INVESTIGATING THE ROLE OF LYSIS PROTEINS OF ENZYMATIC COLICINS IN BACTERIAL COMPETITION

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Abbreviations

BRP	Bacteriocin Release Protein
C domain	Cytotoxic domain
ColA	Colicin A
ColB	Colicin B
ColD	Colicin D
ColE1	Colicin E1
ColE2	Colicin E2
ColE3	Colicin E3
ColE4	Colicin E4
ColE5	Colicin E5
ColE7	Colicin E7
ColE8	Colicin E8
ColE9	Colicin E9
ColK	Colicin K
ColN	Colicin N
ColM	Colicin M
CM	Cytoplasmic membrane
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
g3p	Gene 3 protein (bacteriophage minor coat protein)
IM	Inner membrane
Im5	Immunity protein for colicin E5
Im7	Immunity protein for colicin E7
Im9	Immunity protein for colicin E9

K_d	Dissociation constant
LB	Luria-Bertani Broth
LPS	Lipopolysaccharide
mM	Millimolar
μM	Micromolar
nM	Nanomolar
NDR	Natively disordered region
OD	Optical density
OM	Outer membrane
ORF	Open Reading frame
PAGE	Polyacrylamide gel electrophoresis
Pal	Peptidoglycan associated lipoprotein
PCR	Polymerase chain reaction
PG	Peptidoglycan
PMF	Proton motif force
R domain	Receptor- binding domain
RFU	Relative fluorescence units
RLU	Relative luminescence units
SDM	Site- directed mutagenesis
tRNA	Transfer ribonucleic acid
T domain	Translocation domain
Ta	annealing temperature
Tm	Melting temperature
Tris	2-amino-2-(hydroxymethy)-1,3-propandiol

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Abstract

Colicins (Cols) are plasmid-encoded toxins produced by *Escherichia coli* that kill other *E. coli* and other closely related gram-negative species. Colicins play important role in promoting microbial diversity and provide selective advantage to the producers both as an offensive and defensive weapon.

This study was set out to elucidate a better understanding of the evolution and diversity of the colicin productions within microbial community where there are more than one colicin producing strains. In the first part of the study, the factors that contribute to the observed competitive advantages of ColE9 producers over ColE7 producers were investigated. To measure the degree of cell lysis, mitomycin sensitivity (MMC) assays were carried out which demonstrated that only ColE7 producing cells undergo significant cell lysis due to the induction of ColE7 lysis gene by MMC. Growth curve assays with colicins have shown that ColE7 has faster speed of cell entry and killing than ColE9. Spot tests were carried out to test the synthesis and release of colicins which have shown that ColE7 is produced quicker in greater amounts and released more efficiently than ColE9. The *lux* reporter assays were carried out to test the protection conferred by immunity proteins against cognate and non-cognate colicins. It was suggested that Im9 may have greater protection against non-cognate ColE7 at higher concentrations.

To investigate the roles of lysis genes, the lysis genes of ColE9 and ColE7 were swapped. Interestingly, ColE7 producers containing (ColE9 lysis genes) had competitive advantage over ColE9 producers (ColE7 lysis gene). This reversed outcome strongly suggests that the differences in the level of lysis gene expression may play an important role during the competition between ColE9 and ColE7 producing cells. Biological activity assays and the real-time dual fluorescent reporter assays have suggested that ColE7 is produced faster in larger quantities due to stronger promoter activity of ColE7 which may also account for greater expression of ColE7 lysis gene. A model has been proposed to summarise the factors involved in the competition between ColE9 and ColE7 producing *E. coli* cells in an unstructured well mixed environment.

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1 Introduction

1.1 Colicin overview

Colicins (Cols) are plasmid-encoded protein antibiotics (bacteriocins) that are secreted specifically by *Escherichia coli* during the times of environmental stress. They are active against neighbouring *E. coli* and other closely related gram-negative bacteria and offer selective advantage against other competitors that occupy the same ecological niche. André Gratia identified the first colicin in 1925 when the cultures of *E. coli* V, appeared to be toxic for *E. coli* ϕ (Gratia 1925; Gratia 2000). Afterwards, many types of colicins were characterised from different strains of the enterobacteriaceae family including *Escherichia, Salmonella* and *Citrobacter* (Gratia 1925; Cascales et al. 2007; Riley & Wertz 2002)

It was found that colicins bind specific surface receptors of the sensitive cells prior to the killing and therefore exhibit the narrow spectrum of action (Gratia & Fredericq 1946). During the 1950s, it was shown that DNA damaging agents induce the colicinogenic *E. coli* cells to produce colicin and producing cells were shown to be protected against killing by the colicin due to the presence of a specific immunity protein. (Ozeki et al. 1959; Jacob et al. 1952). Interestingly, colicins were also found to share various properties with bacteriophages such as high specificity of action, requirement for specific OM receptors, exhibition of single-hit kinetics, presence of specific immunity and lethality of production (Lwoff et al. 1952; Jacob et al. 1952). Subsequently, further studies have demonstrated that the various colicins have different modes of action such as depolarisation of lipid bilayer membranes (Lazdunski et al. 1988; Schein et al. 1978), degradation of host nucleic acids (James et al. 2002) or inhibition of protein synthesis (Ogawa et al. 1999; Boon 1971) and cell wall synthesis (El Ghachi et al. 2006). Colicinogenic bacterial strains are widely distributed in natural environment and thought to play an important role in promoting microbial diversity (Riley & Wertz 2002).

1.2 Colicin structure

1.2.1 Domain organisation and role in uptake

Colicins consist of three distinctive functional domains which are responsible for steps involved in import of colicins such as binding to the OM of a sensitive cell, crossing the OM and translocating to a cytotoxic site of action (Figure 1-1). A central receptor binding domain (R) usually occupy around 50% of a colicin molecule and interacts with a β-barrel shaped protein such as BtuB in the OM that is normally involved with the import of essential nutrients such as vitamin B_{12}. An N-terminal translocation domain (<25%) crosses the OM usually via its interaction with secondary receptor (translocator) on OM such as OmpF and forms a translocon through Tol proteins (mutations in which render the cell tolerant of the colicin) or Ton in the host periplasm. The translocator provides access for the T domain to an energized translocation system in the periplasm. The T domain is divided into two parts; a larger structured T-domain (STD) and an intrinsically unstructured T domain (IUTD) due to high degree of flexibility and lack of secondary structure. The C-terminal cytotoxic (C) domain is responsible for cell killing. The cell killing activity of colicins can be categorized into three cytotoxic classes: enzymatic degradation (DNAse or RNAse), membrane- depolarisation such as pore-formers or inhibition of peptidoglycan synthesis in the periplasm (colicin M). (Cascales et al. 2007; Gunasekaran et al. 2003; Ferguson et al. 2002; James et al. 1996).

1.2.2 Colicin classification

Colicins can also be classified by the OM receptor (either primary or secondary) to which they bind or by the mechanism of translocation through the host periplasm according to the Table 1-1. Group A colicins (E1- E9, A, N, K, S4, U, 28b and cloacin DF13) use the Tol dependent translocation system which consists of the proteins TolA, TolB, TolQ, TolR and the peptidoglycan associated lipoprotein (Pal) whereas Group B colicins (5,10, Ia, Ib, B, D and M) use the Ton-dependent translocation system which consist of proteins

TonB, ExbB and ExbD. Both Tol and Ton translocation system are coupled to proton motive force (PMF) across IM, which may be responsible for providing energy for translocation of colicin (Cascales et al. 2007).

Colicin	Outer membrane receptor "primary receptor"	"Translocator" or "secondary receptor"	Mechanism of translocation	Cytoxicity
Group A				
A	BtuB	OmpF	Tol A,B,Q,R	Pore-forming
E2, E7, E8, E9	BtuB	OmpF	Tol A,B,Q,R	DNase
E3, E4, E6	BtuB	OmpF	Tol A,B,Q,R	rRNase
E5	BtuB	OmpF	Tol A,B,Q,R	tRNase
E1	BtuB	TolC	Tol A,R	Pore-forming
N	LPS	OmpF	Tol A,Q,R	Pore-forming
K	Tsx	OmpF	Tol A,B,Q,R	Pore-forming
S4	OmpW	OmpF	Tol A,B,Q,R	Pore-forming
U	OmpA	Omp F, LPS	Tol A,B,Q,R	Pore-forming
28b	OmpA	Omp F, LPS	Tol A,B,Q,R	Pore-forming
Cloacin DF13	IutA	Unknown	Tol A,Q,R	RNase
Group B				
5, 10	Tsx	TolC	TonB, Exb B,D	Pore-forming
Ia, Ib	Cir	Cir	TonB, Exb B,D	Pore-forming
B	FepA	Unknown	TonB, Exb B,D	Pore-forming
D	Fep A	Unknown	TonB, Exb B,D	tRNase
M	Fhu A	Unknown	TonB, Exb B,D	Peptidoglycan synthesis inhibitor

Table 1-1: Colicin classification

Colicins are grouped into Group A and B based on the mechanism of translocation across the periplasm. Adapted from (Cascales et al. 2007; Kim et al. 2014)

1.3 Overview of Colicin import

The model of the colicin import mechanism is shown in Figure 1-1.

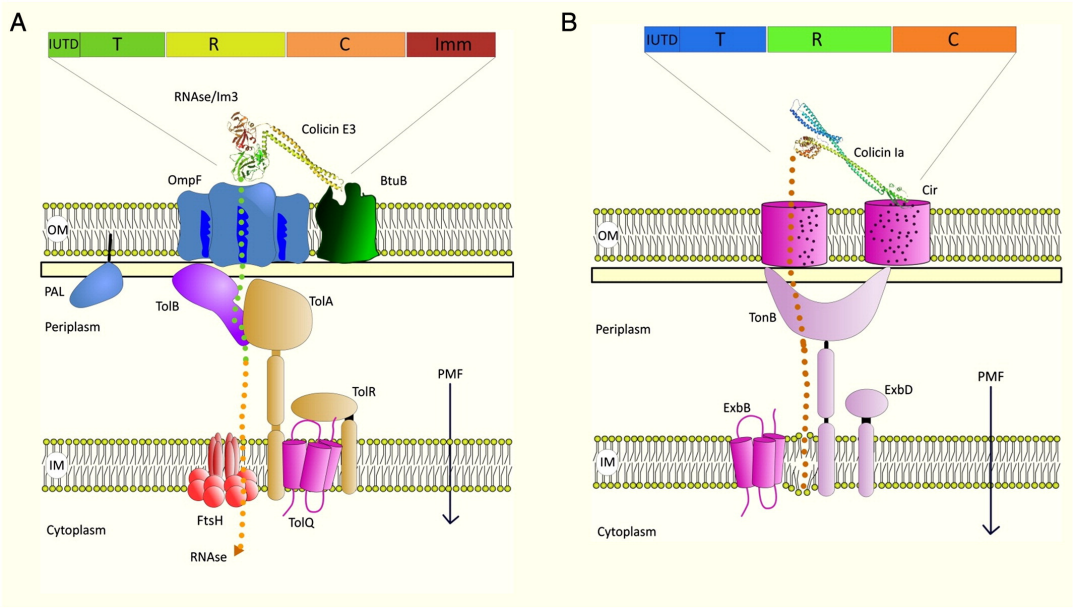

Figure 1-1: Model of the colicin import mechanism. Figure and legends adapted from (Kim et al. 2014).

