	This study
pGEM-T P_{copB}	pGEM-T cloning vector containing the promoter region P_{copB} (Amp^R)	This study
Promoter fusions		
pGEMT $P_{copA::lux}$	pGEMT vector containing the lux reporter $P_{copA::lux}$	This study
pGEMT $P_{copB::lux}$	pGEMT vector containing the lux reporter $P_{copB::lux}$	This study

pHG327 $P_{copA::lux}$	pHG327 vector containing the lux reporter $P_{copA::lux}$	This study
pDM4.2 $P_{copB::lux}$	pDM4.2 suicide vector containing the lux reporter $P_{copB::lux}$	This study

Table 2.3 *Bacterial plasmids that were used in this study*

2.3.8 Polymerase chain reactions (PCRs)

For routine screening PCR was performed using Taq polymerase (NEB) or Q5 High-fidelity DNA polymerase when proof reading was required.

A general 25 µl reaction consisted of 0.1 µl of polymerase, 1.0 µl of template DNA, 2.5 µl of polymerase reaction buffer (NEB), 1.0 µl of 20 mM dNTPs and 1 µl of the forward and reverse primers. Nuclease free molecular grade water was then added to bring the final volume to 25 µl.

The PCR reaction temperatures were dependent on both the length of the primer sequences and also the polymerase used. The temperatures chosen were based on the manufacturers protocols (NEB), the annealing temperatures used for the primers are listed in *table 2.4.*

Primer name	**Sequence F: 5'-3' R: 5'-3'**	**Restriction Site**	**Annealing temperature Q5 (°C)**	**Annealing temperature Taq (°C)**	**Source**
copA-F	CTCTCGAGGCGTAAATTCCG	XhoI	66°C	53°C	This study
copB-R	ACTAGTCCCGGGTATAGAAGCTGTTGC	SpeI SmaI	72°C	59°C	This Study
copB-F	CCACTCGAGCGTACAGTCCAACTCGG	XhoI	72°C	63°C	This study

copB-R	GGACTAGTCTTCCCGGG GGCACGTTTCGATGAGG	SpeI SmaI	72°C	68°C	This Study
LcrV_ comp 005_ KpnI_F	TAGGTACCATTAGAGCC TACGAACAA	KpnI	68°C	54°C	(N. Barratt, 2021 personal communication)
LcrV_ comp 005_ SacI_R	TAGGTACCATTAGAGCT CACGAACAA	SacI	68°C	54°C	(N. Barratt, 2021 personal communication)
luxCRv	CTACAACATCATAAAGG CC	-	58°C	45°C	(J. Puruves, 2022 Personal communication)
YopJ-F	GGTACCTACTAAGCTTC AGGGAGTTGGTACTGTT GTTA	HIII KpnI	72°C	61°C	(V. Garcia 2021 personal communication)
YopJ-R	CGGAGCTCCTCAGTCCG GTTGTGGTGATT	SacI	72°C	65°C	(V. Garcia 2021 personal communication)

Table 2.4: PCR primers that were used in this study. Annealing temperature used for the PCR was dependent on the polymerase used, temperatures for both polymerases were shown for each primer. Underlined sections of the primer sequences denote the respective 6 base restriction cutting sites.

2.3.9 Reducing the plasmid copy number

The use of high copy number plasmids for the study of quorum sensing systems would not be appropriate given that there would only be one copy of the promoter region found exclusively in each virulence plasmid which is itself low copy. Therefore if multiple copies of a plasmid containing the promoter region were present titration effects may affect the normal regulatory process.

Two cloning strategies were therefore used to better emulate the usual numbers of P_{copA} and P_{copB} found in *Y. pseudotuberculosis*. The first strategy would utilize a low copy number plasmid transformed into *Y. pseudotuberculosis* and the second would utilize a single crossover event to incorporate the reporter plasmid directly onto the virulence plasmid through the use of a homologous region. This would be done by utilizing a suicide vector with a λpir dependency for replication which would be grown up in a PIR^+ strain of *E. coli* then conjugated into the *Yersinia* mutants. This limits there to be only a single copy of the integrated plasmid within the bacteria which would be replicated as part of the virulence plasmid and therefore put under the same regulatory mechanisms as the virulence plasmid.

There are both advantages and disadvantages for both cloning strategies. Using a low copy number plasmid is the least invasive of the two since the plasmid remains separate and under its own replication control unlike the suicide vector which is integrated into virulence plasmid at the site of homology. However, utilizing the integrated suicide vector would limit the number of copies of *copA/copB* and give an accurate representation of the activity of the genes under normal expression and control.

2.3.10 Bacterial conjugations and single crossover plasmid integrations

Suicide vector technologies were used for the bacterial conjugations in this project. The basis of which is outlined in figure 2.1. The suicide vector technology relies on the principle that the origin of replication is dependent on the production and binding of π, a protein which is encoded by *pir*. (68) When a *pir* dependent suicide vector such as pDM4.2 is transformed into a pir^- strain such as *Y. pseudotuberculosis*, plasmid replication cannot occur and will not pass onto daughter cells.

Due to this feature, suicide vectors are often used in conjugations to generate single crossover insertions to incorporate plasmids into genomic or other plasmids. After integration occurs, the suicide vector can undergo replication when the genomic DNA or plasmid replicates via its own origin of replication, rendering the inserted plasmid π independent.

Pir containing S-17 1 *E. coli* was transformed with the pir dependent pDM4.2 plasmid containing the appropriate reporter genes, the bacterial transformants were then grown overnight for 16 hours in LB containing ampicillin. The overnight donor cultures were centrifuged (8,000 x g for 4 mins) and gently washed with fresh LB before being mixed with the relevant recipient strain; *Y. pseudotuberculosis* parent, *ΔytbI/ΔypsI* or *ΔytbR/ΔypsR*. Each mating was done at four different ratios 1:1, 2:1, 4:1 and 10:1 (donor: recipient) as described

by Milton (66). 100 µl of the mixture was spot cultured onto a plate to grown overnight on YLB agar.

Two plates were set up for colony counting, a 'low concentration plate' which would contain 100µl of the bacterial suspension and a 'high concentration plate' which would contain 900µl of the bacterial suspension spun down and resuspended with 100µl of fresh YLB. The overnight bacterial colony was then harvested from the plates and resuspended in 1 ml of LB. 100 µl of this resuspended mixture was transferred and spread on a YLA containing ampicillin and nalidixic acid 'low concentration' plate. This low concentration plate was done to ensure countable colonies were seen if high numbers of bacteria were transformed. The remaining 900 µl mixture was centrifuged (8,000 x g for 4 mins), supernatant removed and resuspended in 100 µl fresh YLB and then inoculated on a second 'high concentration' agar plate containing AMP and NAL. This was to ensure that there were countable colonies if transformation numbers were low.

Overnight conjugation plates were then screened for light using the luminometer (Hamamatasu) to screen for light colonies. Light colonies were then picked, subcultured and colony PCRs (using LcrV_ comp 005_ SacI F+R primers) were done to check for the presence of pYV.

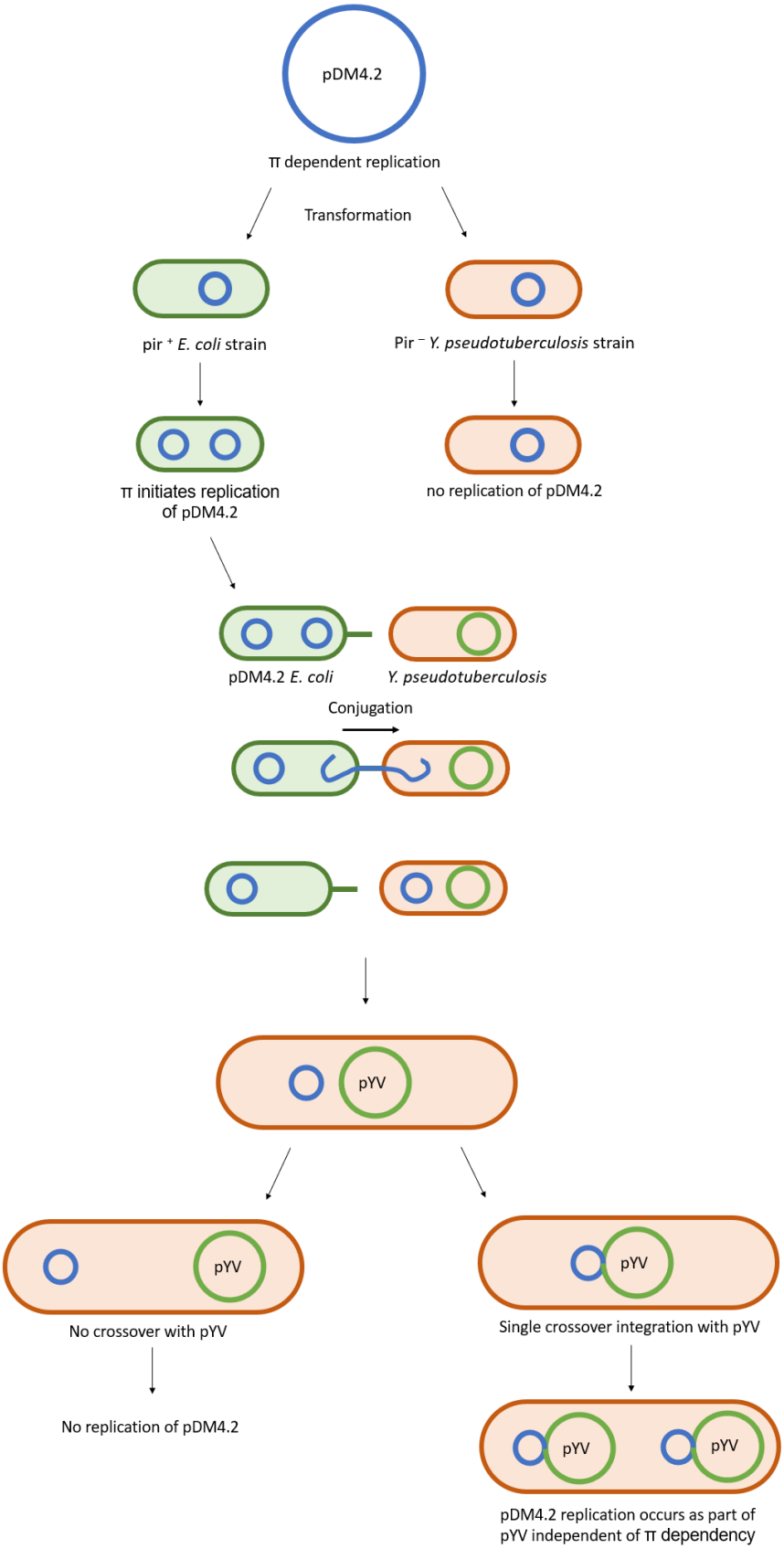


Figure 2.1: outline of the use of π dependent suicide vectors and their use in pir − and pir + strains.

2.4 Reporter activity – luminescence and cell density Tecan spectrophotometer assays

Reporter activity was measured using a luminometer/spectrophotometer Tecan Infinite (Tecan) method modified from Atkinson *et al* (33). Three separate overnight liquid cultures were grown for each of the three *Y. pseudotuberculosis* strains to be tested, taken from the inoculum of fresh overnight plate cultures. The overnight cultures were screened for light using the luminometer (HAMAMATSU), centrifuged (4,000 x g for 4 minutes), washed with fresh YLB and diluted to an OD_{600} between 0.018-0.022. 200 µl of each strain was transferred to individual wells of a clear flatbottomed black-walled 96 well-microtiter plate (Greiner) with the outermost wells being filled with 200µl of sterile distilled water to help prevent excessive dehydration figure 2.2. Due to evaporation of the water within the media results past 16 hours were not taken into consideration.

Absorbance was measured at 600nm (10nm bandwidth 25 flashes) followed by a luminescence measurement (1000ms integration time), automatically using a 30-minute kinetic cycle with a 1s linear shaking phase to agitate the sample and prevent the formation of biofilms before reading.

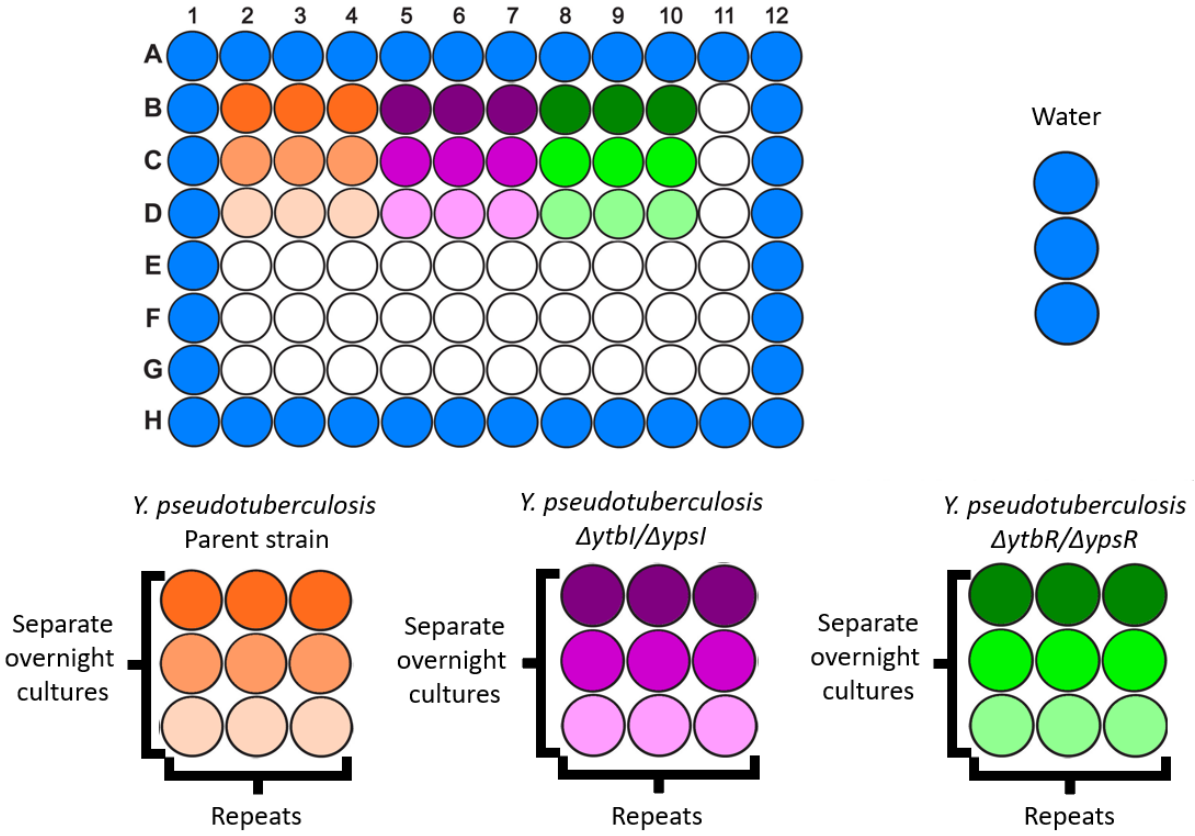


Figure 2.2 96 well plate layout of the reporter activity assay. Each well contains 200 µl of OD 0.018-0.022 overnight cultures. The edge wells of the plates were filled with 200 µl of sterile distilled water to reduce evaporation and drying out of samples. Well colours represent the repeat triplicate samples

2.5 AiiA assay – Luminescence and cell density Tecan spectrophotometer assay

Overnight cultures of *E. coli* containing the pGEMT::*aiiA* for the production of the AHL degrading enzyme AiiA were grown in non-selective YLB media and centrifuged (13,000 x g, 2 minutes). The supernatant was filtered through a 0.2 µm filter to remove bacterial cells and retained. Bioluminescence/absorbance was measured as described in section 2.2 but with the addition of the spent *E. coli* supernatant added to 100 µL of fresh YLB media before inoculation and dilution of the overnight reporter strains.