A. Group A enzymatic colicins such as colicin E3 bind to the OM receptor (BtuB) via their central receptor binding (R) domain at an angle of 45° with respect to the cell surface. This allows the IUTD (green dots) to pass through the lumen of an OmpF monomer and across the OM to bind TolB protein that is normally bound to Pal. The resultant colicin translocon is energized by TolA/TolQ/TolR complex in a PMF dependent manner. Immunity protein (Im3) is released from the ColE3-Im3 complex and the nuclease domain (C) crosses the cell membrane (orange dots) by an unknown mechanism. FtsH dependent processing of the colicin at the IM cleaves the nuclease domain from the colicin in the cytoplasm.

B. Group B pore-forming colicins such as colicin Ia uses two copies of the OM receptor, Cir. First Cir is used for receptor binding and the second one as translocator. Energy generated by the TonB-ExbB-ExbD complex promotes transport of the pore-forming domain into the IM (orange dashes).

1.4 Colicin synthesis

1.4.1 Col plasmids

All colicinogenic strains of *E. coli* contain the pCol plasmids responsible for colicin production. The pCol plasmids are classified into two types based on the molecular weight. The type I are multi-copy plasmids with low molecular weight (6-10kb) that mainly encode group A colicins. The type II are characterised by a single copy plasmid (in excess of 40kb) with many additional genes and they mainly encode group B colicins (Riley & Gordon 1992; Hardy et al. 1973).

1.4.2 Colicin Operon

The genetic organization of colicin operons is summarized in Figure 1-2 (Riley 1993b). In all the colicin operons, the first gene is the colicin structural gene (named *cxa)* that encodes a specific colicin protein, where x is the specific colicin activity. In the operons of a nuclease colicin, the second gene is a specific immunity gene (*cxi*) encoding the immunity protein which is under regulation of two promoters; the LexA promoter and its own constitutive promoter. The last gene is the lysis gene (*cxl*) which encodes the lysis protein that promotes the release of colicins into external medium by cell lysis following the induction of group A colicins (Chak & James 1986; Kuhar & Zgur-Bertok 1999; Wal et al. 1995; A. P. Pugsley 1983; Lloubes et al. 1986). For pore-forming colicins, the immunity gene is located on the opposite DNA strand of the inter-genic space between the colicin structural gene and the lysis genes and is transcribed from its own constitutive promoter (Lloubes et al. 1986; Mark et al. 1984; Cavard & Oudega 1992; James et al. 1996; Chak & James 1985). There are redundancies in many of the colicin operons such as the enzymatic ColE3 and ColE9 operons which carry additional immunity gene for ColE8 and ColE5, respectively (Chak & James 1984; Cooper & James 1984; Lau et al. 1984; Chak & James 1986; Curtis et al. 1989)

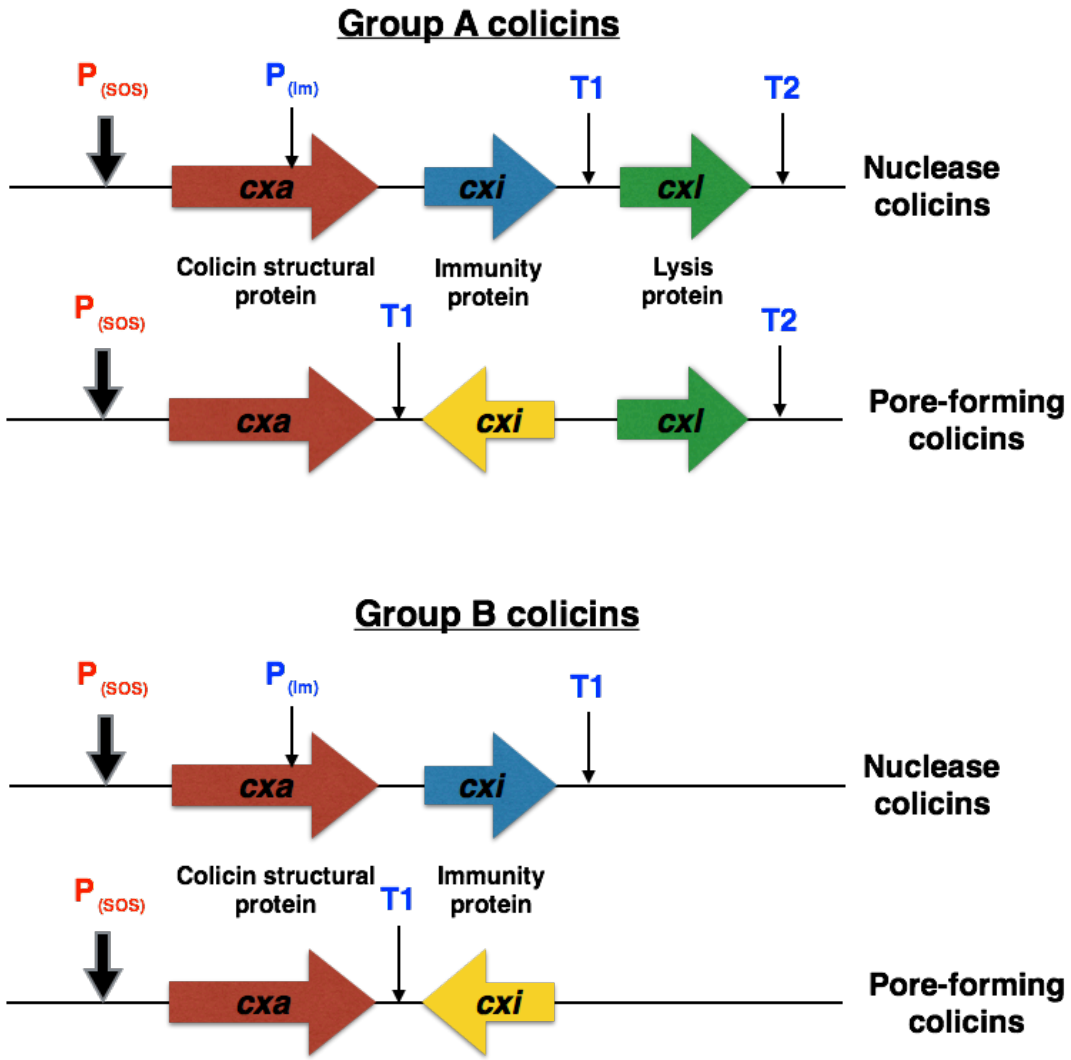

Figure 1-2: Organization of the colicin operons.

The genes encoding the colicin structural protein (cxa), immunity protein (cxi) and lysis protein (cxl) are shown. T=terminators (blue), P (SOS) = promoter of the colicin operon (red) and P (im) = the constitutive promoter of the immunity gene (blue). Figure adapted from (Riley 1993b)

1.5 Colicin expression and release

1.5.1 Colicin expression

Colicin expression is controlled tightly by the LexA and RecA proteins of the SOS response (Lloubes et al. 1986; Gillor et al. 2008). LexA binds to the strong SOS promoter located proximal to the colicin structural gene *cxa* and thereby repressing colicin gene expression. A period of DNA damage activates RecA protein which reverses the LexA repressor (Little et al. 1980) and therefore allowing colicin transcription and associated co-expression of the lysis gene (Suit & Luria 1988). IscR is a global transcriptional regulator which further regulates colicin production. It stabilises LexA on the SOS promoter and prevent the RecA-mediated inactivation of LexA for a short period to ensure colicins are only produced in response to persistent DNA damage and when the damage is beyond the level of DNA repair (Butala et al. 2012). However, it was found that under normal conditions without any external inducing agent, small population of colicinogenic *E. coli* cells were able to produce colicin by autoinduction (Durkacz et al. 1974; Mulec et al. 2003; Anthony P. Pugsley 1983).

1.5.2 Colicin Release

The extracellular release of colicins differ from other extra-cytoplasmic proteins that are released by Gram-negative bacteria as they lack N-terminal signal sequence and do not use protein secretory pathways (Gilson et al. 1990; Pugsley & Schwartz 1984). Release of colicins are non-specific (Cavard et al. 1987) and occur by leakage following lysis or quasi-lysis (dependent on the level of colicin induction). The leakage occurs after expression of the lysis gene, which encodes the lysis protein or Kil protein from the same SOS promoter that regulates colicin production (Cavard & Oudega 1992; Jakes & Zinder 1984). This ensures timely release of colicin following simultaneous synthesis of both colicin and lysis protein and controlled repression of lysis protein synthesis in the absence of colicin expression as the lysis protein can

cause host cell lysis in the absence of colicin production (Cavard et al. 1987; A. P. Pugsley 1983).

The lysis proteins are synthesized as precursor polypeptides (around 4.5 kDa) and all lysis protein precursors share sequence similarity (Riley 1993a; Wal et al. 1995). The mature lysis proteins exist as lipoproteins with similar amino acid sequences (around 30 a.a residues) and were found to be functionally interchangeable suggesting a shared mode of action (Pugsley & Schwartz 1983). The lysis protein is thought to cause localized disruption of the IM and movement of colicin across the IM as the mutations to key residues in the lysis protein prevented any accumulation of the colicin in the periplasm (Chen et al. 2011; Cavard 2004). Colicins do not kill cells immediately after synthesis (Herschman & Helinski 1967); a delay of several hours for a colicin to be exported following a period of induction has been reported (Varenne et al. 1981).

1.6 Colicin Import into sensitive *E. coli* cells

1.6.1 Crossing the outer membrane

In order to exert their cytotoxic effect on the sensitive cells, colicins need to move across the OM, periplasm and across the IM. The first step in colicin import of all colicins except ColN (binds the sugar moieties of LPS), is the binding of R domain to a high affinity OM receptor on the surface of *E. coli* cell (Johnson et al. 2014). High affinity OM receptors are made up of 22-stranded β-barrels with N-terminal globular domain forming the internal plug within the barrel. On the amino terminus of the plug domain, there is a short amino acid sequences called the TonB box that interacts with TonB protein at the periplasmic side of the OM (Pawelek et al. 2006). This interaction of TonB box with TonB causes the movement of the plug domain into and out of the β-barrel to allow the uptake of the natural substrate of the receptors such as cobalamin or iron siderophores (Devanathan & Postle 2007).

Colicins also have similar short consensus sequences in their N-terminal translocation domains called a TonB box (for TonB dependent colicins) or TolB box (for Tol-dependent colicins except colicins N and E1, Table 1-1). The classical model of colicin translocation suggested that binding of the colicins with TonB causes competitive displacement of the plug allowing its penetration through the cavity of the barrel (Wiener et al. 1997; Hilsenbeck et al. 2004). However, the validity of this classical model has been disputed in later studies (Smallwood et al. 2009; Cao & Klebba 2002). Recent studies have provided further insights into the colicin import mechanism across OM. ColN have been found to bind to LPS as its primary receptor and uses OmpF as a translocator to allow IUTD to thread through the lumen of adjacent OmpF barrel using OBS as a pulling mechanism once inside (Johnson et al. 2014; Jakes 2014). On the other hand, it was demonstrated using a chimeric colicin called IaE3R (E3 receptor domain binding BtuB for its killing activity) that two copies of Cir are required for the activity of ColIa; one as the primary high affinity receptor and the second copy as a translocator for insertion of the ColIa IUTD (Jakes & Finkelstein 2010). Alternative model of the colicin

translocation across the OM involving the formation of a colicin translocon has been suggested by analysing the crystal structures of colicins in complex with their primary receptors (Kurisu et al. 2003; Sharma et al. 2007; Zakharov et al. 2012).

1.6.2 Colicin translocon

Studies have shown that the central plug domain did not move significantly to create the pore for the colicin to penetrate through nor induce significant conformation change to accommodate colicins despite the high affinity binding of R-domains of ColE2 or ColE3 to the primary OM. Moreover, the R domain was found to be angled at 45° with respect to the OM surface placing the translocation and cytotoxic domains further away from the primary receptor (Kurisu et al. 2003; Sharma et al. 2007). It has been suggested that R domain of ColE2 and ColE3 acts as a 'fishing pole' for the T domain to recruit its translocator, OmpF to cross the OM through the porin (Figure 1-1) (Sharma et al. 2007). OmpF exists as a trimer of identical 16 stranded β-barrels (Bourdineaud et al. 1990) and is essential for the translocation of E colicins and colicins A, K and N (Benedetti et al. 1989). Studies have shown the evidence for formation of a colicin translocon of intact complex of BtuB-ColE9-Im9-OmpF at the cell surface which was dependent on the presence of IUTD for quaternary complex formation (Housden et al. 2005). Recent studies on ColE9 have revealed that there are two OmpF binding sites (OBS1 and OBS2) of the ColE9 IUTD with binding affinities of 2 μM and 24 μM respectively (Housden et al. 2010). It was found that neither OBS1 nor OBS2 is essential for the cytotoxicity of the enzymatic colicin. However, the presence of two OBS domains enhances the cell killing as they act sequentially to deliver the TolB box to TolB in the periplasm (Housden et al. 2010). More recently, it has been shown that TolB is tethered to the colicin translocon via both OmpF binding sites, which occupy two of the three subunits of OmpF in an antiparallel configuration as OBS1 is able to insert into OmpF in either orientation (Housden et al. 2013).

1.6.3 Immunity release

All *E. coli* cells producing colicins protect themselves by co-synthesizing a plasmid-encoded immunity protein (Im). For enzymatic colicins, the nuclease domains are active on synthesis and would degrade cellular nucleic acids without the neutralization by constitutively expressed immunity protein (A. P. Pugsley 1983). Enzymatic immunity proteins show binding affinities in the femto-molar range against cognate colicins but offer no protection against non-cognate colicins despite showing micro-molar binding affinities *in vitro* (Wallis et al. 1995; Li et al. 2004). Pore-forming colicin immunity proteins form part of the integral IM proteins that either prevent or block the depolarizing channel formation at the cytoplasmic membrane (Espesset et al. 1996).

Nuclease immunity proteins (about 10 kDa) are released from cells as a heterodimer (about 70 kDa) with the colicin (Jakes & Zinder 1974; Sidikaro & Nomura 1974). They inactivate enzymatic colicins either by binding directly to or exosite away from the nuclease active site (Graille et al. 2004; Kleanthous & Walker 2001; Cheng et al. 2002). It has been shown that some part of Im3 protein (up to 38 %) also interacts with T-domain and this bipartite binding stabilizes the colicin in a favourable conformation (Soelaiman et al. 2001; Walker et al. 2004; Krone et al. 1986).

It is believed that the immunity protein is released after primary binding to its high affinity OM receptor but prior to translocation of C-domain across the OM (Krone et al. 1986; Housden et al. 2010; Zhang et al. 2008). It was shown that the formation of colicin translocon (ColE9-BtuB-OmpF) was not sufficient to release Im9 from the ColE9-Im9 complex and that unfolding must occur prior to the release of Im9 (Housden et al. 2005; Zhang et al. 2008). The importance of flexibility and unfolding of the extended helical arms of the R domain as well as global conformational rearrangement across all three domains of ColE9 for immunity release and entry of nuclease domains across the OM have been demonstrated (Kurisu et al. 2003; Sharma et al. 2007; Penfold et al. 2004; Vankemmelbeke et al. 2013). Furthermore, it was demonstrated that the functional Tol proteins and the interaction between TolA

box of TolB protein and TolAIII domain of TolA protein are essential for Im9 release from ColE9-Im9 complex using energy supply from IM PMF (Bonsor et al. 2009; Vankemmelbeke et al. 2009). Recent discovery that Im9 is released from ColE9-Im9 complex following a conformational rearrangement of the DNAse domain in response to low forces (< 20 pN) triggered by a conformational remodelling of the T Domain on binding TolB, is in agreement with the speed of killing and therefore supports the role for the formation of colicin translocon and PMF in immunity protein release (Farrance et al. 2013; Housden et al. 2005; Zhang et al. 2008; Vankemmelbeke et al. 2009).

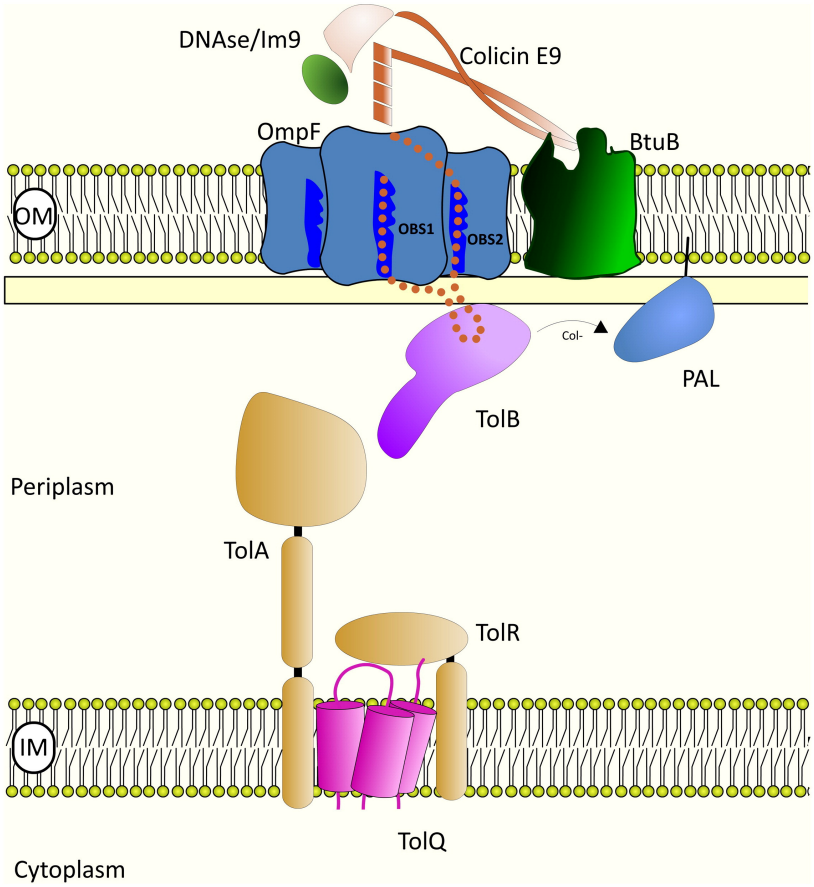

Figure 1-3: Model of the colicin translocon. Adapted from (Kim et al. 2014)

ColE9-Im9 complex binds to its high affinity OM primary receptor BtuB recruiting the translocator protein OmpF via its IUTD (red dots). Both OmpF binding sites (OBS1 and OBS2) within the IUTD of ColE9 occupy two of the three subunits of OmpF in an antiparallel configuration to tether TolB in periplasm to the colicin translocon in a fixed orientation. Allosteric signalling of TolB forces immunity release at the cell surface.

1.6.4 Tol-dependent translocation of nuclease colicins

The Tol-Pal complex are conserved across a variety of bacteria suggesting the important functional significance of the Tol proteins (Sturgis 2001). Although the normal cellular function of Tol system in *E. coli* is still unclear, they appear to play role in maintaining the integrity of the cell wall, transducing energy from the IM, and promoting septal wall formation during cell division (Cascales et al. 2001; Goemaere et al. 2007; Gerding et al. 2007). In addition, external agents such as colicins and filamentous bacteriophage hijack Tol system to gain entry into the cells. The Tol–Pal system consists of five proteins, TolA, TolB, TolR, TolQ and Pal.

TolA is a 44 kDa periplasmic protein organized into three domains; N-terminal TolAI domain anchors to the IM, central domain II spans the periplasm, and the C-terminal TolAIII domain binds to both TolB and Pal (Levengood et al. 1991; Germon et al. 2001; Dubuisson et al. 2002; Walburger et al. 2002; Cascales et al. 2000). TolQ and TolR are transmembrane proteins that are involved in the PMF-dependent activation of TolA (Cascales et al. 2001). TolB is a periplasmic protein that interacts with TolA via its N-terminal domain and with Pal via its C-terminal β-propeller domain (Bouveret et al. 1995; Dubuisson et al. 2002). Colicins interact with TolB via its TolB binding epitopes called TolB box which appears essential for the import of Group A colicins except ColE1, ColN and cloacin DF13 (Table 1-1). TolB box of ColE9 is able to competitively recruit TolB from its physiological interaction with Pal producing a conformational change in TolB that encourages a low affinity interaction of the N-terminus of TolB with TolAIII, which is important for immunity protein release of the ColE9/Im9 complex (Bonsor et al. 2009; Vankemmelbeke et al. 2009).

1.7 Competition of bacteria

Studies have shown that in a spatially structured environment such as on the agar plate, small population of colicin producers are able to grow and invade sensitive neighbouring cells despite having high cost of production. This can be explained by the immediate gain of resources by killing of nearby sensitive cells (Kirkup & Riley 2004; Durrett & Levin 1997; Chao & Levin 1981). However, in a physically unstructured environment involving free cells or mass action where there is random distribution of resources, they are unable to invade an established population of sensitive cells due to the high energy cost of colicin production due to plasmid carriage (Nakamaru & Iwasa 2000; Durrett & Levin 1997; Chao & Levin 1981). It was also shown that colicins can cross induce each other's expression which is positively correlated to their potency. Therefore, more potent colicin will induce the other producers with

stronger expression which may explain abundance of weaker colicin producers in nature (Chao & Levin 1981; Durrett & Levin 1997; Majeed et al. 2013).