CHAPTER 3

Results

3.1 Lux reporter cloning strategy

To explore the relationship between the quorum sensing system and the control of RepA-mediated plasmid replication, reporter genes were constructed that utilized the luxCDABE cassette as a bioreporter to measure gene activity of the genes *copA* and *copB*. The strategy outlined in figure 3.1 shows the initial cloning steps used in the construction of the reporter

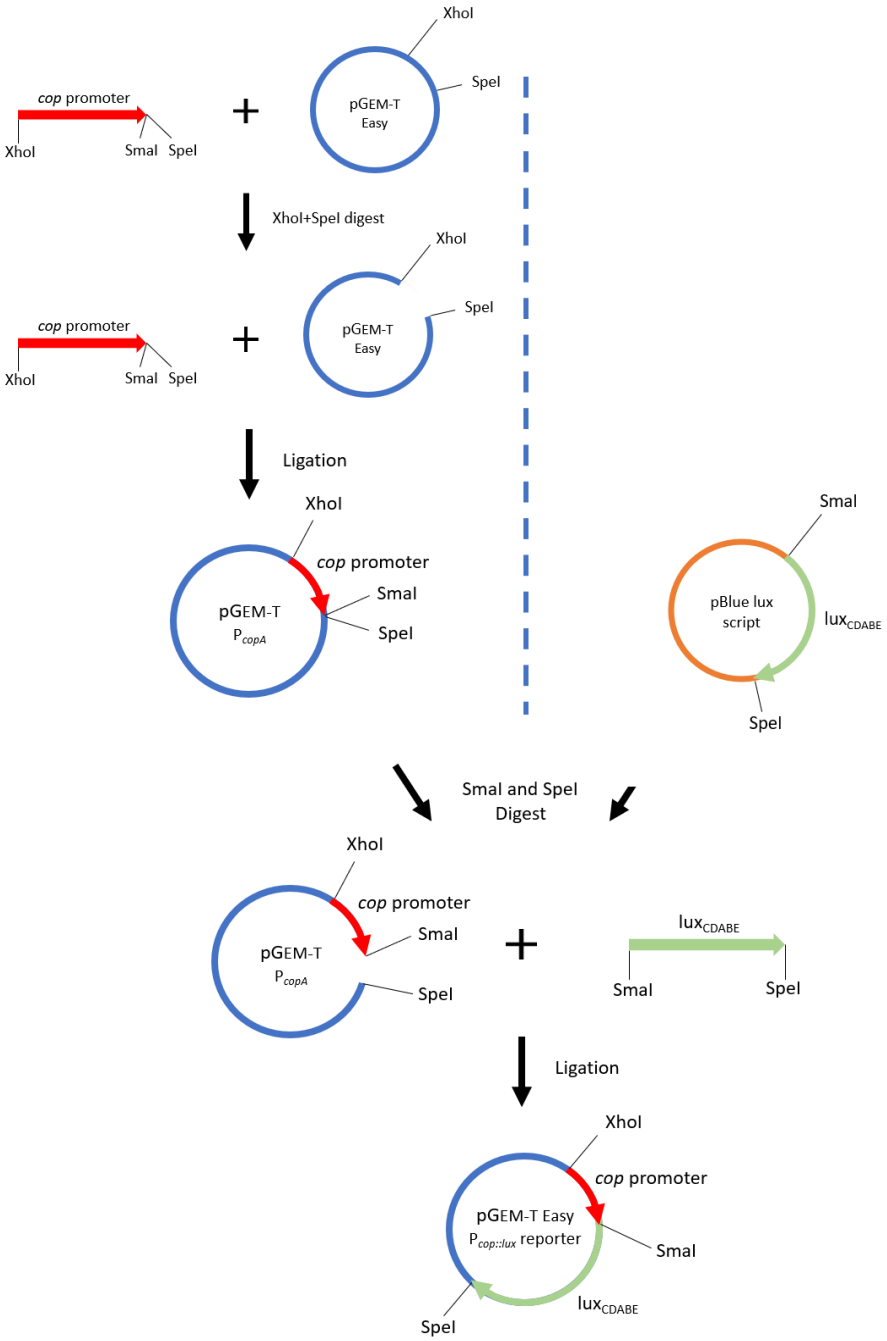


Figure 3.1 The cloning strategy for the production of the $P_{copA::lux}$ and $P_{copB::lux}$ reporter in pGEM-T Easy. The cop promoters were cut and inserted into a linearized pGEM-T Easy vector (XhoI and SpeI). *luxCDABE* was then digested out of pBlue Lux script (SmaI and SpeI) and ligated into the pGEM-T P_{copA} to produce the fully assembled reporter plasmid

sequence. This pGEM-T vector contained a lux reporter consisting of a promoter region of copA/copB and the lux cassette.

3.2 Production of template DNA

Before the assembly of the *cop* reporter began the promoter regions of *copA* and *copB* were PCR amplified using Q5 polymerase to incorporate the XhoI, SpeI and SmaI restriction sites (figure 3.1).

For the template, a genomic DNA extraction was performed on *Y. pseudotuberculosis* YPIII parent yielding 101.4 ng/µL (Nanodrop) that when analysed by agarose gel electrophoresis produced a single large band of >10,000 bp consistent with the presence of chromosomal DNA (figure3.2).

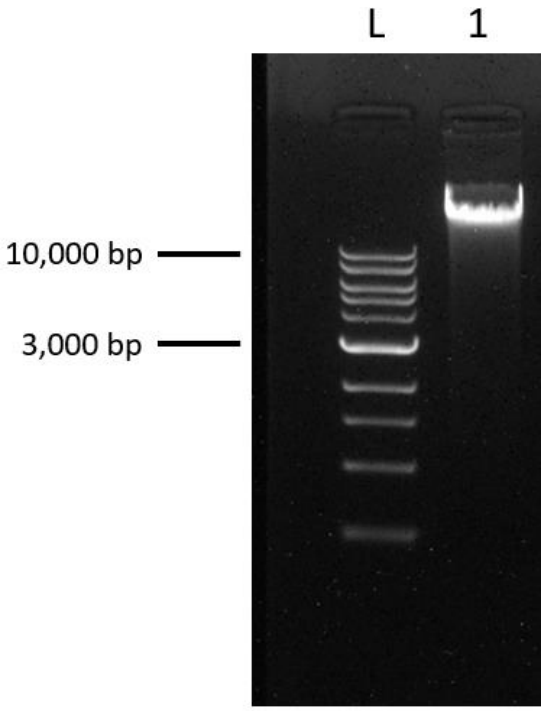


Figure 3.2. 1% agarose gel containing 1x SYBR safe DNA stain 60min 70V Lane 1, NEB Pre-stained 1kb ladder Lane 2, genomic prep extract from *Y. pseudotuberculosis* YPIII which showed a single very large band >10,000kb consistent with the presence of chromosomal DNA.

Additional PCR checks were performed to confirm that the DNA was derived from *Y. pseudotuberculosis* using primers; *copB* (copB-F + copB-R) and *lcrV* (LcrV_ comp 005_ KpnI_F + LcrV_ comp 005_ SacI_R). It was important to confirm the presence of the cop operon as well as the presence of *lcrV* as a marker for the presence of the pYV virulence plasmid. A control PCR was aso performed using the same primer pairs and a previously prepared chromosomal DNA sample (V. Garcia, 2021 personal communication)

Figure 3.3 reveals that both the extracted chromosomal DNA and the control sample contained both the genes *copB* as well as *lcrV*, through their presence of the 328 bp (*copB*) and 900 bp (*lcrV*) PCR, confirming the presence of the pYV virulence plasmid.

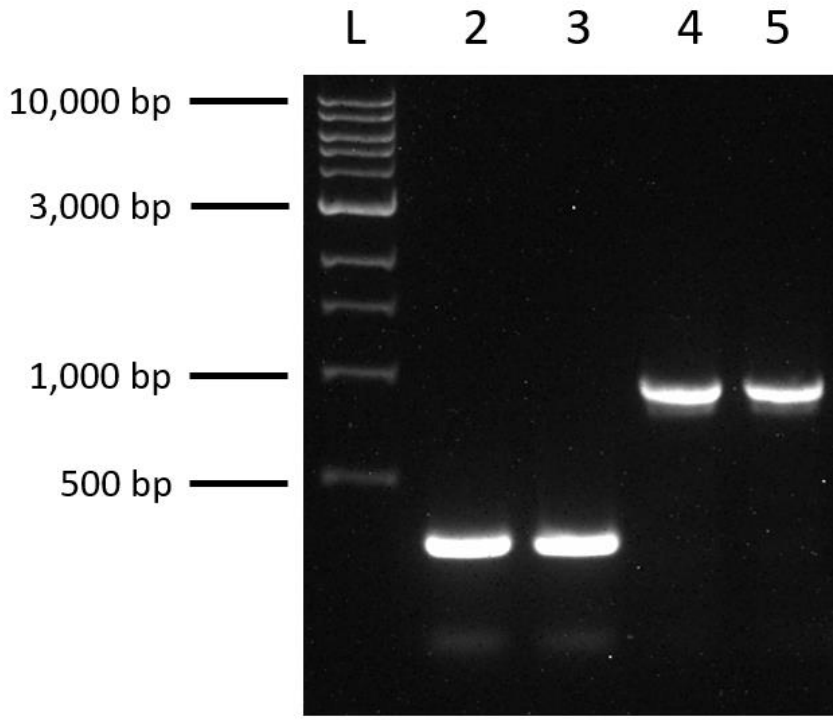


Figure 3.3: 1% agarose gel containing 1x SYBR safe DNA stain 70min 70V

Lane 1, NEB Pre-stained 1 kb ladder Lane 2, *copB* (VG) 328 bp Lane 3, *copB* (JP) 328 bp Lane 4, *lcrV* (VG) 900 bp Lane 5, *lcrV* (JP) 900 bp

The PCR confirmed that the template DNA was correct and also successfully amplified *copB* that was to be used in the construction of the *copB* promoter fusion. The remaining *copB* PCR product was therefore purified and stored. *copA* was then PCR amplified to incorporate the same restriction sites as *copB* (Figure 3.1) and the promoter PCR product purified.

copA and *copB* PCR products were then purified using a gel clean-up kit and analysed by agarose gel electrophoresis which revealed two distinct clean bands of *copA* (180 bp) and *copB* (328 bp) as seen in Figure 3.4.

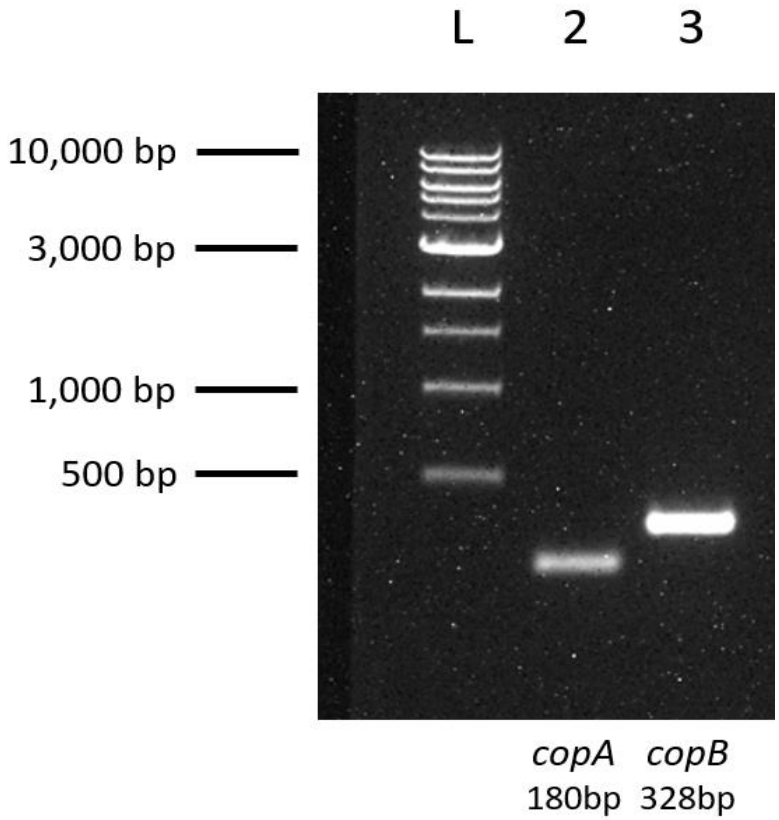


Fig. 3.4: 1 % agarose gel containing 1x SYBR safe DNA stain 60 min 70 V. Lane 1 (L), NEB Pre-stained 1 kb ladder Lane 2, copA purified PCR fragment 180 bp Lane 3, copB purified PCR fragment 328 bp.

3.3 Cloning copA and copB

After purification the copA and *copB* PCR product promoter regions were cloned into pGEM-T Easy which contains a single thymine 3' overhang at either end of the plasmid facilitating ligation to a complementary 5' adenine.

To generate the 5' A overhang necessary for this ligation to occur the purified promoter sequences would need to be A-tailed with a taq DNA polymerase, since the Q5 family DNA polymerase generates blunt ends without the single adenine overhang common to non-proofreading taq polymerases. Once A-tailed, pcopA and pcopB were cloned into pGEM-T Easy and grown on X-gal ampicillin YLB agar. White colonies were picked for subculturing and screened using a colony PCR, using primer pairs copA-F + copA-R (*copA*) and copB-F + copB-R (*copB*) to confirm the presence of P_{copA} and P_{copB}. Plasmid minipreps from PCR positive colonies were checked by restriction analysis (Figure 3.5) and confirmed the presence of *copA* and *copB* in pGEM-T Easy which were subsequently named pJKEA and pJKEB.

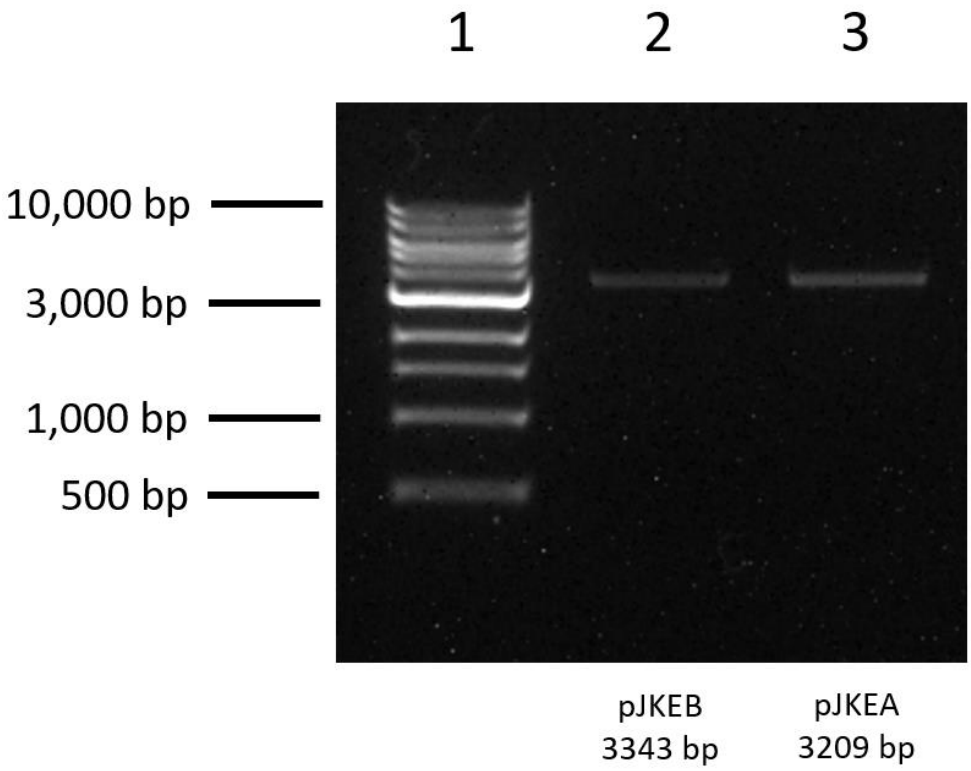


Figure. 3.5: 1 % agarose gel containing 1x SYBR safe DNA stain 25 min 80 V. Lane 1, NEB Pre-stained 1 kb ladder Lane 2, purified pJKEB 3343 bp linearized (XhoI) Lane 3, copA purified pJKEA 3209 bp linearized (XhoI).

3.4 Assembly of the lux reporter gene

In order to clone *luxCDABE* downstream of P_{copA} or P_{copB} the *luxCDABE* cassette was excised from pBlue*lux* as a SpeI and SmaI fragment and analysed by agarose gel electrophoresis (figure 3.6). The band corresponding to the 5798bp luxCDABE fragment was excised, purified and cloned into identically digested pJKEA and pJKEB.

Due to the large difference in size between the vector (3 kb) and lux cassette (5.7 kb) a series of 5 insert to vector ratios (1:1, 2:1, 3:1, 5:1, 10:1) were set up to facilitate ligation. After plating on X-gal and ampicillin YLA colonies were screened for bioluminescence using a Hamamatsu high sensitivity luminometer. Light producing colonies were picked, subcultured and screened using copA-F + copA-R (P_{copA}) and copB-F + copB-R (P_{copB}).

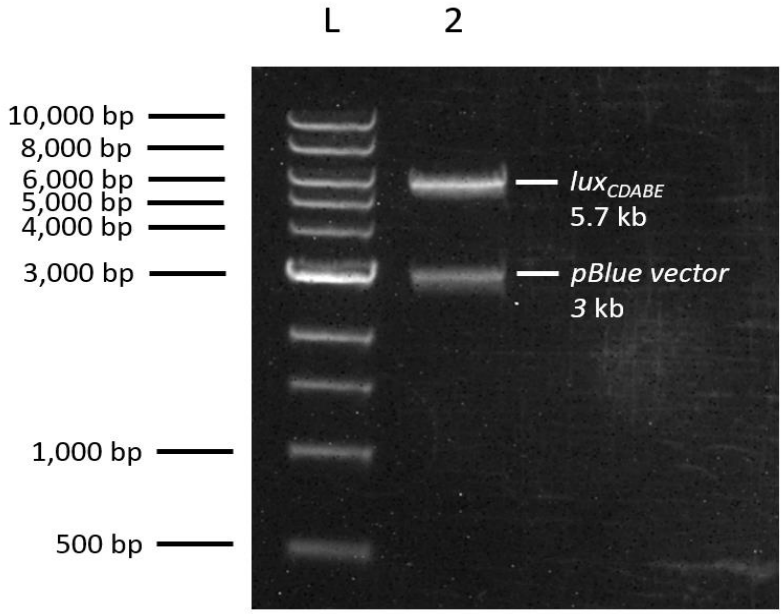
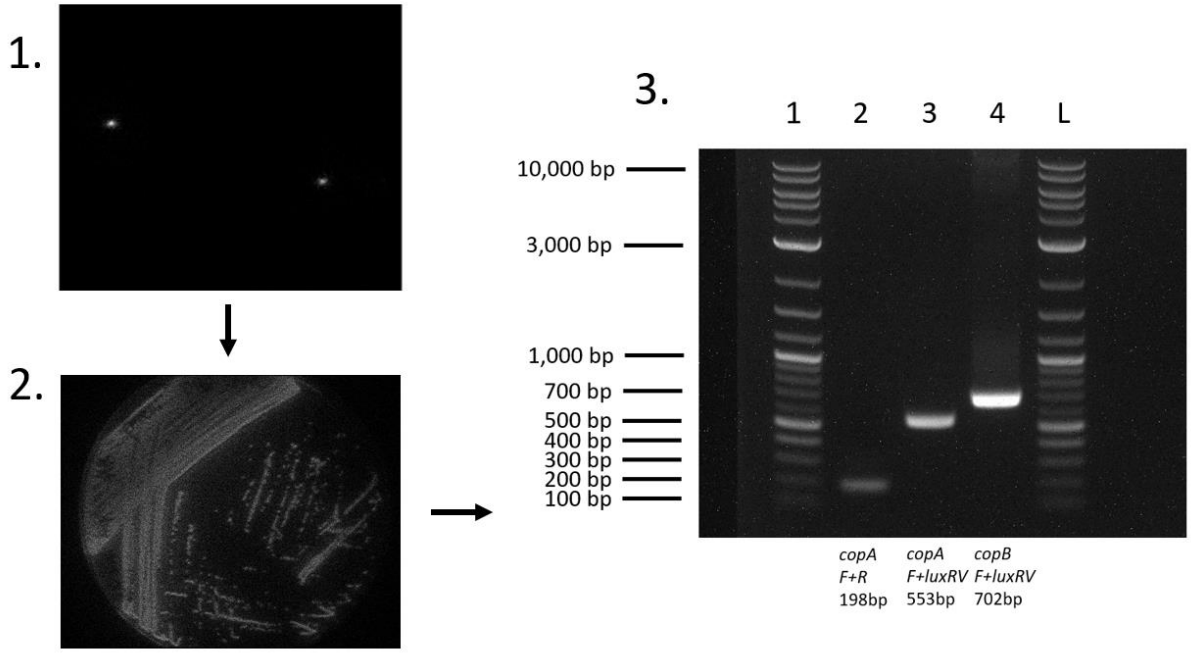


Figure 3.6. 0.8 % agarose gel containing 1x SYBR safe DNA stain 60 min 70 V. Lane 1, NEB Pre-stained 1 kb ladder Lane 2, pBlue luxscript plasmid cut with the two enzymes SmaI and SpeI. The larger 5.7 kb band contained the dropped out luxCDABE cassette, while the smaller 3 kb band contained the pBluelux backbone.

Additionally, a second PCR screen was performed (Figure 3.7) using the forward primers copA-F (P_{copA}) and copB-F (P_{copB}) which would anneal to the P_{copA} / P_{copB} and luxCRv (*luxC*) to generate a PCR product which contains the entirety of the P_{copA}/P_{copB} region and the first 355 bp of the *luxC*. The use of this secondary PCR allows for both the conformation of the $P_{cop::luxCDABE}$ fusion and for confirming the orientation of the inserted lux cassette.



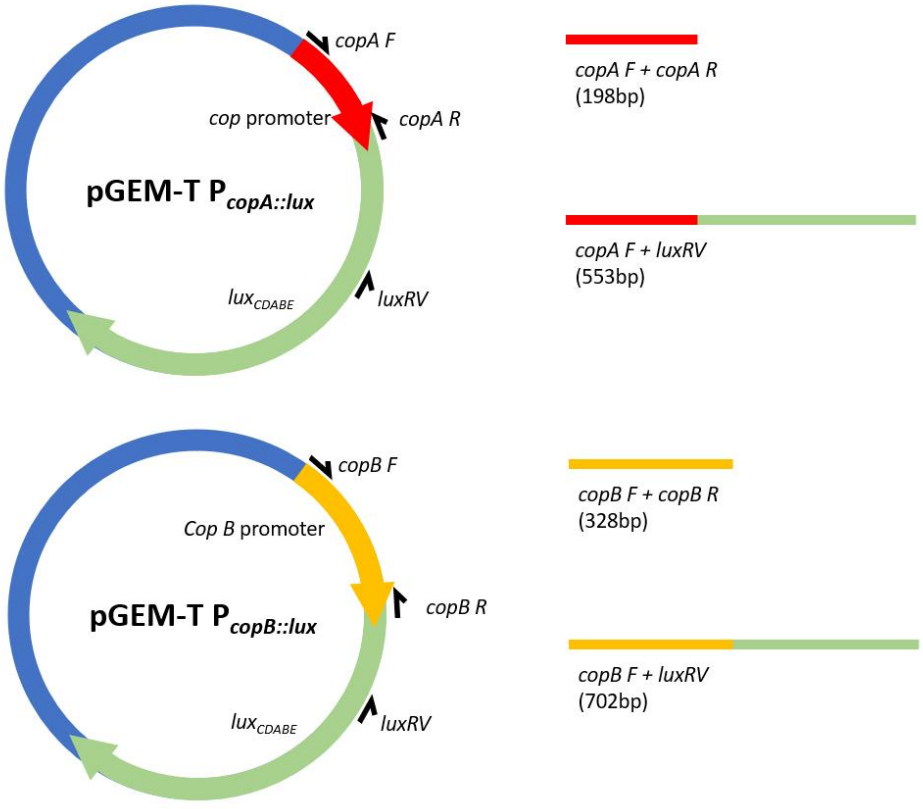


Figure 3.7: Screening process for $P_{cop::lux}$ 1. Two transformed colonies containing the lux reporter plasmid. 2. Subculture plate from original transformed colony. 3. Colony PCR screen using forward and reverse primers. Example screen; lane 2 shows the 198 bp fragment of $P_{copA::lux}$ produced from copAF+R primers, Lane 3 contains a PCR of $P_{copA::lux}$ containing copA F+*luxRv* primers, the increase in size is due to the additional 355 bp of the *luxC* region. Lane 4 contains the generated 702 bp $P_{copB::lux}$ fragment using copB F +lux R primers.

3.5 Incorporation of the lux reporter into PDM4.2

To stably integrate $P_{cop::lux}$ reporter gene into pYV the suicide vector pDM4.2 was digested with XhoI and SpeI. pGEM $P_{copA::lux}$ and pGEM $P_{copB::Lux}$ reporter plasmids were similarly digested and cloned into the complementary sites. Positive colonies were screened for bioluminescence followed by a colony PCR using the primer pairs copA-F/copB-F and luxCRv to confirm the presence of the $P_{cop::lux}$ reporter (figure 3.8).

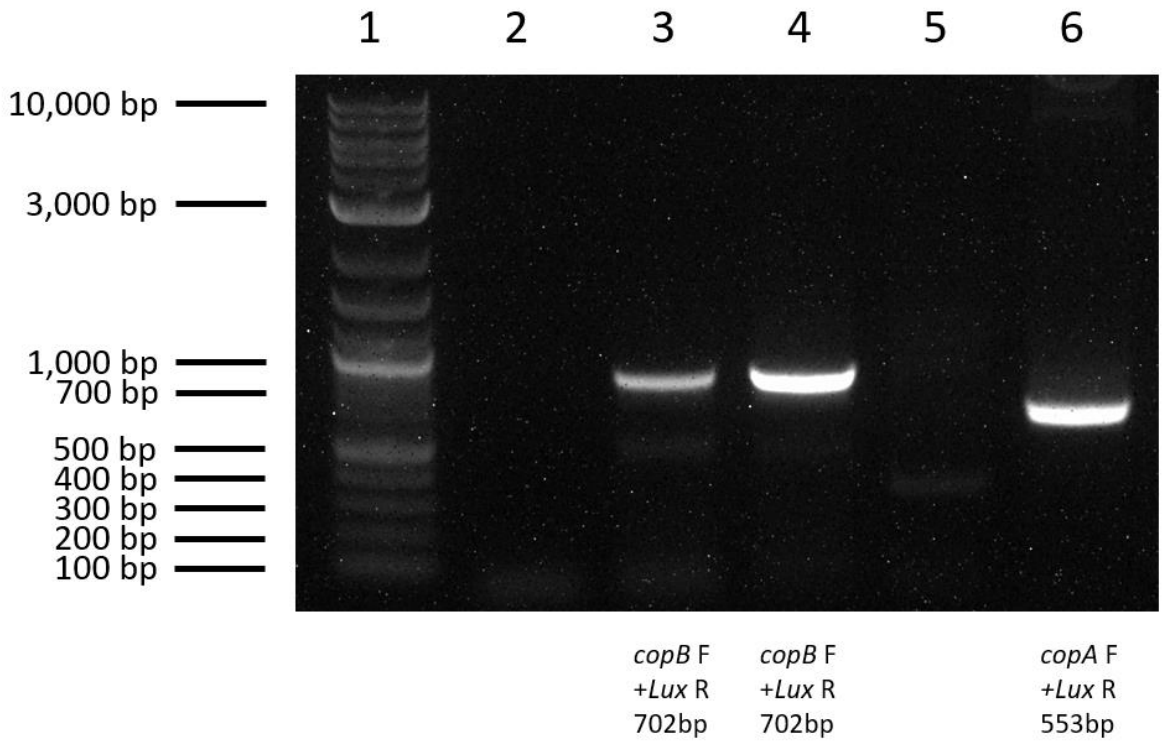


Figure 3.8 0.8 % agarose gel containing 1x SYBR safe DNA stain 60 min 70 V. Lane 1, NEB 1kb ladder plus. Lane 2, colony negative for $P_{copB::lux}$ reporter. Lane 3, 702bp $P_{copB::lux}$ positive colony. Lane 3, 702bp $P_{copB::lux}$ positive colony. Lane 4, negative colony for $P_{copA::lux}$. Lane 5, 553bp $P_{copA::lux}$ positive colony.

pGEM CopA::Lux and pGEM CopB::Lux reporter plasmids were then digested using XhoI and SpeI to drop out the reporter genes. pDM4.2 was also treated with XhoI and SpeI to generate the complementary overhangs for ligation. The pDM4.2 *cop::lux* products were ligated and transformed into S-17-1 λpir *E. coli* and grown on 100 µg/mL ampicillin containing agar. Colonies were then screened for lux activity followed by a colony PCR to confirm the presence of both the *cop* (328 bp) and *cop*+lux (702 bp) fragments.

3.6 Conjugation of pDM4.2 copA::lux / copB::lux

To stably integrate pDM4.2 *cop::lux* into the pYV virulence plasmid as a single cross over event conjugations were performed between the pDM4.2 *cop::lux* S-17 *E. coli* doner strains and the WT Parent, *ΔytbI/ΔypsI* and *ΔytbR/ΔypsR Y. pseudotuberculosis* recipients.

Bioluminescent positive colonies were screened by colony PCR for the presence of *copA::lux/copB::lux* using copA-F/copB-F + luxCRv for *Yersinia yopJ* using YopJ-F + YopJ-R (Figure 3.9). This confirmed the presence of both the *pDM4.2 cop::lux* reporter plasmid and the pYV virulence plasmid.

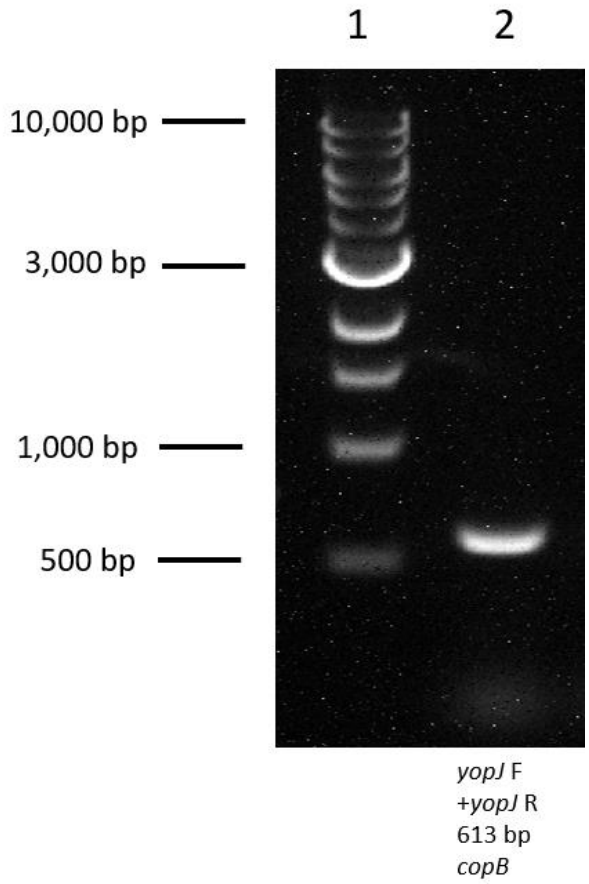


Figure 3.9 0.8 % agarose gel containing 1x SYBR safe DNA stain 60 min 70 V. Lane 1, NEB Pre-stained 1 kb ladder Lane 2, 613bp *yopJ* PCR fragment confirming the presence of the pYV virulence plasmid in *pDM4.2 copB::lux* strain.

pDM4.2 *copB::lux* was successfully incorporated to each of the *Yersinia* strains with each of the conjugation plates containing >50 colonies. However, despite repeated attempts *pDM4.2 copA::lux* failed to conjugate into any of the mutants and the conjugation plates did not produce any colonies.