1.8 Hypothesis and project aims

This work set out to elucidate a better understanding of the evolution and diversity of the colicin productions within microbial community where there are more than one colicin producing strains. A preliminary data using dual fluorescent system to monitor the competition between two E colicins have shown that ColE9 producing cells had competitive advantage against ColE7 producers in an unstructured well mixed environment (Bano 2010).

The first part of the project was focussed on investigating the factors that might contribute to the observed competitive advantage of ColE9 producing cells over ColE7 producers. In order to test the hypothesis that the observed competitive advantage of ColE9 producers is due to the significant cell lysis caused by ColE7 lysis genes on induction, the lysis genes of ColE9 and ColE7 were swapped to see if the outcome of the competition may change.

2 Methods

2.1 General chemicals, reagents and buffers

The general laboratory chemicals were purchased from Sigma unless otherwise stated. Buffers were prepared in distilled water as aqueous solutions according to (Sambrook & Russell 2001). Solutions were sterilised by autoclaving.

2.2 Bacterial strains, plasmids and media

The bacterial strains and the plasmids used in this study are described in Table 2-1 and Table 2-2 respectively. *E. coli* cells containing pSBM15 (pColE9-J::Kan^r) or pSBM20 (pColE7-K317:: Kan^r) were used for the expression of ColE9 and ColE7 respectively. *E. coli* JM83 containing the plasmid pACYC184 was used as a negative control. The host strain for cloning and mutagenesis was *E. coli* JM83 (Invitrogen) unless otherwise stated. Bacterial cultures were grown in Luria-Bertani (LB) broth or on LB agar. Selection for the recombinant clones was done on media supplemented with the appropriate concentrations of antibiotics: 100 μg ml^{-1} ampicillin (AP), 25 μg ml^{-1} chloramphenicol (CMP) and 50 μg ml^{-1} kanamycin (Kan). The *E. coli* DPD1718 strain was used for the luminescence reporter assays.

Bacterial Strains	**Description/ Genotypes**	**Source/ References**
E. coli JM83	General laboratory strain of *E. coli*. Genotype: F^-, *ara, rpsL, ara Δ[lac-pro*AB*]* Φ80*lacZ* ΔM15	Invitrogen
E. coli DPD1718	General laboratory strain of *E. coli*. Genotype: A fusion of *E. coli* recA promoter region to the *Photorhabdus luminescens luxCDABE* reporter integrated into the *LacZ* locus of *E.coli* DPD1692	(Vankemmelbeke et al. 2005)

Table 2-1: Bacterial strains used in this study are described with their genotypes and sources.

Plasmid	Description	Source/ Reference
pColE9-J	Native ColE9 encoding plasmid	(Cooper & James 1984)
pColE7-K317	Native ColE7 encoding plasmid	(Chak et al. 1991)
pACYC184	P15A, Cm^r, Tc^r	(Chang & Cohen 1978)
pJP23	pACYC184- *sulA*Ω*gfpmut2*, Cm^r	(McCool et al. 2004)
pSBM13	pJP23, constitutive promoter::*gfpmut2*, Cm^r	A constitutive promoter cloned at the place of ColE9p into pJP23 (Bano 2010)
pSBM15	pColE9-J:: Kan^r	1416bp kanamycin cassette cloned in the unique *Pvu* I site at 5283bp into native ColE9-J plasmid (Bano 2010)
pSBM16	pJP23, constitutive promoter, *mCherry*, Cm^r	A constitutive promoter cloned at the place of ColE9p into pJP23, ColE9 (Bano 2010)
pSBM20	pColE7-K317:: Kan^r	1416bp kanamycin cassette cloned at *Pvu* I site at 5283bp into native pColE7-K317 plasmid (Bano 2010)
pYC10	pColE9-J:: Kan^r	pSBM15 with *Pst* I site inserted (this study)
pYC11	pColE7-K317:: Kan^r	pSBM20 with *Pst* I site inserted (this study)
pYC12	pColE9-J:: Kan^r	pSBM15 with *Pst* I and *Bam*H *I* sites inserted (this study)
pYC13	pColE7-K317:: Kan^r	pSBM20 with *Pst* I and *Bam*H I sites inserted (this study)
pYC14 (E9VE7I)	pColE9-J :: Kan^r	pSBM15 with ColE9 structural and immunity gene replaced by that of ColE7 (this study)
pYC15 (E7VE9I)	pColE7-K317:: Kan^r	pSBM20 with ColE7 structural and immunity genes replaced by that of ColE9 (this study)

Table 2-2: Plasmids used or made in this study are described with their description and sources. Recombinant clones were identified by screening on LB agar supplemented with 50 $\mu g\ ml^{-1}$ kanamycin (Kan).

2.3 DNA manipulation techniques

2.3.1 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was carried out according to the standard protocol using an Eppendorf Mastercycler Gradient thermocycler (Saiki et al. 1985). PCR amplifications were carried out in 50 µl with 50 ng DNA template and 10 pmol of each of the respective primers ordered from Sigma- Aldrich. The summary of reagents used in a standard 50 µl reaction and the typical reaction conditions are shown in Table 2-3 and Table 2-4 respectively.

Volume	Components
5 µl	Template DNA (50ng/µl)
5 µl	Forward primer (10 mM)
5 µl	Reverse primer (10 mM)
5 µl	dNTPs (2mM)
5 µl	x10 PCR reaction buffer
25 µl	Pure water (Sigma)
0.5-1 µl	DNA polymerase
50 µl	Total reaction volume

Table 2-3: A standard PCR reaction mixtures

Steps	Cycles	Temperature	Time
Step 1	1	95°C	1 min
Step 2	35	95°C 55°C 72°C	45 s 1 min 2 min
Step 3	1	72°C	30 min
Step 4		4°C	HOLD

Table 2-4: A standard PCR reaction conditions

2.3.2 Oligonucleotide primers

The oligonucleotide primers used in this study are listed in the Table 2-5.

Primers	Sequence (5'-3')	Description
CNP 512	GACGGGTACTTTTTGATCTGCAGATAAAACCAGTGG	Forward primer to introduce *Pst* I site in pSBM15 (pColE9-J: Kan^r)
CNP 513	CCACTGGTTTTATCTGCAGATCAAAAAGTACCCGTC	Reverse primer to introduce *Pst* I site in pSBM15
CNP 514	AATATCCCTCCCTGCAGTGACAGAGAAAATAATGG	Forward primer to introduce *Pst* I site in pSBM20 (pColE7-K317:: Kan^r)
CNP 515	CCATTATTTTCTCTGTCACTGCAGGGAGGGATATT	Reverse primer to introduce *Pst* I site in pSBM20
CNP 516	AAGGATGAATGACTGGGATCCTTTCACAACAAGGAG	Forward primer to introduce *Bam*H I sites in pSBM15 and pSBM20
CNP 517	CTCCTTGTTGTGAAAGGATCCCAGTCATTCATCCTT	Reverse primer to introduce *Bam*H I sites in pSBM15 and pSBM20

Table 2-5: Oligonucleotide primers used in this study are listed. The recognition sites for *Pst* I and *Bam*H I are underlined in red.

2.3.3 QuikChange site-directed mutagenesis

The QuikChange site-directed mutagenesis was carried out using an Eppendorf Master cycler according to the manufacturer's instructions (Stratagene). The typical reaction mixtures and the cycling parameters are shown in the Table 2-6 and Table 2-7 respectively. Briefly, pSBM15 and pSBM20 were used as the dsDNA template with the mutagenic oligonucleotides CNP 512-515 in the Table 2-5, to introduce *Pst* I sites. The parental DNA template (methylated, nonmutated) were digested with *Dpn* I by incubation at 37°C for 1 h leaving behind only mutated plasmids containing *Pst* I sites (pYC10 and pYC11). The subsequent transformation into JM83 super-competent cells were carried out according to the manufacturer's instructions (**see section 2.4**). Same method

was used to introduce the *Bam*H I sites to pYC10 and pYC11 into produce pYC12 and pYC13 respectively.

Volume	Components
1 µl	dsDNA template (5-50ng)
1 µl	Forward primer (125ng)
1 µl	Reverse primer (125ng)
1 µl	dNTPs (2mM)
5 µl	10x reaction buffer
40 µl	Pure water (Sigma)
50 µl	Total reaction volume
+ 1 µl	*pfuTurbo*® DNA polymerase

Table 2-6: A typical reaction mixture for QuikChange site-directed mutagenesis

Segment	Cycles	Temperature	Time
Step 1	1	95°C	30s
Step 2	12-18	95°C 55°C 68°C	30 s 1 min 1min/ kb of plasmid length
Step 3		4°C	HOLD

Table 2-7: A typical cycling parameters for QuikChange site-directed mutagenesis

2.3.4 Mini/Midi scale isolation of *E. coli* plasmid DNA

A 5 ml overnight culture of *E. coli* JM83 was grown in LB broth supplemented with the appropriate selective antibiotics. Plasmid DNA was extracted using the Wizard Plus SV Miniprep kit (Promega) according the manufacturer's instructions. Large scale circular plasmid DNA isolation was conducted using spin columns QIAGEN midi prep kit. Purified DNA plasmids were stored at -20°C.

2.3.5 Restriction digestions of plasmid DNA

Endonuclease digestion of plasmid DNA was carried out according to the protocol (Sambrook & Russell 2001). High-fidelity restriction endonuclease enzymes (*Pst* I-HF and *Bam*H I- HF) from New England BioLabs (NEB) were used with 1x CutSmart ® buffer in double digestion reactions according the manufacturer's instructions.

2.3.6 Ligation of plasmid DNA fragments

Both rapid ligation and overnight ligation were performed using T4 DNA ligase (Promega) according to the manufacturer's instruction. Recombinant plasmids produced by ligations were transformed into *E. coli* JM83 unless stated otherwise.

2.3.7 Agarose gel electrophoresis

PCR products were isolated by gel electrophoresis using 1 % (w/v) agarose gel in 50 ml TAE buffer (242 g Tris, 57.1ml acetic acid and 100 ml 5 M EDTA). 5 µl of SYBR® Safe DNA gel stain was then added for visualization. DNA samples were loaded into the gel with 5x loading dye along with DNA ladder (100 bp or 1 kb; NEB). The DNA bands were then visualized via a Safe Imager™ Blue-Light Transilluminator and digital images were saved on the computer. DNA bands of interest were excised and purified using the QIAquick ® gel extraction kit (QIAGEN) according to the manufacturer's recommendations.

2.3.8 DNA quantification

Plasmid DNA samples were quantified on NanoDrop ND-1000 spectrophotometer by measuring absorbance at 260 nm. The purity was estimated by the ratio of absorbance of nucleic acids (260 nm) to amino acids (280 nm)

2.3.9 DNA sequencing

Plasmid DNA samples including pYC plasmids were sent to Source, BioScience (Nottingham) for sequencing with sequencing primers shown in Table 2-6.

Primers	Sequence (5'-3')	Description
pColE9-J No1	TTTTGTGGCCCGC TCTGCGT	Forward primer to pSBM15 (pColE9-J: Kan^r) prior to *Pst* I site
pColE9-J No2	GCAAAACCTACC GAACGTCC	Forward primer to E9 structural gene in pSBM15
pColE9-J No3	CAGTTGGTGATAA ATGGCTG	Forward primer to E9 structural gene in pSBM15
pColE9-J No4	CAGATTTGCATGA CGTTGAA	Forward primer to pSBM15
pColE9-J No5	AAAAGCGCATCA GAATCAGC	Reverse primer to pSBM15
pColE7-K317No1	TCCATGAGTGACT CCTCCGA	Forward primer to pSBM20
pColE7-K317No2	GTATTACGGAGG ATAAAGGC	Forward primer to pSBM20
pColE7-K317No3	CCTGTTCCAGATC GTATAGC	Forward primer to pSBM20
pColE7-K317No4	GCGCGTCAGAAT CAGCCTGA	Reverse primer to pSBM20

Table 2-8: The sequencing primers used in this study are listed

2.4 Bacterial transformation

2.4.1 Preparation of chemically competent *E. coli* JM83 cells

Chemically competent *E. coli* JM83 cells were prepared according to (Cohen et al. 1972). An overnight culture of *E. coli* JM83 was diluted 1 in 100 in fresh LB and was allowed to grow at 37 °C until OD_{600} of around 0.6 - 0.8. The culture was cooled on ice for 10 min prior to harvesting by centrifugation at 4,000 xg for 10 min at 4 °C. The cell pellet was re-suspended in 10 ml of ice cold 100 mM $CaCl_2$, 20 % (v/v) glycerol. Cells were then incubated for 15 min before harvesting again as described above. Cells were finally re-suspended into 2 ml of ice cold 100 mM $CaCl_2$, 20% (v/v) glycerol solution and incubated on ice for at least 2 h to induce the competency. Cells were divided into small aliquots of 100 µl and stored at -80 °C until needed.

2.4.2 Heat shock transformation of *E. coli* with plasmid DNA

Heat shock transformation was carried out by addition of 50 ng of plasmid DNA (typically 1-2 µl) to an aliquot of 100 µl of chemically competent *E. coli* cells. After incubation on ice for 30 min, the cells were then heat shocked at 42 °C for 45 s followed by cooling in ice for 2 min. The cells were then mixed with 400 µl of pre-warmed fresh LB and incubated at 37 °C for 1h. 100-200 µl of the mixture was plated on the LB agar plate with antibiotic selection.

2.4.3 Preparation of electro-competent *E. coli* JM83 cells

To make electro-competent *E. coli* JM83 cells, an overnight culture was diluted 1:100 into fresh LB supplement with appropriate antibiotics and incubated at 37 °C with shaking until OD_{600} reached around 0.6 - 1.0. The culture was chilled on ice for 30 min and then harvested by centrifugation at 4,000 xg for 15 min at 4 °C. The supernatant was discarded and the cell pellet was re-suspended in an equal volume of pre-chilled sterile distilled H_2O. The cell suspension was again centrifuged and the supernatant was discarded. This process was repeated using 4 ml of ice cold, sterile 10 % (v/v) glycerol

solution. Cells were pelleted by centrifugation and finally re-suspended into 0.4-0.6 ml of pre-chilled sterile 10 % (v/v) glycerol. Cells were aliquoted into 55 µl and stored at -80 °C until needed.

2.4.4 Transformation of *E. coli* cells by electroporation

The electroporation of plasmid DNA into *E. coli* cells was carried out according to (Dower et al. 1988). An Aliquot of 55 µl of electro-competent cells was thawed on ice and 1-2 µl of plasmid DNA was added. The mixture was incubated on ice for 1 min before transferring into a pre-chilled 2 mm electroporation cuvette (BIO-RAD). A Bio-Rad Gene Pulsar was used according to the manufacturer's instructions, to deliver an electric pulse of 2.5 kV. The cuvette was immediately removed and 1 ml of fresh LB was quickly added. After incubating at 37 °C for 1-2 h, 100-200 µl of the mixture was plated on the LB plate supplemented with the appropriate antibiotic for selection of transformed cells.

2.5 Colicin activity assay

2.5.1 Preparation of extracellular and intracellular colicin extracts

Colicin from antibiotic treated cultures was obtained according to (Chak & James 1984) with few modifications. Briefly, an overnight culture of *E. coli* JM83 containing appropriate plasmid for ColE9 or ColE7 production was diluted 1:100 with fresh LB and grown at 37 °C to an OD_{600} of around 0.2-0.4. The colicin inducing agent, mitomycin C (MMC) was then added at concentration of 0.5 µg ml^{-1}. An aliquot of 1 ml culture at a particular post treatment time point was collected and centrifuged at 6,000 x*g* for 2 min. The supernatant and cell pellet were separated by centrifugation. The supernatant was again centrifuged at 10,000 x*g* for 15 min and filter sterilized to be used as extracellular colicin extract. Meanwhile the pellet was re-suspended into 50 µl sterile PBS and followed by incubation at 4 °C for at least 20 min after the addition of 50 µl of chloroform. The sample was then centrifuged at 10,000 x*g*

for 15 min. The aqueous phase was removed and used immediately as intracellular colicin.

2.5.2 Spot test

The spot test was performed as previously described by (Penfold et al. 2000). Briefly, 7 ml of molten 0.7 % (w/v) soft top agar containing 80 – 100 µl of an overnight culture of the colicin sensitive indicator strain *E. coli* JM83 was poured onto the LB agar and then 5 µl of the test samples (appropriate dilution were made where necessary) were spotted. The plate was incubated at 37 °C overnight. The following day, the colicin activity of the test sample was scored by observing a clear zone of inhibition of the growth of the indicator cells.

2.6 Mitomycin C (MMC) sensitivity assay

Overnight cultures of the colicin producing strains (test sample) and a non-producer (control) *E. coli* strains were diluted 1:100 in 10 ml of fresh LB broth containing the appropriate concentration of antibiotics. The cultures were then grown in the shaking incubator at 37 °C to OD_{600} of around 0.3. Mitomycin C (MMC) concentration of 0.5 µg ml^{-1} was then added and OD_{600} indicating the growth of the bacteria was measured every 30 min. This concentration of MMC did not inhibit the normal growth of the control strain as it was considered a sub minimum inhibitory concentration (MIC) of MMC for *E. coli* cells.

2.7 Luminescence reporter assay

2.7.1 Experimental set-up

This assay makes use of an SOS-inducible chromosomal lux operon to detect DNA damage induced by the addition of ColE9 or ColE7 to *E. coli* DPD1718 reporter cells. *E. coli* DPD1718 contains a fusion of the *E. coli recA* promoter region and the *Photorhabdus luminescens lux CDABE* reporter integrated into

the *lacZ* locus of *E. coli* DPD1692. The assay was performed as described previously (Vankemmelbeke et al. 2005). All assays were performed in a microtitre plate luminometer (Lucy 1; Anthos Labtech, Salzburg, Austria) at 37 °C. The luminometer, microtiter plate and media were pre-warmed to 37 °C to prevent induction of a stress response due to cooling. Plasmid constructs including pSBM15, pSBM20, pACYC184 were transformed into *E. coli* DPD 1718 cells and grown in LB broth overnight in presence of appropriate antibiotics including 25 μg ml^{-1} chloramphenicol (CMP) and 50 μg ml^{-1} kanamycin (Kan). Overnight cultures of the luminescent reporter strain were diluted 1:100 with minimal media supplemented and grown at 37 °C until OD_{600} reached around ~0.35 - 0.4. The mimimal medium broth was prepared as described (Sambrook & Russell 2001). The cells were then diluted 1:2 with minimal media in a total volume of 100 μl into a 96-well black microtiter plate with an optical bottom (Nunc). Purified ColE9 or ColE7 was added to each well. Induction of luminescence was followed over a period of 3 h, with readings taken every 300 s. The cell density was also monitored by measuring OD_{492} values. Luminescence was represented as relative luminescence unit (RLU).

2.7.2 Data analysis for the lux assay

Data analysis for the luminescence assay was carried out as previously described by (Vankemmelbeke et al. 2005). For quantitative analysis, the gamma value was defined as the luminescence induced for any given sample concentration minus the luminescence of the control cells at the same time point, divided by the luminescence of the control cells at that time point as shown by equation below.

$$\gamma = \frac{L_{\text{sample}} - L_{\text{control}}}{L_{\text{control}}}$$

The arbitrary time point of 50 min for the calculation of gamma value was chosen as previously described (Vankemmelbeke et al. 2005). The protection against ColE9 or ColE7 by non-cognate immunity proteins were assessed by

dividing the gamma values of the ColE9 or ColE7 producing cells (pSBM15 or pSBM20) treated with ColE9 or ColE7 by the gamma value of control cells treated with ColE9 or ColE7. All assays were performed at least twice with three replicates for each condition.

2.8 Colicin competition assays

2.8.1 Competition experiments in a liquid (mixed) environment

Cells from an overnight culture of a ColE9 producing strain (*E. coli* JM83 pSBM15) labelled with green fluorescent protein (GFP) and a ColE7 producing strain (*E. coli* JM83 pSBM20) labelled with mCherry were collected by centrifugation at 4,000 xg for 5 min. The supernatant was discarded and the cell pellets were re-suspended in the same volume of fresh medium. The OD_{600} of the both cultures were adjusted before the cultures were mixed in a 1:1 ratio by volume. To estimate the ratio of both types of cells, an aliquot of 100 µl of the mixed culture was immediately removed and mixed with 900 µl of molten 0.7 % soft agar. Then 20 µl of the mixture was spread on to the glass slide, allowed to set and then analysed by confocal microscopy. Simultaneously, the mixed culture was diluted 1:100 into fresh medium, in a total volume of 50 ml and allowed to grow at 37 °C with shaking. After 24 h, cells were centrifuged and re-suspended into the same volume of fresh medium before analysis by confocal microscopy. The remaining culture was further diluted and grown at 37 °C with shaking for another 24 h and the measurements by confocal microscopy were repeated.

2.8.2 Confocal microscopy

A glass slide carrying the test culture was placed into the universal slide holder of an inverted Carl Zeiss LSM 700 laser scanning microscope (Germany) and confocal images and Z-stacks were captured using the ZEN software. The objective used was a Zeiss C- Apochromate 40x/ 1.2 water lens. The Apochromate 40x/1.2 water working objective lens was chosen to enable good

sample depth penetration in addition to visualizing reasonable sized areas of field of interest. The fluorescent filters and tracking were configured to enable the detection of one or more fluorescent proteins. For GFP detection, the 488 nm laser was used to excite the specimen and the short pass (SP) 555 nm emission filters to capture the signal. For simultaneous detection of mCherry the 561 nm laser was used.

2.8.3 Fluorescence reporter assay

A single colony of the *E. coli* JM83 pACYC184 (plasmid lacking *gfpmut2* gene = negative control), pSBM15, pSBM20, pSBM13+pSBM15 and pSBM16+ pSBM20 was grown overnight in LB at 37 °C. Cultures were collected by centrifugation at 4,000 xg for 5 min. The supernatant was discarded and the cell pellets were re-suspended in the same volume of fresh medium. The OD_{600} of the cultures were adjusted and then 100 µl of each culture was added to each well of a pre-warmed 96-well microtiter plate (Nunc) and then mixed either with same volume of LB or competing cultures to make up the total volume of 200 µl. The fluorescence measurements for GFP and mCherry were taken every 30 min automatically using the Tecan Infinite 200 for up to 24 h. All assays were performed with three replicates for each condition.