3.7 Incorporation of lux reporter into pHG327 – large Klenow fragment cloning

Due to the lack of unique cloning sites between the reporter gene and *pHG327* a different approach was taken to cloning which is outlined in figure 3.10, utilizing large Klenow fragment a cleaved component of DNA polymerase I.

Because of the conjugation issues with *copA* in pDM4.2, the *copA* reporter was cloned into the low copy number vector, pHG327. The *copA::lux* reporter was excised from *pGEM copA::lux* using XhoI and SpeI and pHG327 was linearised with EcoRI. Both pHG327 and the *copA::lux* fragment were then treated with Klenow and dNTPs to blunt both ends of the DNA strands. Following ligation, positive colonies were screened for light production and the presence of *copA::lux* confirmed by PCR using the primers copA-F and luxCRv.

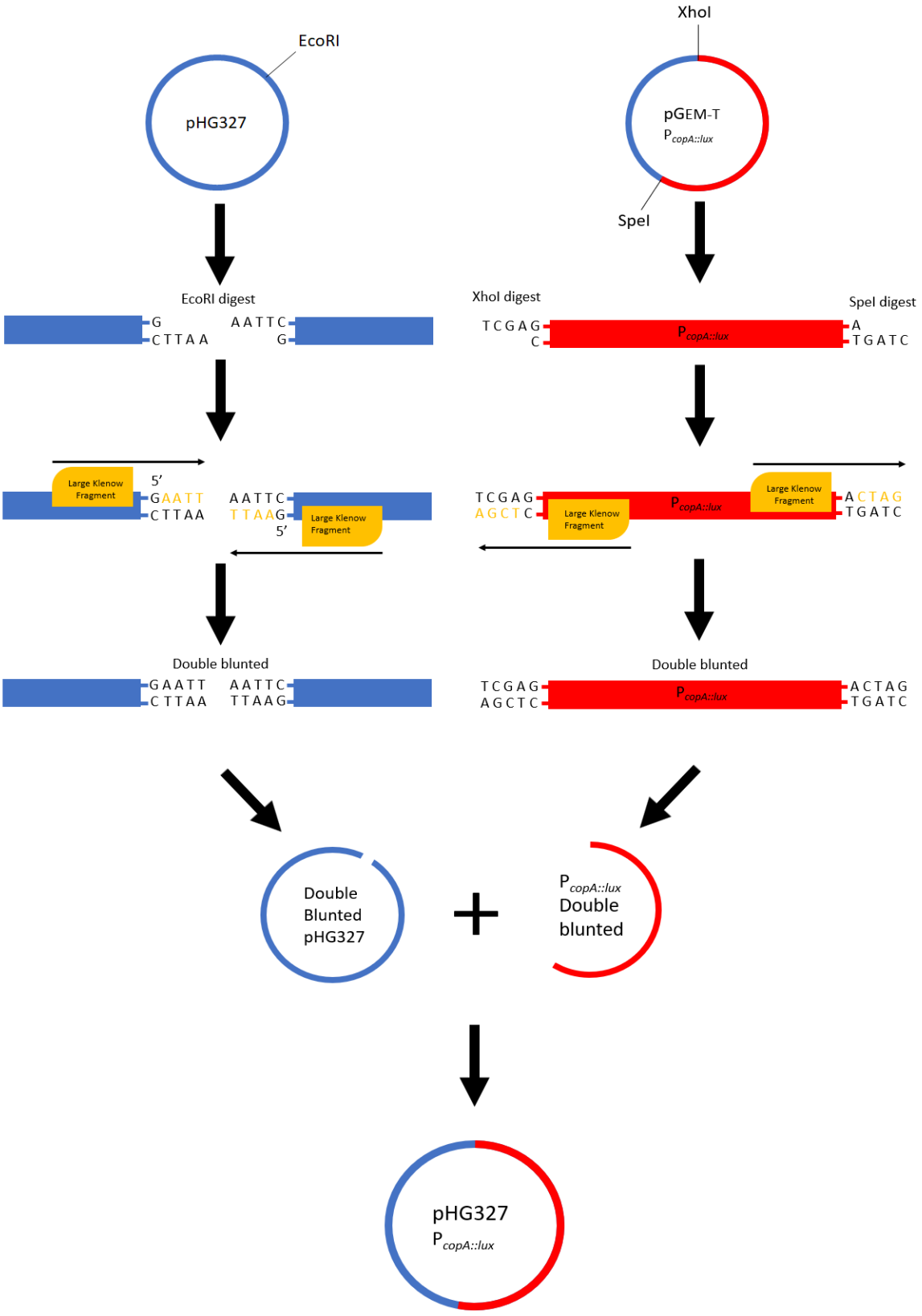


Figure 3.10 Cloning strategy for Large Klenow Fragment cloning of $P_{copA::lux}$ into pHG327

The XhoI and SpeI sites were removed due to the process of cloning using Klenow, making screening using XhoI and SpeI impossible. *pHG327* P*copA::lux* was instead, digested with BalI and BamHI and analysed by agarose gel electrophoresis (figure 3.11) which produced the expected 5.9 kb band of $P_{copA::lux}$ and the 3.3 kb pHG327 backbone fragment.

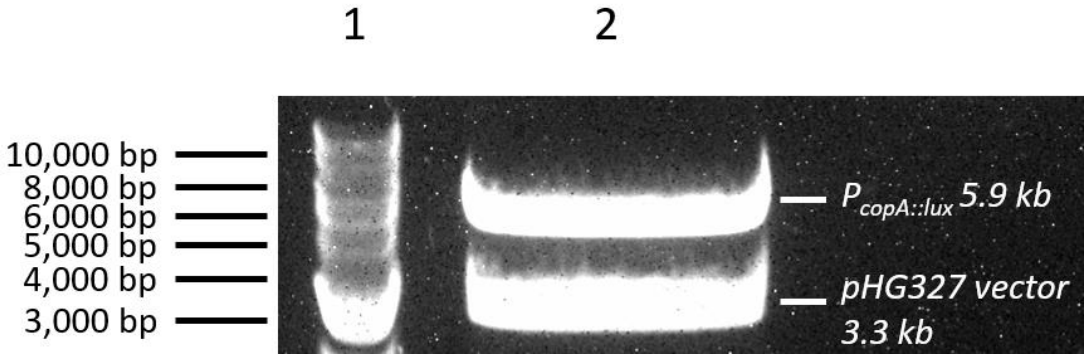


Figure 3.11: 1 % agarose gel containing 1x SYBR safe DNA stain 60 min 70 V. Lane 1, NEB Pre-stained 1 kb ladder Lane 2, pHG327 $P_{copA::lux}$ plasmid cut with the two enzymes BalII+ BamhI. The larger 5.9 kb band contained the dropped out $P_{copA::lux}$, while the smaller 3.3 kb band contained the pHG327 vector backbone.

A final screen was done before transformation using *copA* F+R and *copA* F+luxRv to confirm the insertion of the reporter gene into pHG327 (figure 3.10) which yielded the expected bands for *copA* (198 bp) and *copA*+*luxC* (553 bp).

pHG327 copA::lux was then transformed into the three chemically competent *Y. pseudotuberculosis* strains: parent, *ΔytbI/ΔypsI* and *ΔytbR/ΔypsR* which were screened for bioluminescence.

The reporter gene for copA $P_{copA::lux}$ was prioritized for cloning into the pHG327 vector and used to test and troubleshoot this cloning strategy, with the later intent of cloning *copB* into pHG327. Unfortunately, due to time constrains $P_{copB::lux}$ was not cloned into the pHG327 vector as was originally intended.

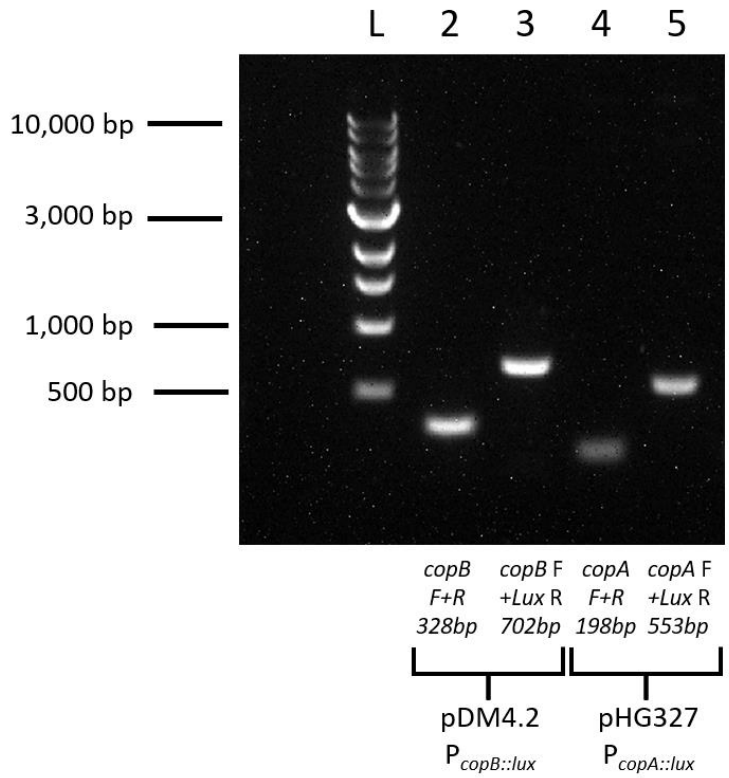


Figure 3.12: PCR screens of both the pDM4.2 $P_{copB::lux}$ and pHG327 $P_{copA::lux}$ reporters. L contained NEB 1 kb ladder. 2. 328 bp *copB* fragment from copB F+R primers. 3. 702 bp *copBluxC* fragment from copB F+luxCRv primers. 4.198 bp copA fragment from copA F+R primers. 4. 553 bp *copAluxC* fragment from *copA* F+luxRV primers.

3.8 Reporter activity Tecan assays

Reporter activity was measured using a Tecan spark microplate reader with OD_{600} and Luminosity (RLU) measured over 18 hours with RLU/OD_{600} providing a measurement of promoter activity with regards to population density.

Promoter activity was measured at two different temperatures; 26°C and 37°C. These temperatures were chosen to emulate the different temperature ranges that *Y. pseudotuberculosis* populations occupy when inside and outside a host.

3.8.1 *Y. pseudotuberculosis* $P_{copB::lux}$ pDM4.2 growth experiments

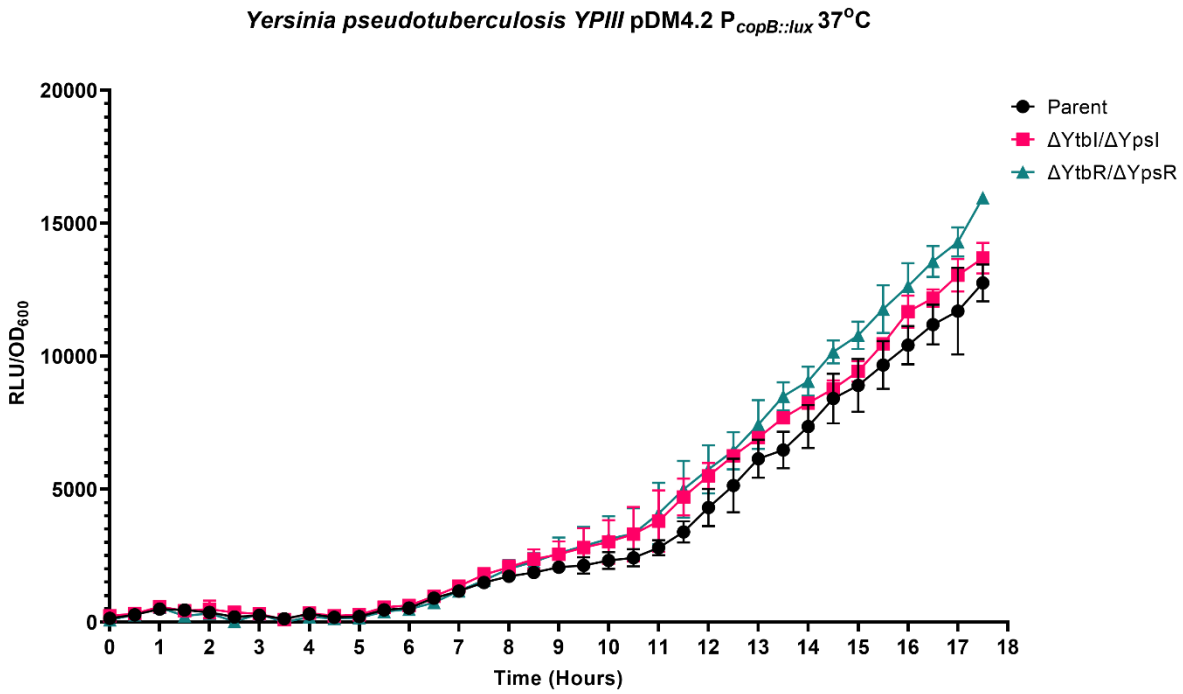


Figure 3.13: Activity assay of pDM4.2 $P_{copB::lux}$ in the three *Y. pseudotuberculosis* strains; Parent, *ΔytbI/ΔypsI* and *ΔytbR/ΔypsR* grown at 37°C over 18 hours. Luminosity (RLU) and Cell density (OD measured at 600nm) was measured every 30 minutes over 36 times points. RLU/OD_{600} was used to measure activity of the reporter genes based on lux activity per given cell density. Vertical bars represent standard deviation. OD_{600} data shown in appendix 1.

copB activity was measured at 37°C in the *Y. pseudotuberculosis* parent and quorum sensing mutants carrying pDM4.2 $P_{copB::lux}$ and revealed that $P_{copB::lux}$ activity was similar in all three *Y. pseudotuberculosis* strains. During the first 7 hours of growth the activity of $P_{copB::lux}$ was low, there was a noticeable increase in the activity of $P_{copB::lux}$ around 7 hours (Figure 3.13) which continued to increase until the end of the experiment at 18 hours.

copB was measured at 26°C in the *Y. pseudotuberculosis* parent and quorum sensing mutants carrying pDM4.2 $P_{copB::lux}$ and revealed that *copB* was inactive throughout the entire 18 hours. The measured values obtained from this experiment fell within the levels for background noise and have been plotted in figure 3.14 for comparison with the 37°C data set shown in figure 3.13.

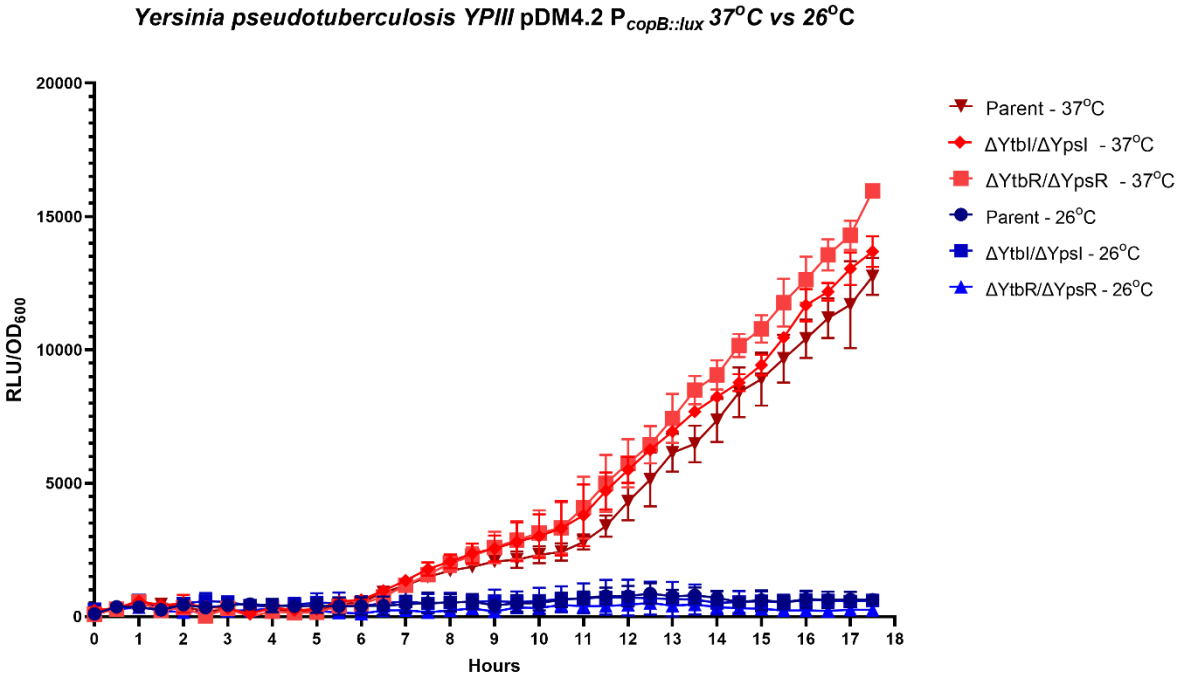


Figure 3.14: comparison of $P_{copB::lux}$ activity when grown at 26°C and 37°C in the three *Y. pseudotuberculosis* strains; Parent, *ΔytbI/ΔypsI* and *ΔytbR/ΔypsR*. Data from figure 3.13 was plotted with the data from the temperature experiment to compare the difference in activity of the promoter at 26°C and 37°C. . Vertical bars represent standard deviation. 37°C and 26°C OD_{600} data shown in appendix 1 and 2.