3 Results

3.1 ColE9 producers show selective advantage over ColE7 producers in an unstructured mixed environment

A fluorescent reporter assay was developed in the previous study by (Bano 2010) to study the evolution and diversity of the colicins within microbial community where there are more than one colicin producing strains. A dual fluorescent system was used to visualise the competition between two E colicin strains producing two different DNase domains, ColE9 and ColE7. From the preliminary data, it was suggested that ColE9 producing strain had competitive advantage against ColE7 in a well-mixed environment. In order to confirm this, the dual fluorescent system was repeated with one set of cells expressing ColE9 and GFP (green) and another set expressing ColE7 and mCherry (red) over 48 h and was visualised using the confocal microscopy. After 48 h, the confocal image appeared predominantly green indicating ColE9 producing strains were the dominant strain and had clearly outcompeted ColE7 producing strains. These data confirm that in an unstructured environment of mixed cultures, ColE9 producing cells exhibit a competitive advantage over ColE7 producing cells.

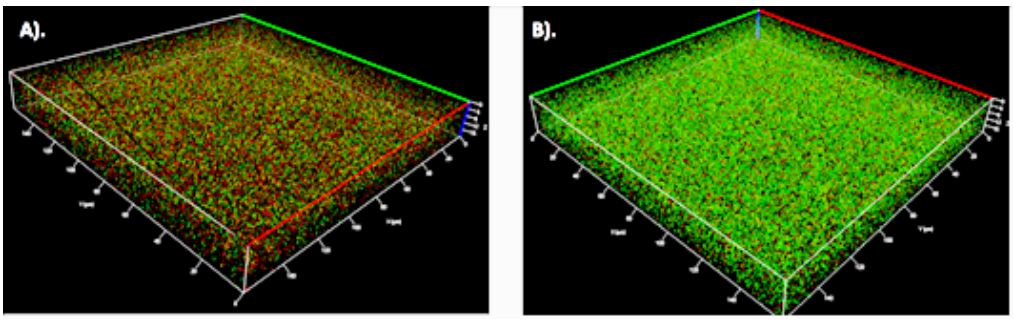

Figure 3-1: Confocal microscopy images showing the result of competition between ColE9 and ColE7 producing cells from this study.

A). Time = 0 h (at the time of mixing, both cultures for competition in well mixed environment), there is an equal ratio of ColE9 (labelled green) and ColE7 (labelled red) producer strains.

B). After 48 h, the confocal image became predominately green indicating the selective growth advantage of the ColE9 producing cells.

3.2 Investigation of the reasons for the competitive advantage of ColE9 producing cells over ColE7 cells

In this study, several factors that may contribute to the observed competitive advantage of ColE9 producers over ColE7 producers are considered including;

1). Differential cell lysis of ColE9 and ColE7 operons upon MMC induction

2). Difference in the speed of cell entry and killing by ColE9 and ColE7

3). Difference in synthesis and release of ColE9 and ColE7 operons upon MMC induction

4). Difference in protection conferred by Im9 and Im7 against a non-cognate E colicin

3.2.1 ColE7 operon causes significant cell lysis upon MMC induction whereas ColE9 operon does not

Colicin induction is a lethal event for the producing cell and is regulated by the SOS response and involves co-expression of colicin structural gene and the colicin lysis gene (Spangler et al. 1985). MMC sensitivity assay was carried out to investigate whether prolonged exposure to strong DNA damaging agent such as MMC, can induce SOS promoters in ColE9 and ColE7 operons to express their lysis genes and therefore result in the lysis (death) of the host *E. coli* JM83 cells. This assay investigates the differences in the level of cell lysis between *E. coli* JM83 cells carrying pSBM15 (pColE9-J with kanamycin resistance) and pSBM20 (pColE7-K317 with kanamycin resistance) and pACYC184. As shown in Figure 3-2, *E. coli* JM83 cells carrying pSBM20 had reached the stationary phase at OD_{600} values of ~0.7 after 2 h of MMC induction whereas OD_{600} values increased constantly in *E. coli* JM83 pSBM15 despite MMC induction. No change was observed in *E. coli* JM83 pACYC184 cells (control) that do not contain colicin genes indicating that the observed effect may be due to the differences in the lysis of ColE9 and ColE7 cells. The

data suggests that ColE7 operon causes significantly greater level of cell lysis upon MMC induction compared to ColE9 operon. This difference in cell lysis may explain the observed competitive advantage of ColE9 producers over ColE7 producers. ColE9 released by ColE9 producing cells may induce the ColE7 operon to cause significant cell lysis (death) whilst ColE7 may have limited induction on ColE9 operon resulting in smaller degree of cell lysis.

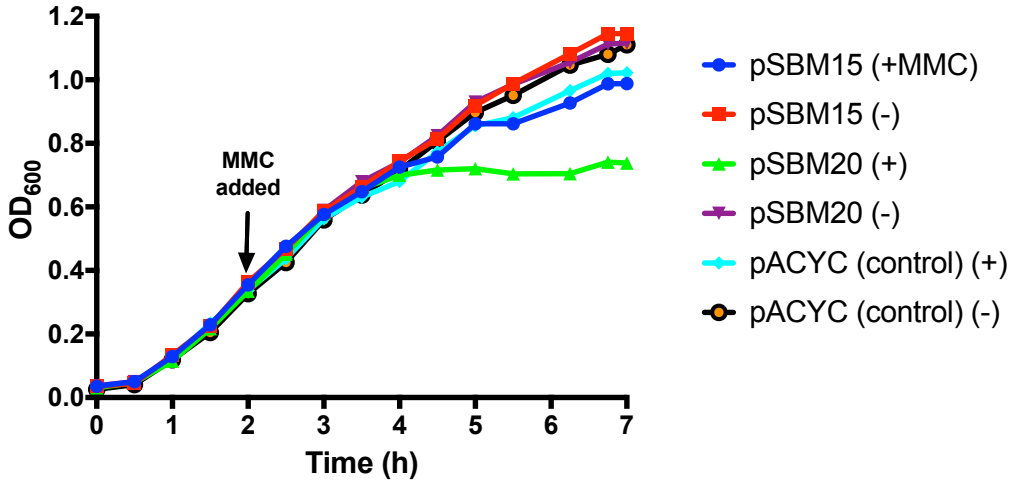

Figure 3-2: MMC sensitivity assay showing changes in cell density of *E. coli* JM83 cells carrying pACYC184 (control), pSBM15 and pSBM20

Following 2 h induction with sub-MIC concentration (0.5 µg ml^{-1}), *E. coli* JM83 cells carrying pSBM20 had reached stationary phase at OD_{600} values of ~ 0.7 indicating cell lysis whilst *E. coli* cells carrying pSBM15 did not show any decrease in OD upon MMC induction. The assays were repeated three times with similar results.

3.2.2 ColE7 has faster speed of cell entry and killing than ColE9

In this study, growth curve analysis was carried out on *E. coli* JM83 cells carrying pSBM20 and pSBM15 to investigate the speed of cell entry and killing on adding external ColE9 and ColE7. Figure 3-3 shows that the reduction in culture turbidity relative to the control strains was observed within 1 h of addition of ColE7 to *E. coli* JM83 pSBM15 cells whereas similar decline was seen after 1 h of addition of ColE9 to JM83 *E. coli* pSBM20 cells. This suggests that ColE7 may have faster cell entry and killing than ColE9.

Interestingly, despite an earlier decline in the relative cell density of ColE9 producers (pSBM15) by externally added ColE7, cells producing ColE9 had eventually reached plateau around OD_{600} of 0.5. On the other hand, despite slower action of ColE9, OD_{600} for *E. coli* JM83 cells carrying pSBM20 reached lower than 0.4. This may be due to the greater extent of ColE7 cell lysis upon prolonged induction with ColE9 which agrees with the result from MMC sensitivity assay. No decline in cell turbidity was seen when external colicin was added to cells expressing cognate immunity i.e. Im9 and Im7 provide full protection against externally added cognate ColE9 and ColE7 respectively.

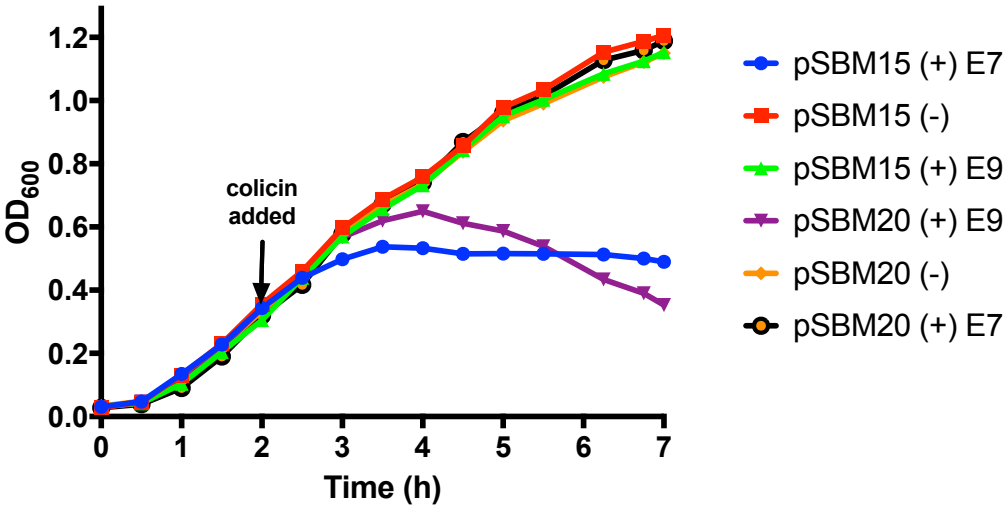

Figure 3-3: Growth curve assay showing colicin mediated decline in cell growth.

Growth curve assay showing changes in cell density of *E. coli* JM83 carrying pSBM15 and pSBM20 after addition of either 4 nM ColE7 or ColE9 at 2 h. There was reduction in culture turbidity relative to the control strains which was observed within 1 h of addition of ColE7 to *E. coli* JM83 pSBM15 cells whereas similar decline was seen after 1 h of addition of ColE9 to JM83 *E. coli* pSBM20 cells. This suggests that ColE7 may have faster cell entry and killing than ColE9. Despite slower action of ColE9, OD_{600} for *E. coli* JM83 cells carrying pSBM20 reached lower than 0.4. This may be due to the greater extent of ColE7 cell lysis upon prolonged induction with ColE9. The assays were repeated twice with similar results.

3.2.3 ColE9 is produced in smaller amount than ColE7 and its release requires MMC induction

The spot test was performed to compare the amount of intracellular and extracellular ColE9 and ColE7 on MMC induction. The amount of colicin present within the *E. coli* JM83 cells was used to measure the intracellular titre and the amount of colicin exported from cells into the culture media was used as the extracellular titre. The colicins caused zones of inhibition on the LB agar plate as they killed colicin-sensitive indicator strain and colicin titre was then determined as shown in the Figure 3-4. After 1 h of MMC induction, the zones of inhibition were seen for both intracellular and extracellular ColE7 up to the 10^{-1} dilution but there was greater amount of ColE7 intracellularly (Figure 3-4A). Similar amount of intracellular ColE7 was also observed in the un-induced sample but the amount of extracellular ColE7 was slightly less than the induced sample. There was no intracellular or extracellular ColE9 on un-induced sample while there was very faint zone of clearance for the undiluted sample of ColE9 after 1 h of MMC induction.

After 4 h of MMC induction, both intracellular and extracellular ColE9 killing was observed up to the 10^{-2} dilution. The zones of clearance produced by intracellular and extracellular ColE9 at the dilution of 10^{-2} was comparable to that of ColE7 at 10^{-4} dilution indicating approximately 100-fold greater production of ColE7 than ColE9 (Figure 3-4B). Comparison between the induced and un-induced samples of ColE9 reveals that the both synthesis and release of ColE9 are significantly increased by prolonged exposure to MMC. The synthesis and release of ColE7 is increased by small extent on MMC induction but ColE7 is produced and released even without MMC induction.

The results suggest that the ColE7 is produced quicker in greater amounts and released more efficiently than ColE9. This suggests that in the early phase of competition between ColE7 and ColE9 producing cells, ColE7 cells may have the upper hand as ColE7 is produced and released faster to initiate killing of ColE9 cells. However, induction of ColE9 operon by ColE7 cause production and release of ColE9 which in turn causes significant cell lysis of ColE7

producers over the longer period of time accounting for the observed competitive advantage of ColE9 producers after 48 h.

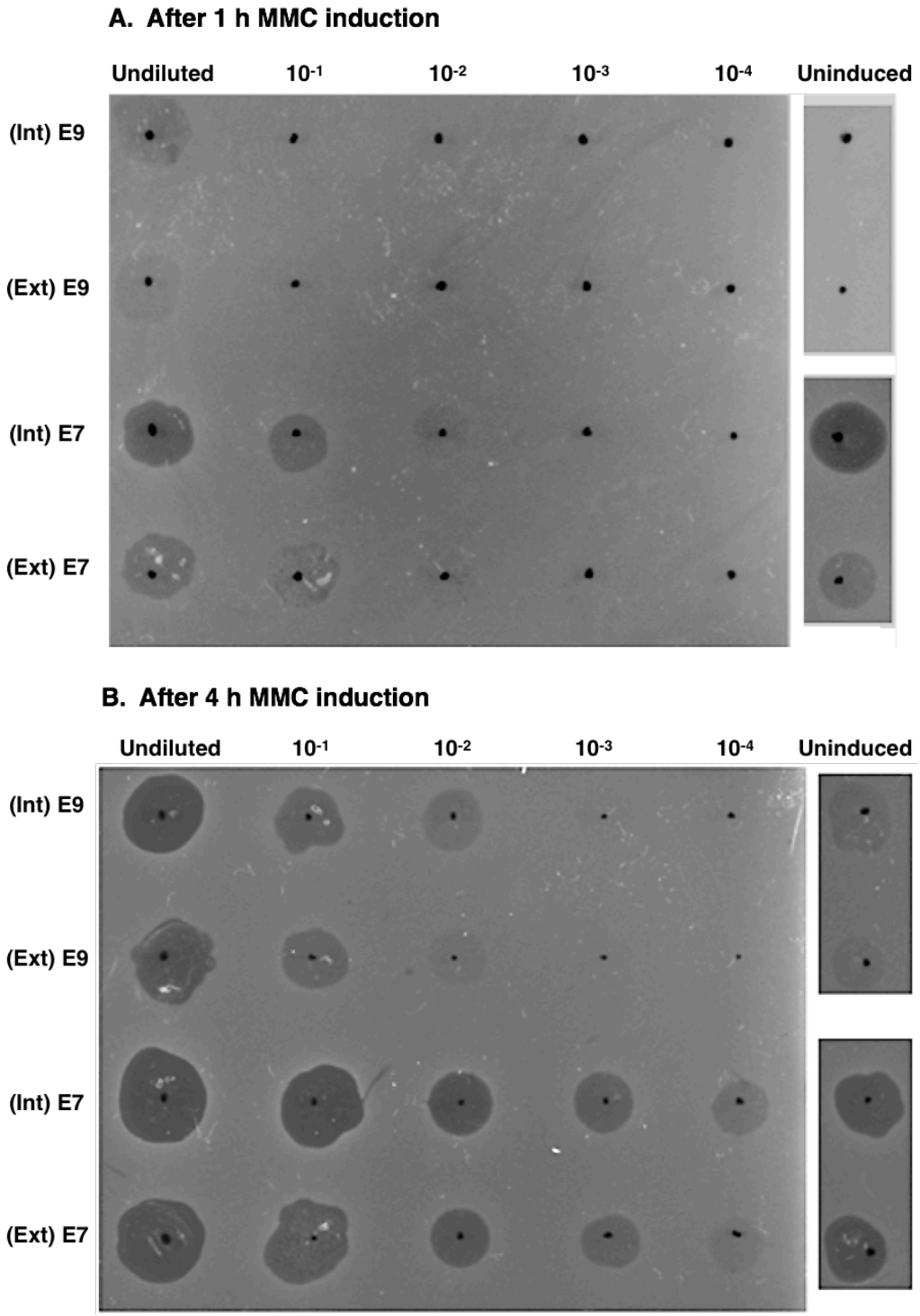

Figure 3-4: Spot tests to determine the intracellular and extracellular colicin titres on MMC induction tested on JM83 *E. coli* cells. A). 1 h of MMC induction: the zones of inhibition are seen for both intracellular and extracellular ColE7 up to the 10^{-1} dilution whereas there was no intracellular or extracellular ColE9 on un-induced sample and very small zones

for the undiluted ColE9. This indicates the ColE7 is synthesized and released without MMC induction.

B). 4 h of MMC induction. Both intracellular and extracellular ColE9 was observed up to the 10^{-2} dilution. The zones of clearance produced by intracellular and extracellular ColE9 at the dilution of 10^{-2} was comparable to that of ColE7 at 10^{-4} dilution indicating approximately 100-fold greater production of ColE7 than ColE9. This suggests that the ColE7 is produced quicker in greater amounts and therefore released more efficiently than ColE9.

3.2.4 Im9 may have greater protection than Im7 against non-cognate E colicins at higher concentrations

The luminescence reporter assay (*lux*), which detects DNA damage induced by the addition of colicins as luminescence, was carried out to investigate the differences in the protection conferred by cognate and non-cognate immunity proteins against externally added ColE7 or ColE9; pSBM20 expressing Im7 challenged with ColE9 or pSBM15 expressing Im9 challenged with ColE7 at concentrations of 0.4 nM, 4 nM and 40 nM (Figure 3-5). As expected, Im9 and Im7 had provided full protection against their cognate colicins (ColE9 and ColE7 respectively) which was shown by no increase in the luminescence values compared to the control cells without addition of any colicin (Figure 3-5A and Figure 3-5C). The experiments with ColE7 were hampered by the fact that the control cells treated with 4 nM or 40 nM ColE7 mounted a very low response (very low luminescence values) due to effective cell killing (Figure 3-5C and Figure 3-5D). This made it difficult to calculate the gamma values accurately at these concentrations. Similar problem was seen when ColE9 was added to the control cells at the concentration of 40 nM. Control cells treated with 40 nM had produced lower level of RLU compared to the control cells treated with ColE9 at concentration of 4 nM (Figure 3-5A and Figure 3-5B). Therefore, the experiments were repeated using a lower concentration range (0.1 nM and 0.4 nM) of ColE7 and ColE9 (Figure 3-6). The controls cells showed dose-dependent increase in RLU values when ColE9 or ColE7 were added at the concentrations of 0.1 nM and 0.4 nM. There was no change in RLU values for pSBM20 + ColE7 or pSBM15 + ColE9 compared to the

control cells without addition of colicins (Figure 3-6). This indicates that there is full protection by immunity proteins against addition of cognate colicins; Im7 vs ColE7 and Im9 vs ColE9. For pSBM15 + ColE7 or pSBM20 + ColE9 (addition of colicins against non-cognate immunity proteins), there was colicin dose dependent increase in RLU values over time. However, the RLU values were significantly less when compared to the controls cells treated with same concentration of colicins. These results indicate that the presence of immunity protein offer some degree of protection against non-cognate ColE9 and ColE7.

The results of the percentage protection from number of *lux* assays are summarised in Table 3-1. The percentage protection values for Im9 vs ColE9 (pSBM15 + ColE9) and Im7 vs ColE7 (pSBM 20 + ColE7) were calculated to be 99–100 % which indicate that immunity proteins offer full protection against cognate colicins. At colicin concentrations at 0.1 nM and 4 nM, Im9 was shown to have higher protection against ColE7 compared to Im7 against ColE9 (75% vs 56% at and 66% vs 58% respectively). Unexpectedly, Im7 was found to have higher protection against ColE9 at 0.4 nM concentration than Im9 against ColE7 (64±7 % vs 53±12 %). However, the protection values were shown to have high standard error values. Overall, the results suggest that both Im9 and Im7 provide some protection against non-cognate colicins and Im9 may have higher protection than Im7 at higher concentration of non-cognate colicins.