3.8.2 *Y. pseudotuberculosis* $P_{copA::lux}$ pHG327 37°C growth experiments

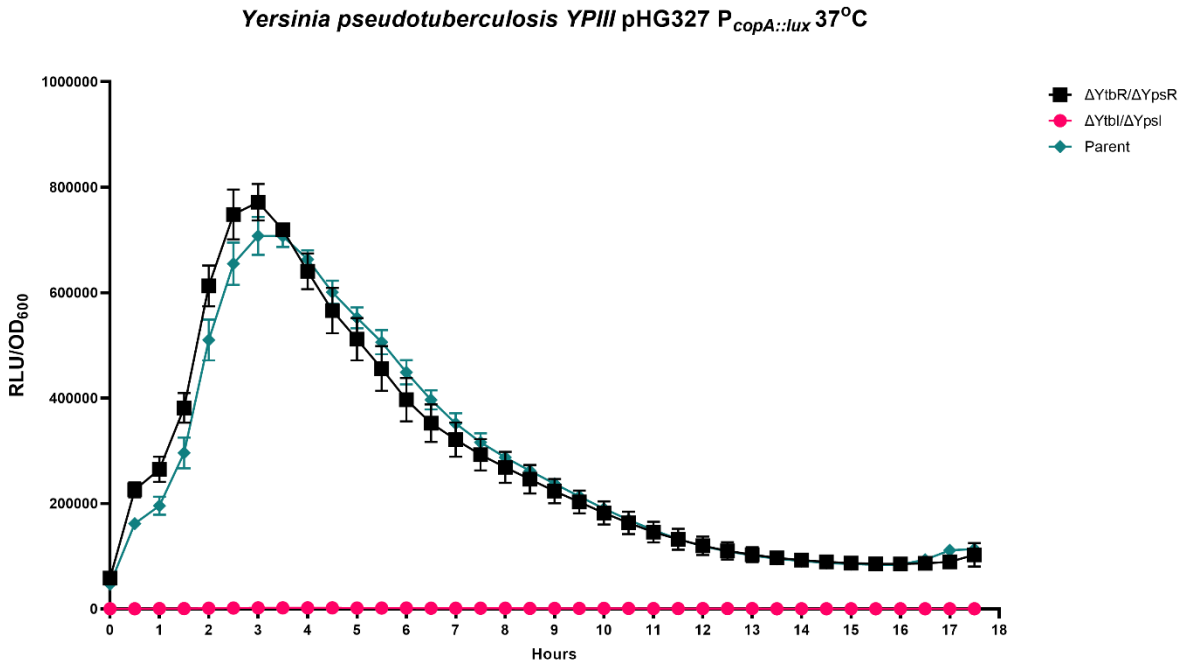


Figure 3.15: Activity assay of pHG327 $P_{copA::lux}$ in the three *Y. pseudotuberculosis* strains; Parent, *ΔytbI/ΔypsI* and *ΔytbR/ΔypsR* grown at 37°C over 18 hours. Luminosity (RLU) and Cell density (OD measured at 600nm) was measured every 30 minutes over 36 times points. RLU/OD_{600} was used to measure activity of the reporter genes based on lux activity per given cell density. Vertical bars represent standard deviation. OD_{600} data shown in appendix 3.

copA activity was measured at 37°C in the *Y. pseudotuberculosis* parent and quorum sensing mutants carrying pHG327 $P_{copA::lux.}$. In the parent and *ΔytbR/ΔypsR* the activity of $P_{copA::lux}$ showed a rapid increase to approximately 3 hours, after which activity fell up to 12 hours and then stabilised to the end of the experiment (figure 3.15). However, $P_{copA::lux}$ in *ΔytbI/ΔypsI* appears to be extremely low when compared to the parent and the *ΔytbR/ΔypsR* mutant (figure 3.15).

When $P_{copA::lux}$ activity in *ΔytbI/ΔypsI* is plotted alone the profile of the curve was comparable to the parent and *ΔytbR/ΔypsR* suggesting that there is the same rates of change of $P_{copA::lux}$ activity but just at much lower level (figure 3.16).

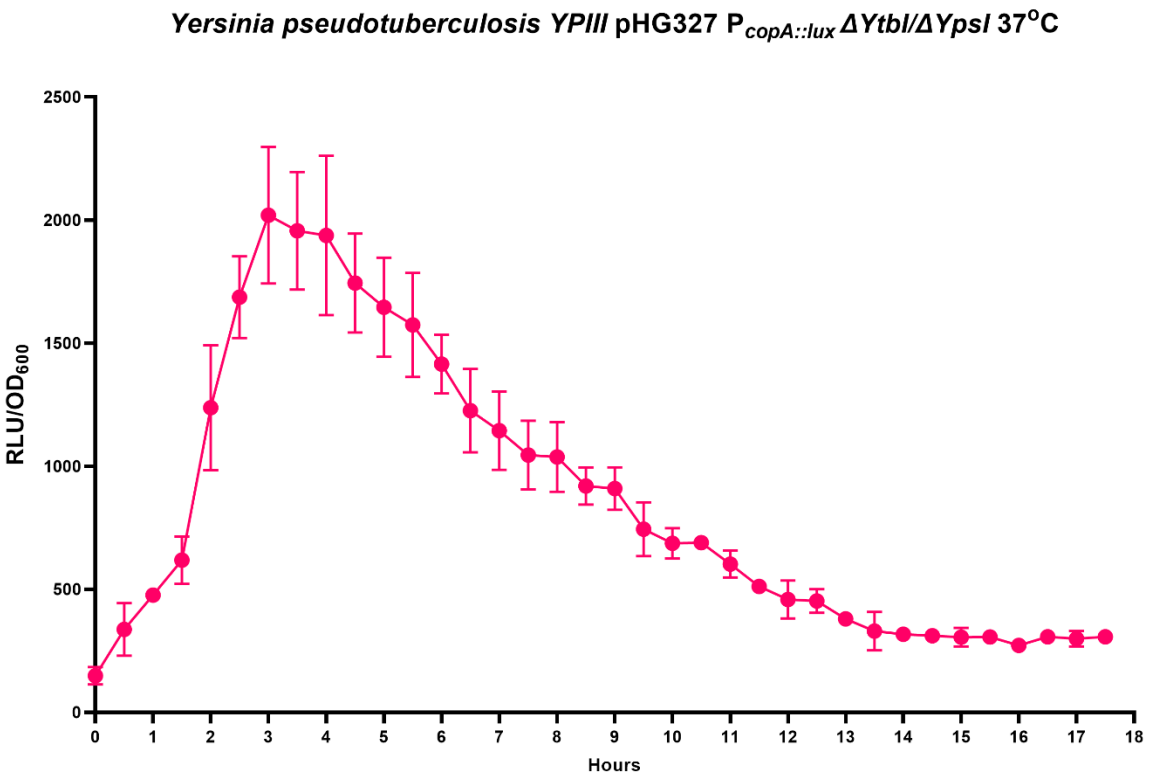


Figure 3.16: Activity assay of pHG327 $P_{copA::lux}$ in *ΔytbI/ΔypsI*. Luminosity (RLU) and Cell density (OD measured at 600nm) was measured every 30 minutes over 36 times points. RLU/OD_{600} was used to measure activity of the reporter genes based on lux activity per given cell density. *ΔytbI/ΔypsI* was plotted independently of the parent and *ΔytbR/ΔypsR* to show the activity profile of the strain which is consistent with the parent and *ΔytbR/ΔypsR* just at much lower levels. Vertical bars represent standard deviation. OD_{600} data shown in appendix 3.

3.8.3 *Y. pseudotuberculosis* $P_{copA::lux}$ pHG327 26°C growth experiments

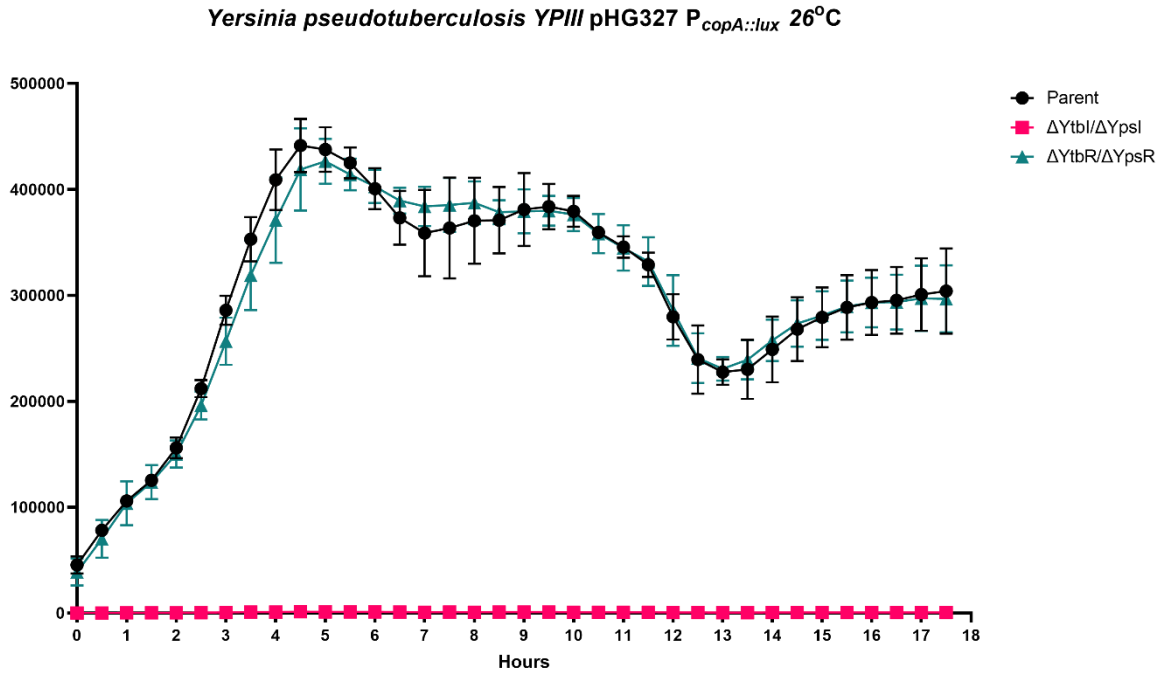


Figure 3.17: Activity assay of pHG327 $P_{copA::lux}$ in the three *Y. pseudotuberculosis* strains; Parent, *ΔytbI/ΔypsI* and *ΔytbR/ΔypsR* grown at 37°C over 18 hours. Luminosity (RLU) and Cell density (OD measured at 600nm) was measured every 30 minutes over 36 times points. RLU/OD_{600} was used to measure activity of the reporter genes based on lux activity per given cell density. Vertical bars represent standard deviation. OD_{600} data shown in appendix 4.

copA activity was measured at 26°C in the *Y. pseudotuberculosis* parent and quorum sensing mutants carrying pHG327 $P_{copA::lux.}$. In the parent and *ΔytbR/ΔypsR* the activity of $P_{copA::lux}$ showed a rapid increase to approximately 5 hours, after which activity remained high and fluctuated before stabilising at hour 14 (figure 3.17). Again, $P_{copA::lux}$ in *ΔytbI/ΔypsI* appears to be extremely low when compared to the parent and the *ΔytbR/ΔypsR* mutant (figure 3.17).

Similarly to the results at 37°C, when plotted alone $P_{copA::lux}$ activity in *ΔytbI/ΔypsI* shows a profile comparable to the parent and *ΔytbR/ΔypsR* suggesting again that there is the same rates of change of $P_{copA::lux}$ activity but just at much lower level (figure 3.18).

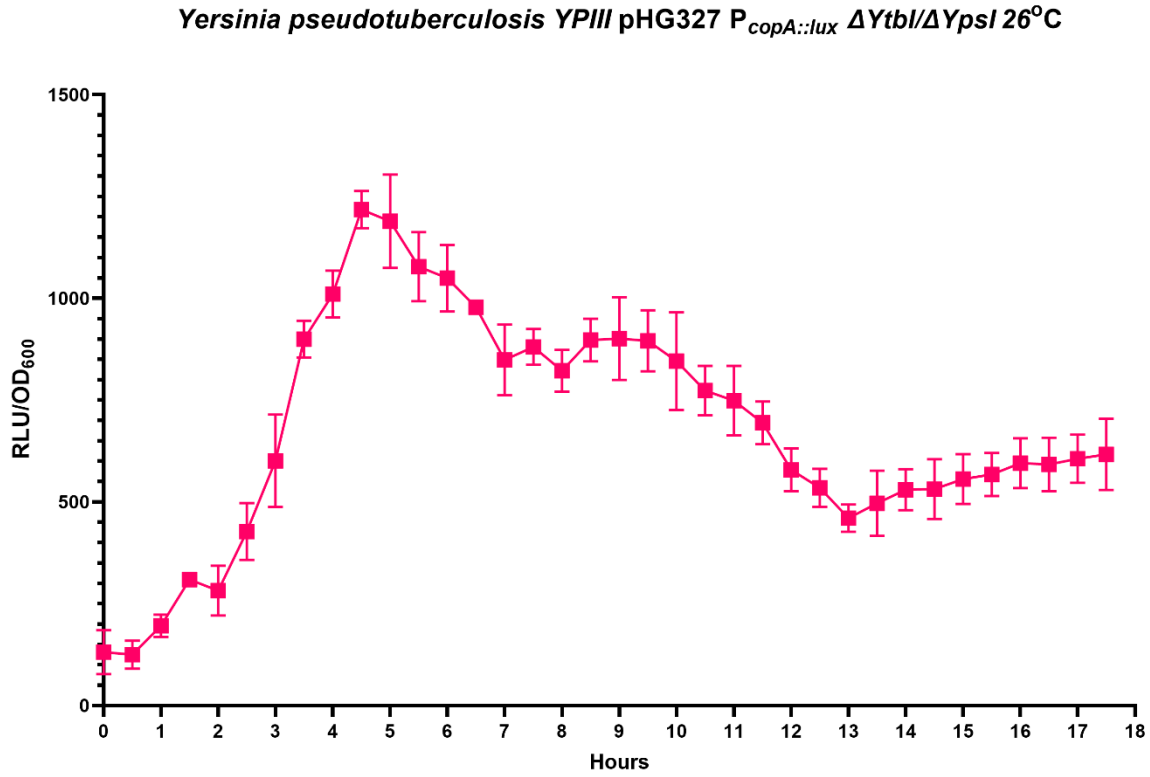


Figure 3.18: Activity assay of pHG327 $P_{copA::lux}$ in *ΔytbI/ΔypsI*. Luminosity (RLU) and Cell density (OD measured at 600nm) was measured every 30 minutes over 36 times points. RLU/OD_{600} was used to measure activity of the reporter genes based on lux activity per given cell density. *ΔytbI/ΔypsI* was plotted independently of the parent and *ΔytbR/ΔypsR* to show the activity profile of the strain which is consistent with the parent and *ΔytbR/ΔypsR* just at much lower levels. Vertical bars represent standard deviation. OD_{600} data shown in appendix 4.

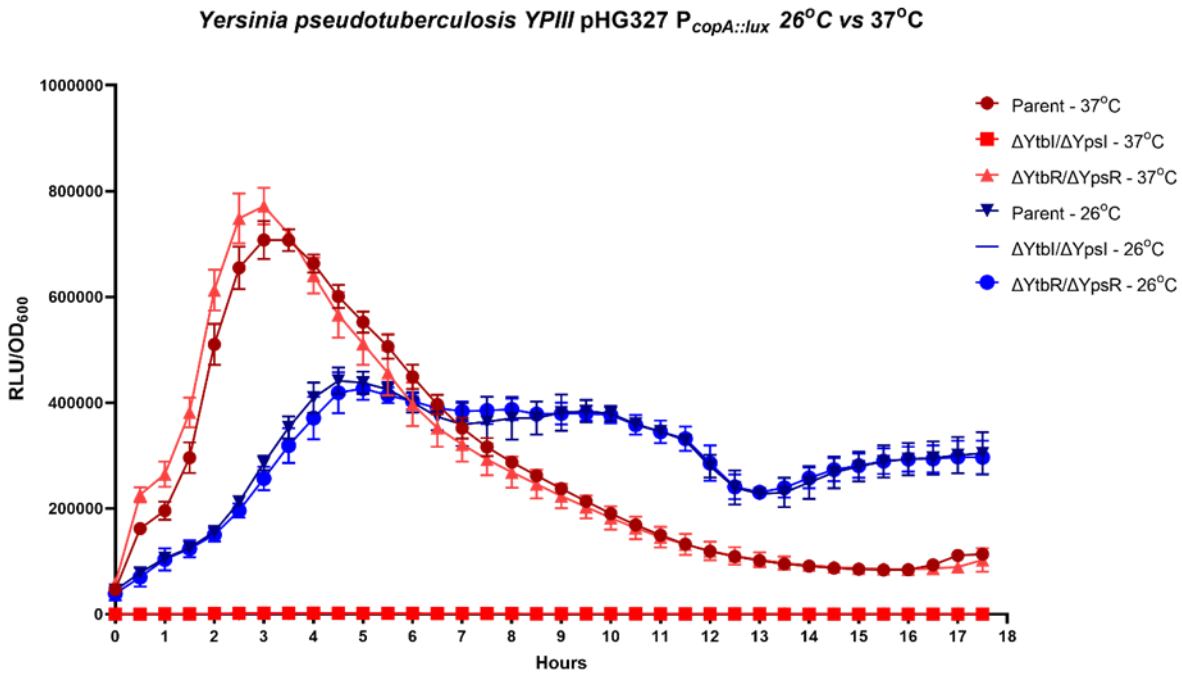


Figure 3.19: comparison of $P_{copA::lux}$ activity when grown at 26°C and 37°C in the three *Y. pseudotuberculosis* strains; Parent, *ΔytbI/ΔypsI* and *ΔytbR/ΔypsR*. Data from figures 3.15 and 3.17 were on the same graph to show the difference in activity of the promoter between the two temperatures. Vertical bars represent standard deviation. 37°C and 26°C OD_{600} data shown in appendix 3 and 4.

3.9 Inactivation of *Y. pseudotuberculosis* parent AHLs using AiiA

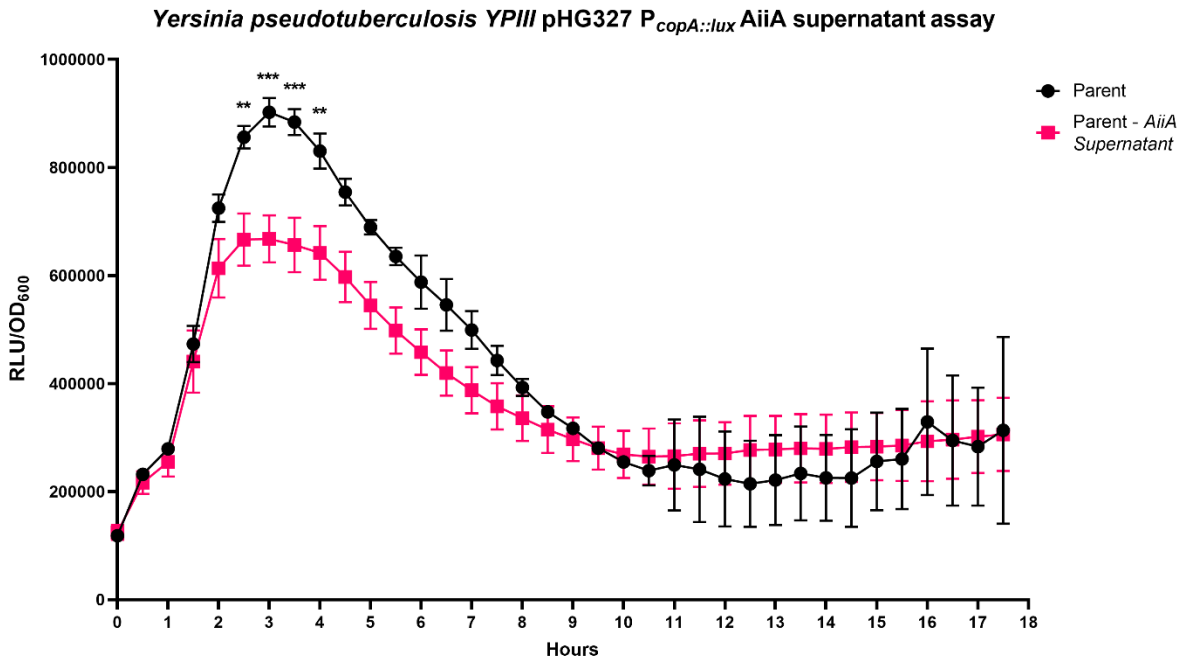


Figure 3.20: Activity assay of pHG327 $P_{copA::lux}$ in the *Y. pseudotuberculosis* Parent strain comparing the difference in promoter activity with the presence of AHL degrading enzyme AiiA; Parent vs Parent with added AiiA. Luminosity (RLU) and Cell density (OD measured at 600nm) was measured every 30 minutes over 36 times points. RLU/OD_{600} was used to measure activity of the reporter genes based on *lux* activity per given cell density. Vertical bars represent standard deviation. OD_{600} data shown in appendix 5.

In order to confirm that the lack of AHLs in *ΔytbI/ΔypsI* was responsible for reduced activity in $P_{copA::lux}$, supernatants taken from *E. coli* expressing *aii*A were combined with $P_{copA::lux}$ in a Tecan assay at 37°C. The results shown in Figure 3.20 revealed that both samples had an increase in $P_{copA::lux}$ activity up to 3 hours which then fell and stabilised by hour 11. However there was a reduction in $P_{copA::lux}$ in the parent when grown in the AiiA containing spent supernatant. This was seen clearest at 3 hours where the activity of the AiiA containing supernatant was lower compared to the AiiA free sample.

CHAPTER 4

Discussion

4.1 pDM4.2 Conjugations

During the early stages of the project single crossover conjugation events between pDM4.2-$P_{copA::lux}$ and the three *Y. pseudotuberculosis* strains parent, *ΔytbI/ΔypsI* and *ΔytbR/ΔypsR* were not successful but the reason was unclear given that the $P_{copA::lux}$ plasmid was confirmed to be correctly assembled, using PCR, restriction analysis and the fact that the S17-1 *E. coli* donor strain produced light. One potential explanation for the lack of success could be related to the integration of the reporter into the *copA* locus as a single crossover event. Although it was not anticipated that integration would have a negative effect on *copA* activity, any disruption to the normal function of CopA may have had a detrimental effect on cell viability. This is consistent with previous observations in which the inactivation of the ancillary pre-transcriptional inhibitor *copB* leads to a run-away lethal phenotype due to the uncontrolled replication of the virulence plasmid and associated excessive metabolic load on the cells (3). Given that *copA* is the stronger repressor of *repA* (69), a similar lethal phenotype may have been generated from the integration of the reporter into the virulence plasmid.

4.2 *copB* activity is temperature dependent

At 37°C and 26°C the activity of *copB* indicates that it is regulated by a temperature dependent cellular process or pathway. Although there was no difference in *copB* activity between the parent, *ΔytbI/ΔypsI* and *ΔytbR/ΔypsR* at either 37°C or 26°C, there was a very large difference in activity between the two temperatures. This can be seen easier when comparing the two data sets on the same graph, figure 3.12 reveals that at 26°C *copB* activity was below the readable threshold of the spectrophotometer, while at the temperature of the host 37°C there was a rapid continual increase in activity after a 6-hour period. Between the parent, *ΔytbI/ΔypsI* and *ΔytbR/ΔypsR* there was minimal differences between *copB* activity, likely suggesting that *copB* activity is temperature dependent but quorum sensing independent.

With *copB* being largely inactive at lower temperatures, the function of *copB* may play a significant role within the host environment Previously it was identified that *copB* played a largely inhibitory role in the regulation of RepA by binding to the *repA* promoter and preventing transcription (70). It is therefore unclear how, the increase in *copB* activity from 6 hours (figure 3.13) at 37°C would be of benefit given that the result may be reduce plasmid replication which, in an infection context may be detrimental. Wang *et al* showed at higher temperatures that *Y. pseudotuberculosis* increases virulence plasmid copy numbers within the cells and is required for correct function of the T3SS. Additionally they showed through the overexpression of CopA that a single copy of the virulence plasmid was insufficient to cause systemic infection within the host (71).

The temperature dependent nature of *copB* is reminiscent of the expression of some Yop and needle assembly apparatus genes which is low at environmental temperatures but increases rapidly when grown at host permissive temperatures.

One hypothesis could be that *copB* may play a role with how the pYV plasmid copy number is increased within the host and at higher temperatures. Larsen *et al* (1984) showed in *E. coli* that regulation of plasmid replication was lost when incorporating a temperature-controlled promoter upstream of P_{copB} (72), which lead to a runaway in plasmid replication as temperature was increased. Since the temperature-controlled promoter was in proximity upstream of P_{copB} would likely mean that the resulting transcript would produce the entire P_{copB} mRNA sequence (figure 1.5) containing *copB*, copT, *tap* and *repA*. As a result of the transcription of the complete P_{copB} transcript, RepA can be transcribed even with the complete inhibition of its promoter P_{repA} from the production of its inhibitor CopB.

4.3 The potential dual function of *copB*

While low *copB* expression is sufficient to inhibit *repA* expression even at low levels, high levels of *copB* expression may drive the opposite effect due to the presence of the transcribed *repA* sequence as part of the transcribed the P_{copB} mRNA strand (figure 1.5). The effects on plasmid replication have previously been shown to the increase when P_{copB} + *copB* were removed and drastically increased when a mutation was introduced in *copB* while leaving P_{copB} intact (58) as seen in figure 1.8.

If the expression of *copB* is controlled by a temperature dependent system as the data in this study implies, and if the transcriptional replication of *copB* causes a rapid increase in plasmid number, then the latent activity of *copB* seen during this late stage of growth at 37°C may be playing a role in the propagation and increase in plasmid copy number and increase in virulence factors that we see in *Y. pseudotuberculosis* when growing at 37°C.

Figure 4.2 outlines this proposed hypothesised dual functionality of *copB* at both host and low temperatures. At low temperatures the temperature dependent regulation of *copB* exhibits low levels of transcription and produces small quantities of CopB which is sufficient for regulating *repA* due to the singular nature of the pYV plasmid coupled with the post-transcriptional regulatory effects of *copA*. This maintains low plasmid copy numbers within the cell by restricting the initiation of plasmid replication.

At higher temperatures the transcriptional activity of *copB* increases which allows synthesis of both CopB and RepA from the P*copB* mRNA transcriptional sequence (as seen in figure 1.5). The increased rates of CopB synthesis continue to inhibit P_{repA} and due to reduction of *copA* activity at higher temperatures RepA is synthesised and initiates plasmid replication through the binding of RepA to ORI, increasing the plasmid copy numbers of pYV.

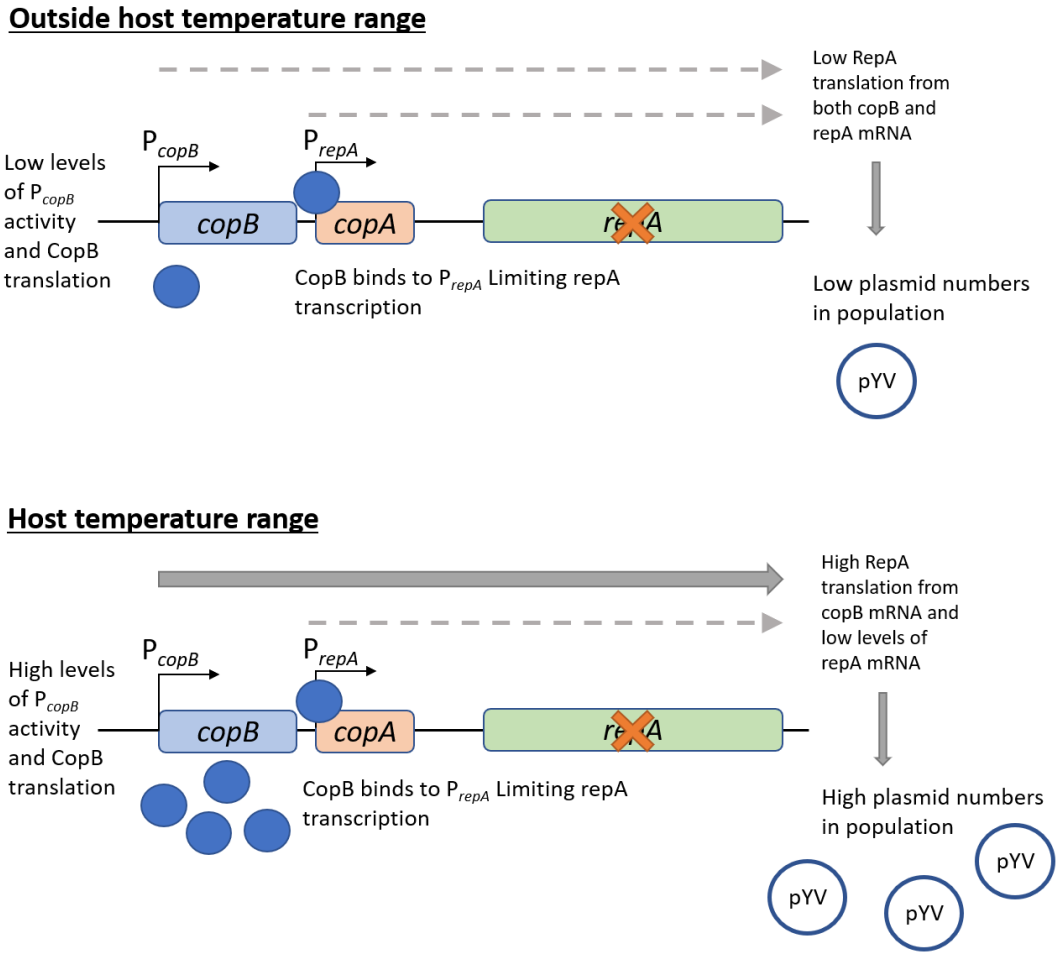


Figure 4.2: Proposed dual regulatory function of copB as a repressor and initiator of pYV replication based on temperature. The thickness of the arrows represents the levels of transcription of the specific mRNA sequence. At low temperatures copB acts as a repressor of plasmid replication with low transcription of the gene producing small quantities of CopB which repress the activity of P_{repA} preventing RepA. At higher temperatures the transcription of copB increases resulting in increased RepA translation from the copB transcript resulting in initiation of plasmid replication and propagation.

4.4 *copA* is dependent on components of the quorum sensing system

The role of *copA* in virulence plasmid regulation has been well studied as a strong post transcriptional inhibitor of *repA* (73), through the binding of its non-coding antisense RNA sequence known as copT, which prevents translation and facilitates RNAse III digestion of the *repA* mRNA (55) as shown in figure 1.6.

At both 26°C and 37°C, expression of $P_{copA::lux}$ was severely reduced in the AHL synthase deficient *ΔytbI/ΔypsI* when compared to the parent and *ΔytbR/ΔypsR*. Temperature also appeared to play a role since the overall activity profile of $P_{copA::lux}$ at 26°C showed a much lower peak at 4 hours when compared to 37°C.

Traditionally *lux* homologue pairs like *ytbI/ytbR* and *ypsI/ypsR* have interactions between the autoinducers produced from the synthases and the binding to the AHL domains of the luxR homologue which then bind to lux boxes to regulate gene activity (74).

The difference in activity could suggest that *Yersinia* has additional *luxR* homologues besides *ytbR/ypsR* which play a role in the regulation of virulence plasmid replications. *luxR* homologues that are not genetically linked to an AHL synthase have been described in other bacterial species such as with *rhiR* in *Rhizobium leguminosarum* (75) and with the *ycoR* in *Y. enterocolitica* , but in all cases *luxR* family genes are highly conserved in both the AHL and DNA binding domains (76). Given that highly conserved orthologous sequences were originally used in the discovery and identification of both *ytbI/ytbR* and *ypsI/ypsR* in *Y. pseudotuberculosis* (61) it is unlikely that there exists any additional 'traditional' *luxR* homologues within the genome. However, this apparent lack of response from the *ΔytbR/ΔypsR* mutant may suggest that there could exist a non-traditional quorum sensing component that bears the AHL binding domain but lacks the conserved DNA binding orthologous sequence which is used to search for lux homologues.

If this were true there may exist other types of AHL associated quorum sensing components, which could be identified through the conserved sequences of the AHL binding domain, which if found could then be investigated using the same techniques employed in this study, through the construction of reporter genes to study the gene regulation in the parent and the QS mutants.

Given that *copA* is a post transcriptional inhibitor of *repA* mRNA, the higher peak and rapid falloff after initial growth would cause an increase in frequency of *repA* translation as *copA* activity decreases. This coupled with the data from the $P_{copB::lux}$ experiments which showed a linear increase in *copB* activity after the initial growth stages could be what drives an increase in plasmid copy number; through the decrease in *copA* and an increase in the transcription of the *repA* containing *copB* mRNA, resulting in an increase in RepA translation and increase in plasmid replication.

Additionally, the 26°C data showed that $P_{copA::lux}$ was expressed more consistently over the 18 hours remaining high relative to its initial peak. This consistently higher activity of P_{copA} and the complete lack of activity of P_{copB} at lower temperatures may provide an explanation to

why lower plasmid copy numbers are observed in *Y. pseudotuberculosis* and *Y. pestis* at these environmental temperatures.

4.5 Effects of AiiA AHL digestion on *copA* activity

Since its discovery in 2000 (77), AiiA has been used in multiple AHL related studies to examine the effects of AHL degradation on cell processes (36, 78) and phenotypes such as biofilm formation in *P. aeruginosa* (79) and *Enterobacter cloacae* (80). The aim of this experiment was to see if the addition of spent culture supernatants taken from *E. coli* carrying plasmid-borne *aiiA* would cause a reduction in the activity of $P_{copA::lux}$. This was to serve as supporting evidence for the observation that was seen in the $P_{copA::lux}$ activity experiments where the AHL negative *ΔytbI/ΔypsI* mutant showed significantly reduced activity when compared to the parent and *ΔytbR/ΔypsR* mutant.

The results of the AiiA spent supernatant experiment showed a drop in $P_{copA::lux}$ by approximately 30 % when compared to the same overnight culture without the spend culture supernatant. Statistical significance was determined by a two-way ANOVA. This data, while supporting the hypothesis that a reduction in AHLs would reduce activity of *copA* require further investigation but were beyond the scope of this study due to the time constraints.

Using a spent supernatant as a source of AiiA may have introduced errors due to the reduced nutrient availability of the media, pH effects and other cellular by-products introduced to the culture. As such the data should be seen as an initial proof of concept which given time would have led to other controlled experiments.

One way this could be done would be through cloning *aiiA* into a compatible medium/high copy number inducible vector which would be transformed into the *Y. pseudotuberculosis* parent $P_{copA::lux}$ pHG327. This would produce a parent strain with a fully functional quorum sensing system but with the AHL profile of the *ΔytbI/ΔypsI* mutant. The negative control strain for this would be the parent containing $P_{copA::lux}$ with the selected empty vector.

Alternatively, purified AiiA could be added to the cultures in a dose dependent manner to study the AHL thresholds that induce P_{copA} activity. However, protease activity might affect the concentration of AiiA and return the strain to its parental AHL profile.

4.6 *copA* and *copB*: 2 regulators, 4 conditions 2 states

The cop regulatory system is a dual control system of *repA* by the post-transcriptional repressor *copA* and the pre-transcriptional promoter inhibitor *copB.* The results of this study suggest that *copA* transcription is regulated by AHLs while *copB* may act independent to the quorum sensing system and is primarily controlled by temperature. A summary of the effects on *copA* and *copB* in all three strains can be seen in figure 4.4 as well as a proposed effect of *repA* activity based on the activity of the *cop* system.

Yersinia pseudotuberculosis WT and ΔytbR/ΔypsR

	Low Temp	High Temp
Low cell density	CopA ↑ CopB ↓ RepA -	CopA ↑ CopB ↓ RepA -
High cell density	CopA ↑ CopB ↓ RepA -	CopA ↓ CopB ↑ RepA +

Yersinia pseudotuberculosis ΔytbI/ΔypsI

	Low Temp	High Temp
Low cell density	CopA - CopB ↓ RepA +	CopA - CopB ↓ RepA +
High cell density	CopA - CopB ↓ RepA +	CopA - CopB ↑ RepA ++

Figure 4.4: The effects of temperature and cell density on copA, copB and their proposed effects on RepA. The first table shows the parent and *ΔytbR/ΔypsR* which showed identical *copA* and *copB* activity across all experiments. The second table shows *ΔytbI/ΔypsI* strains which lacked activity of copA when compared to the parent and *ΔytbR/ΔypsR*.

With *copA* expressing quorum sensing dependency any reduction in population will result in increase in activity of *copA*. This may provide a survival benefit to the bacteria by reducing the metabolic burden that is placed on the cell through the production and management of the 68 kb virulence plasmid. The metabolic burden of plasmids has been previously documented to cause a decrease in cell growth rates (81) as well as an increase in bacterial stress responses at high plasmid numbers (82). By removing the burden of maintaining high plasmid numbers throughout the population, the growth rate would be elevated, and the bacterial population would increase.

Away from human host permissive temperatures, the bacterial behaviour of increasing and maintaining *copA* at higher levels could be advantageous to the bacteria by limiting the plasmid replication rate in these environments and instead dedicating these resources to growth instead of producing unnecessary virulence plasmids. This may explain the difference in growth rates which were observed during the experiments, where *ΔytbI/ΔypsI* which lack *copA* activity show lower growth rates when compared the parent or the *ΔytbR/ΔypsR* which both have high activity of $P_{copA::lux.}$

In the case of a low cell density and high temperature, the elevated *copA* activity may play a role in host immune evasion. By preventing the replication of the plasmid, this also prevents the virulence factors from causing an elevated host immune response to virulence related cell deaths from the growing bacteria; allowing the bacteria to increase the cell numbers by stealth and reach a population size at which point *copA* activity decreases and the increase in *copB* increases driving up the pYV copy number within the population.

Figure 4.5 proposes how the temperature and quorum sensing regulation of *copA* and *copB* may give a survival advantage within the context of disease. By having replication of pYV limited in low populations and elevated at high populations, it allows for coordinated expression of virulence factors at a higher population which would be more capable of overcoming the host defences, something that may be otherwise ineffective or even detrimental to the bacteria at lower cell density populations.

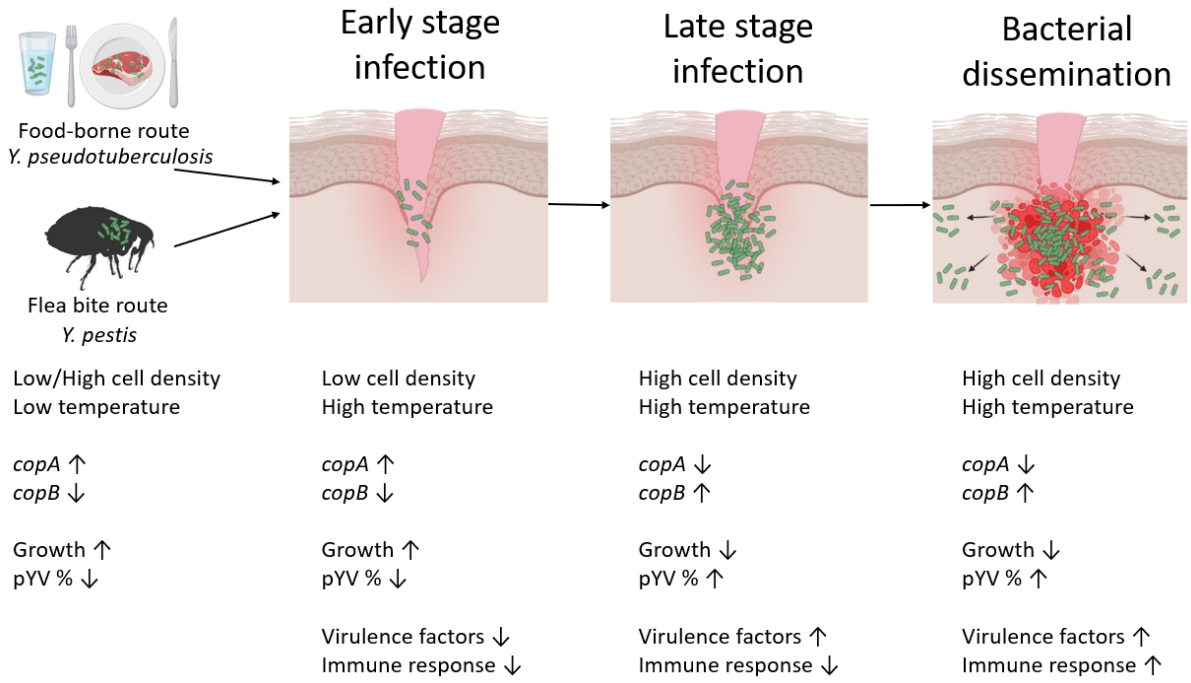


Figure 4.5: Proposed effects of temperature/population on *copA/copB* activity and the resulting effects on pYV, virulence factors and host response.

Given their strong genetic homology between *Y. pseudotuberculosis* and *Y. pestis*, the observed activity of both *copA* and *copB* may be similar in *Y. pestis* and given that the body temperature of fleas matches that of their environments (83), the activity of *copA* and *copB* maybe similar to the low temperature experiments described here, with elevated *copA* expression in both low/high cell densities limiting the replication of the virulence plasmid and dedicating those resources to cell growth.

4.7 Plasmid loss and its relation to cop regulation

The initial observations on pYV loss in *Y. pseudotuberculosis* using Flow cytometry analysis (FACS) showed that plasmid loss was earlier in the *ΔytbI/ΔypsI* mutant than in either the parent or *ΔytbR/ΔypsR* mutant.

The results of this study may also propose an explanation for the reduction of plasmid presence in *ΔytbI/ΔypsI* strains and not in the parent and *ΔytbR/ΔypsR*. This hypothesis proposes that the difference seen between the parent and *ΔytbR/ΔypsR* and the *ΔytbI/ΔypsI* is due to the inability of the AHL synthase mutant to correctly regulate its plasmid replication machinery due to the lack of *copA* activity and the decrease in pYV positive members is the result of slower growth rates and potentially lethal runaway replication of the virulence plasmid. As proposed in section 4.6 there may be an innate survival advantage in the regulation of pYV given the potential reduction in metabolic burden.

With the cultures being grown overnight at 37^{o}C and each day the previous overnight cultures were being used to seed the next cultures, the culturing process might be causing an unintentional regulation of plasmids by preventing the cells from ever reaching the late stages of growth at 37^{o}C where *copB* transcription is at its highest levels.

By standardising the OD_{600} to 0.05 each day the cultures are being effectively reduced back to an early growth phase culture. This could have a large effect on the activity of *copA* transcription which has been shown in this study to have a higher activity in the earlier stages of growth and by extension have a large effect on RepA translation. By constantly lowering the cell density after overnight growth, the RepA activity may be lowered due to the higher activity of *copA* in the fresh cultures reducing the time that the cultures are growing at high cell densities where copA is at its lowest and RepA translation from transcribed *copB* mRNA is at its highest. In the AHL synthase producing WT parent and in *ΔytbR/ΔypsR, copA* is fully functional and this effectively regulates plasmid replication through binding to *copT* and post-transcriptional RNAse III regulation of *repA.*

However, in the *ΔytbI/ΔypsI* mutant the regulatory activity of *copA* is completely absent due to its reliance of a functional AHL synthase and as a result *repA* in the early stages of growth is not regulated by *copA* or *copB* which has been shown to have minimal to no activity in the early stages of growth. Previously it has been shown that inactivation of *copA* (69) or increase in the activity/efficiency of *copB* (57) during late stage growth phases results in uncontrolled runaway replication of their plasmids resulting in unhealthy or even lethal phenotypes due to the large metabolic burden resulting from the overproduction of the 68 kb plasmid. Members of the population lacking the pYV plasmid do not have this increased metabolic pressure so will increase their numbers at a greater rate than the pYV containing population. This means at the end of each day the proportion of pYV negative bacteria will increase when compared to previous days and with pYV replication potentially being higher in the *ΔytbI/ΔypsI* strains due to reduction in RepA regulation would explain why the populations see a faster loss of the plasmid in the *ΔytbI/ΔypsI Y. pseudotuberculosis* strain.

CHAPTER 5

Conclusion

5. Conclusion

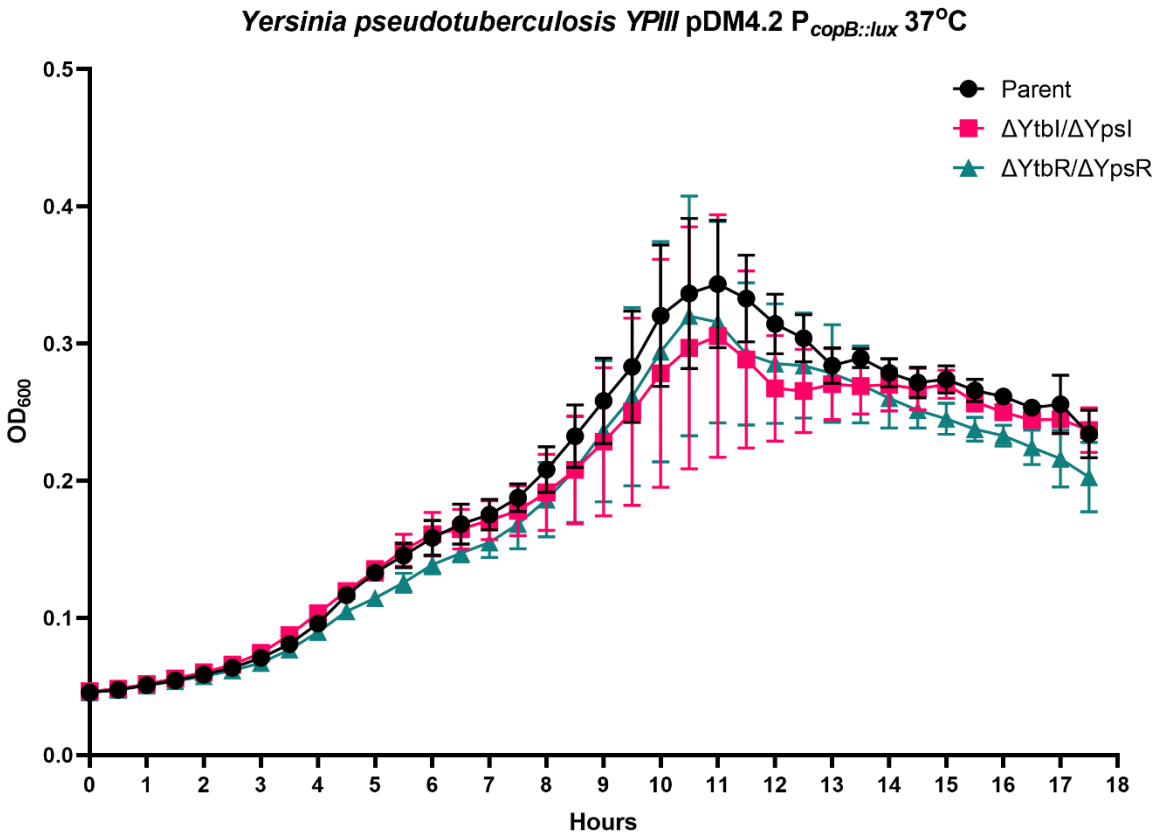
The aim of this study was to establish whether the quorum sensing system in *Y.pseudotuberculosis* plays a role in the regulation of the pYV virulence plasmid. The effects of the quorum sensing system were studied in relation to their interaction with the master post-transcriptional repressor of pYV CopA and its ancillary pre-transcriptional repressor *copB*, using constructed reporter genes which were able to determine the activity of the genes under different conditions.

The results of this study have presented data that suggests that the two regulators of *repA, copA* and *copB* are controlled by two separate regulatory systems, with the quorum sensing system being necessary in the expression of *copA* and a separate temperature related system regulating the activity of *copB*. The split in the regulation of RepA between a temperature dependent system and a population might suggest how the regulation of the pYV plasmid in *Y. pseudotuberculosis* is controlled in the variety of environments which it can inhabit.

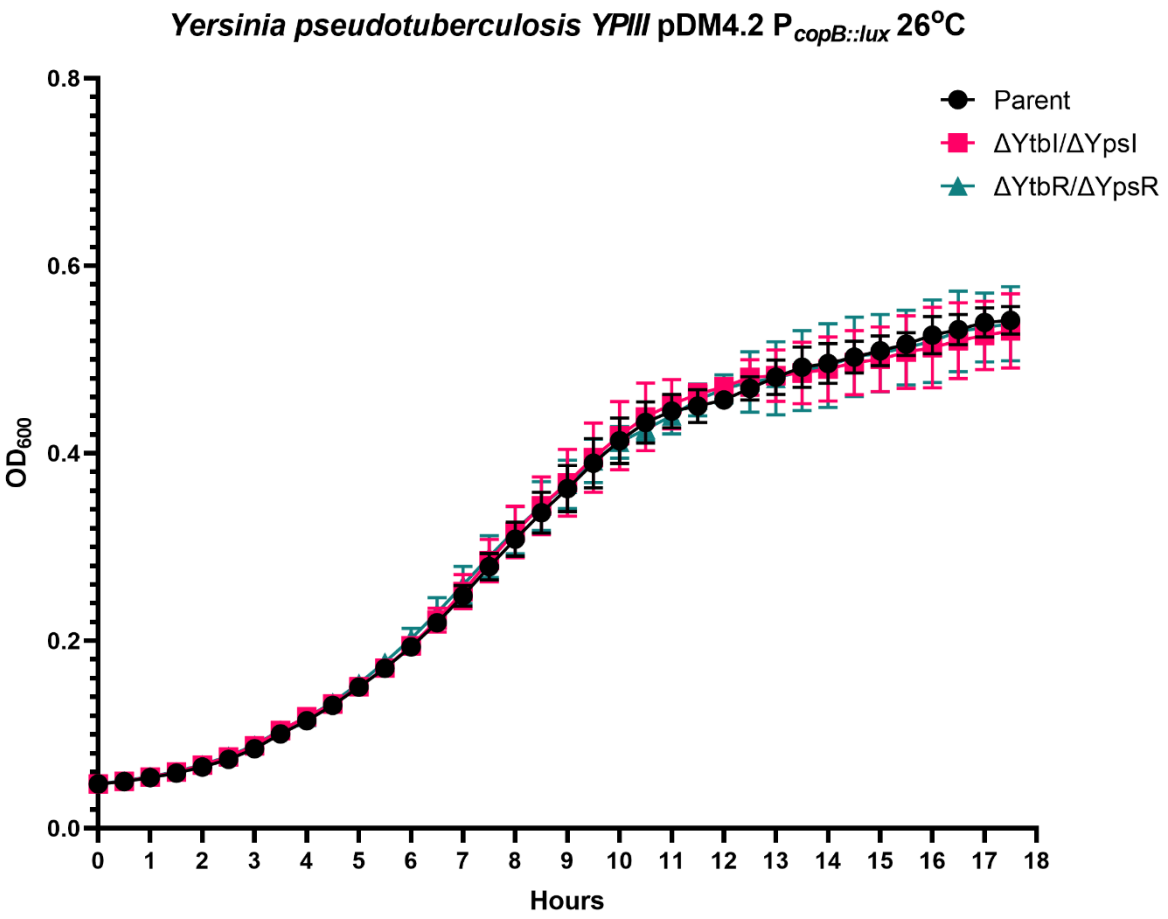
Ultimately there are still many questions that need answering surrounding the transcriptional factors of both *copA* and *copB,* such as the mystery to why knocking out the AHL synthase component of the quorum sensing system caused such a large reduction in activity of *copA* but knocking out its paired *ytbR/ypsR* appeared to have no effect. A suitable next step may be to search for the presence of additional AHL binding domains within *Y. pseudotuberculosis* independently from the DNA binding domain found within traditional *luxR* homologues. This may reveal additional proteins which interact with the quorum sensing system and uncover new targets for knockout for study with *copA*.

The scope of this study could also be expanded to investigate if *copA* and *copB* activity is similar in the closely related species *Y. pestis*, which could be investigated using the same reporter genes in quorum sensing mutants *YepI/YpeI* and *YepR/YpeR* due to the identical sequence of *copA* and *copB*. The results of which may further our understanding of how *Y. pestis* regulates its virulence plasmid at host temperatures.

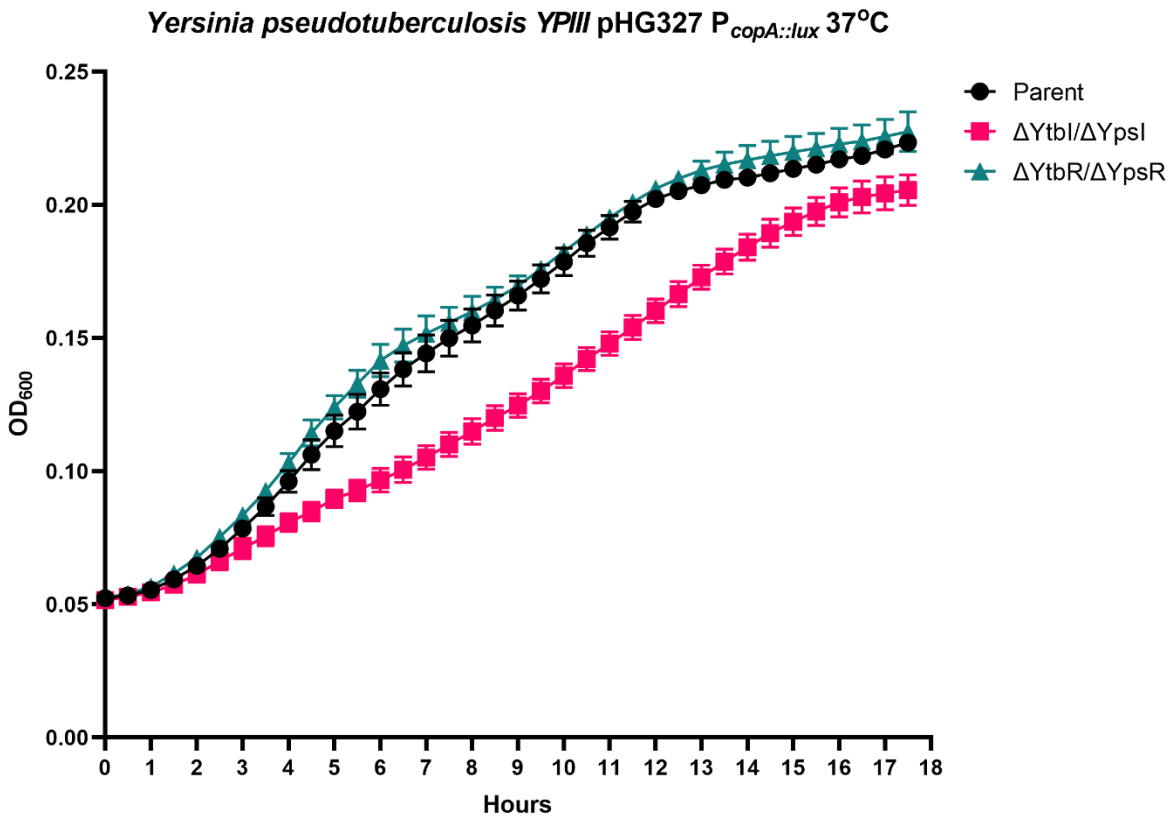
Appendix



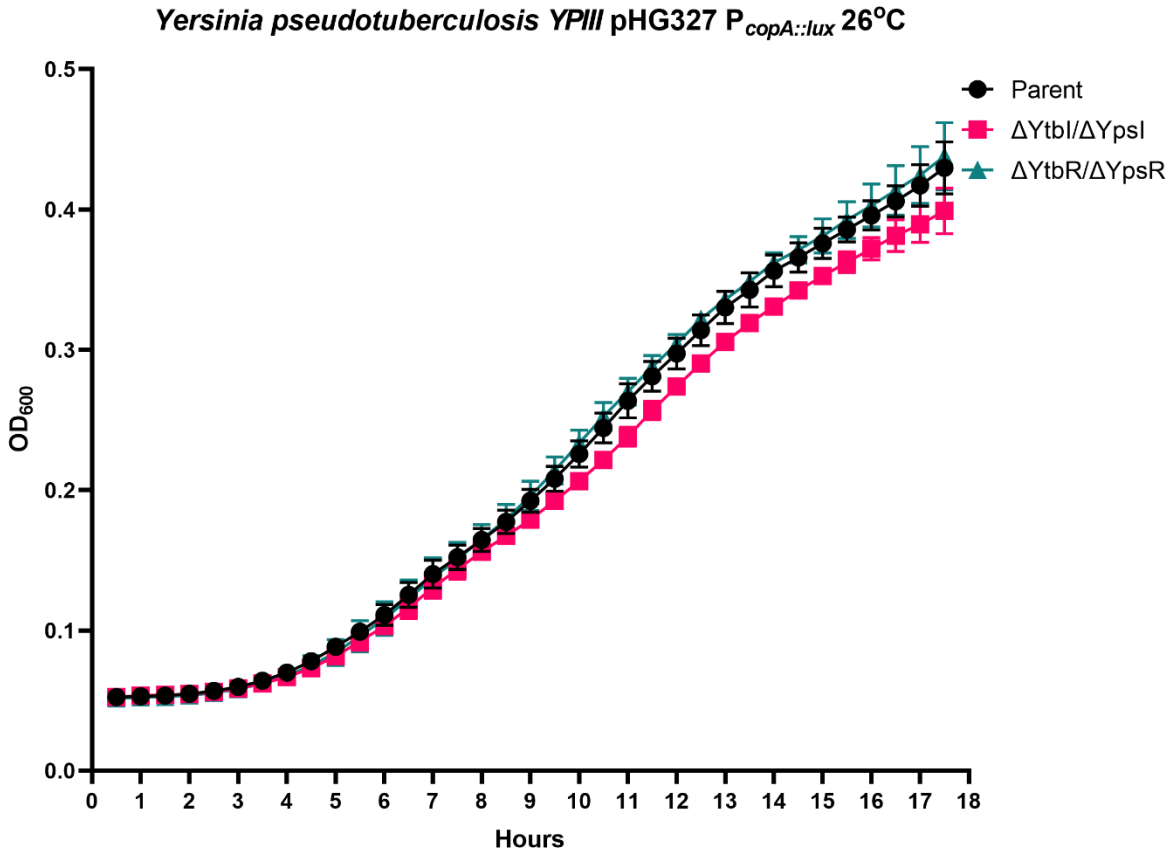
Appendix 1: *Y. pseudotuberculosis* pDM4.2 $P_{copB::lux}$ OD_{600} 37°C growth data from figure 3.13 and figure 3.14.



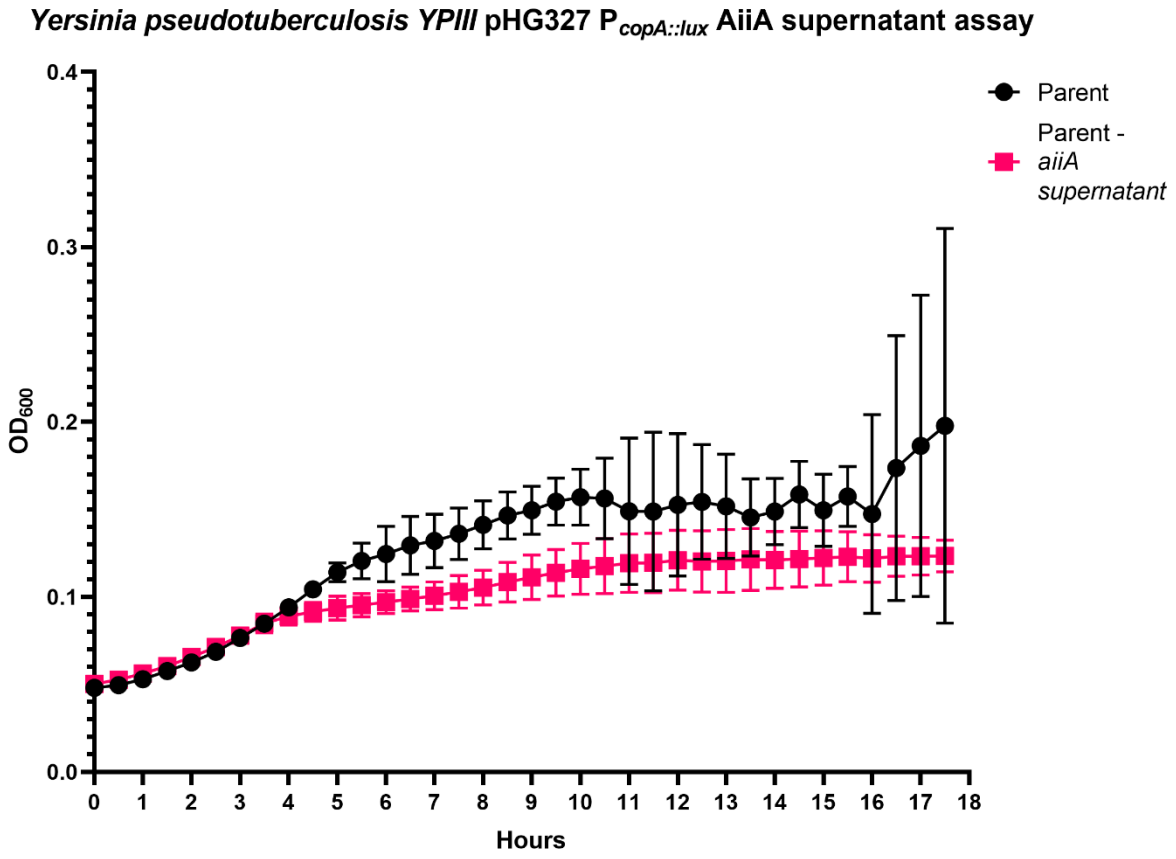
Appendix 2: *Y. pseudotuberculosis* pDM4.2 $P_{copB::lux}$ OD_{600} 26°C growth data from figure 3.14.



Appendix 3: *Y. pseudotuberculosis* pHG327 $P_{copA::lux}$ OD_{600} 37°C growth data from figure 3.15, figure 3.16 and figure 3.19.



Appendix 4: *Y. pseudotuberculosis* pDM4.2 $P_{copA::lux}$ OD_{600} 26°C growth data from figure 3.17, figure 3.18 and figure 3.19.



Appendix 5: *Y. pseudotuberculosis* pHG327 $P_{copA::lux}$ AiiA supernatant assay OD_{600} 37ºC growth data from figure 3.20.

References

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