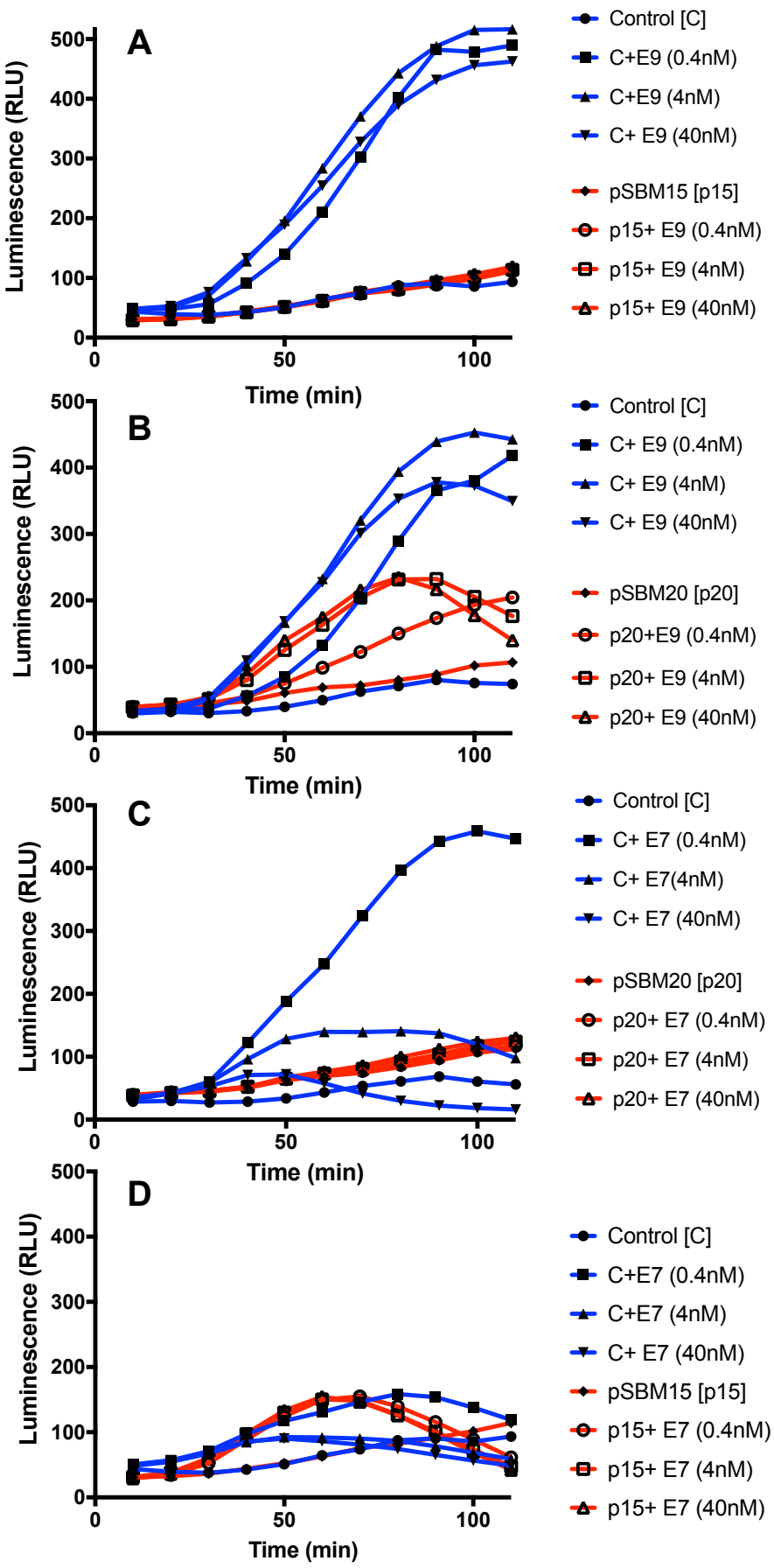
A
Luminescence (RLU)
Time (min)
Control [C]
C+E9 (0.4nM)
C+E9 (4nM)
C+ E9 (40nM)
pSBM15 [p15]
p15+ E9 (0.4nM)
p15+ E9 (4nM)
p15+ E9 (40nM)
B
Luminescence (RLU)
Time (min)
Control [C]
C+ E9 (0.4nM)
C+ E9 (4nM)
C+ E9 (40nM)
pSBM20 [p20]
p20+E9 (0.4nM)
p20+ E9 (4nM)
p20+ E9 (40nM)
C
Luminescence (RLU)
Time (min)
Control [C]
C+ E7 (0.4nM)
C+ E7(4nM)
C+ E7 (40nM)
pSBM20 [p20]
p20+ E7 (0.4nM)
p20+ E7 (4nM)
p20+ E7 (40nM)
D
Luminescence (RLU)
Time (min)
Control [C]
C+E7 (0.4nM)
C+E7 (4nM)
C+ E7 (40nM)
pSBM15 [p15]
p15+ E7 (0.4nM)
p15+ E7 (4nM)
p15+ E7 (40nM)

Figure 3-5: Luminescence reporter assay (*lux*) to investigate the differential protection conferred by cognate and non-cognate immunity against externally added ColE7 or ColE9 at concentrations of 0.4 nM, 4 nM and 40 nM.

A). Im 9 versus ColE9. ColE9 producing cells (pSBM15) with Im9 show no increase in the relative luminescence compared to the control cell. This shows that there is full protection of Im9 against cognate ColE9.

B). Im7 versus ColE9. There was increase in luminescence when ColE9 was added to ColE7 producing cells but there was far less luminescence when compared to control cells with same concentration of ColE9 added. This indicates that Im7 offer some degree of protection against ColE7.

C). Im 7 versus ColE7. ColE7 producing cells (pSBM20) show no increase in the relative luminescence compared to the control cell. This shows that there is full protection of Im7 against cognate ColE7

D). Im9 versus ColE7. Control cells treated with 4 nM or 40 nM ColE7 mounted a very low response due to effective cell killing which made it that made it difficult to calculate the percentage protection for Im9 against ColE7 at these concentrations.

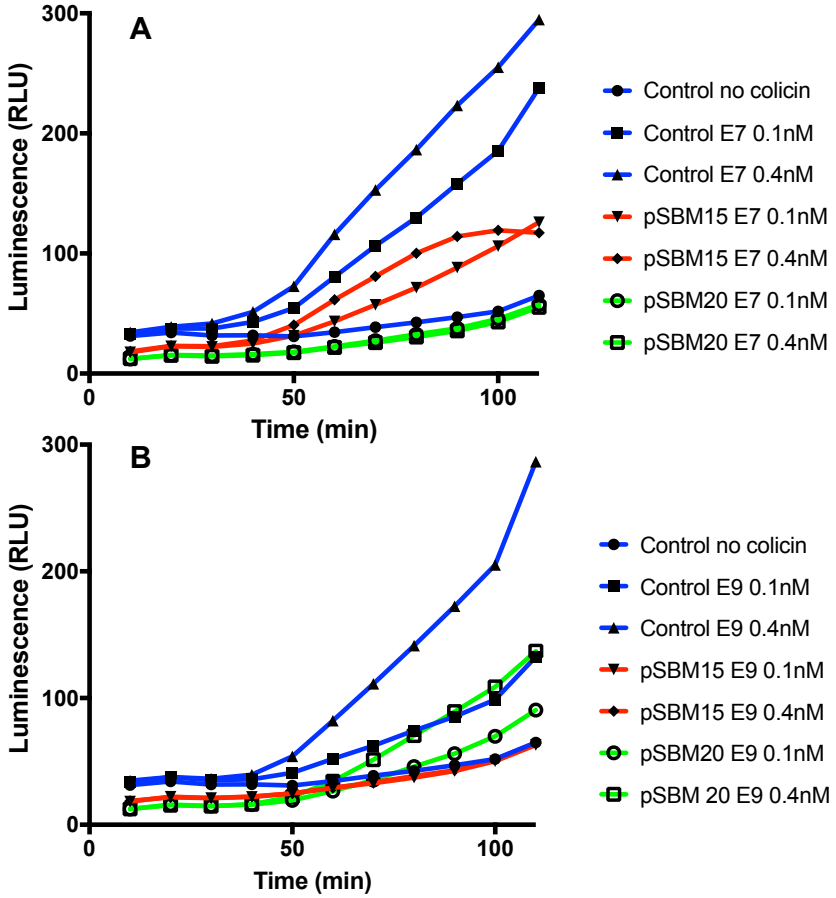

Figure 3-6: Luminescence reporter assay (*lux*) to investigate the differential protection conferred by cognate and non-cognate immunity against externally added ColE7 or ColE9 at concentrations of 0.1 nM and 0.4 nM.

A). ColE7 producing cells (pSBM20) show no increase in RLU indicating that Im7 offers full protection against ColE7 added. On the other hand, ColE9 producing cells (pSBM15) show increase in RLU values over time in a dose dependent manner but show less relative RLU compared to the control cells that contain no immunity protein. This indicates that Im9 offer some degree of protection against ColE7.

B). ColE9 producing cells (pSBM15) show no increase in RLU indicating that Im9 offers full protection against ColE9 added. ColE7 producing cells (pSB20) start to increase in RLU values after 60 min compared to control cells which show rise in RLU value after 50 min. This suggests that Im7 offer some degree of protection against ColE9.

conditions	% protection			
	0.1 nM	0.4 nM	4 nM	40 nM
pSBM15 + ColE9	99	99±1	100±0	100±0
pSBM15 + ColE7	75±0	53±12	66	NA
pSBM20 + ColE9	56	64±7	58±5	47±10
pSBM20 + ColE7	99	99±1	99±1	100

Table 3.1. The summary of average percentage protection values from several *lux* assays are shown with the standard error of the mean.

The percentage protection values for Im9 vs ColE9 (pSBM15 + ColE9) and Im7 vs ColE7 (pSBM 20 + ColE7) were calculated to be 99–100 % which indicate that immunity proteins offer full protection against cognate colicins. At colicin concentrations at 0.1 nM and 4 nM, Im9 was shown to have higher protection against ColE7 whereas Im7 was found to have higher protection against ColE9 at 0.4 nM concentration.

3.3 ColE7 producing strains are winner against ColE9 producers in the early phase of competition

Following the observation of selective advantage of ColE9 producing cells over ColE7 cells after 48 h under confocal microscopy, an attempt was made to monitor the competition between ColE9 and ColE7 producers in real time using a dual fluorescence report system under Tecan. ColE9 producing strains labelled with GFP (pSBM13+ pSBM15) and ColE7 producing strains labelled with mCherry (pSBM16 + pSBM20) were mixed and the competition was monitored over 24 h (Figure 3-7). ColE9 (pSBM 15 + pACYC184) and ColE7 (pSBM20+pACYC184) producing strains without any fluorescence were used as the negative controls.

As shown in Figure 3-7A, the GFP values increase constantly for cells carrying pSBM (13/15) indicating unlimited growth of ColE9 producing strains over time when there is no competition. On the other hand, the GFP value for the

competing mixture (pSBM13/15 + pSBM16/20) initially increases at the similar rate but reaches a plateau after 2 hours. This suggests that there may be inhibition of further growth of ColE9 producing cells due to the antagonistic effect of ColE7 producers. The mCherry values for non-competing *E. coli* cells carrying pSBM (16/20) also increase over 24 h period as shown in (Figure 3-7B). The result also shows that mCherry values for the competing mixture (pSBM13/15 + pSBM16/20) increases consistently over time to the values ~ 30,000.

This indicates that during the early phase of competition in a stationary mixture, ColE7 producing cells seem to have a selective advantage over ColE9 producing cells for first 24 h. This may be because ColE7 is produced and released faster by ColE7 producing cells as shown in the previous biological activity assay (Figure 3-4). It is also important to note that incubating conditions are different for two methods (confocal microscopy and Tecan) used for monitoring competition. For confocal microscopy, the competing cells were made up to total volume of 50 ml and were grown at 37 °C with shaking (well-mixed environment) and mixed culture was diluted 1:100 into fresh LB medium at 24 h whereas in tecan, the competing cells were made up the total volume of 200 µl and were incubated without constant shaking (non-mixed environment). It is possible that without shaking (non-well mixed environment), the ColE9 and ColE7 producer cells are closer to each other allowing ColE7 (synthesized quicker and in larger quantities than ColE9) to diffuse relatively shorter distance to exert its killing activity on ColE9 producing cells.

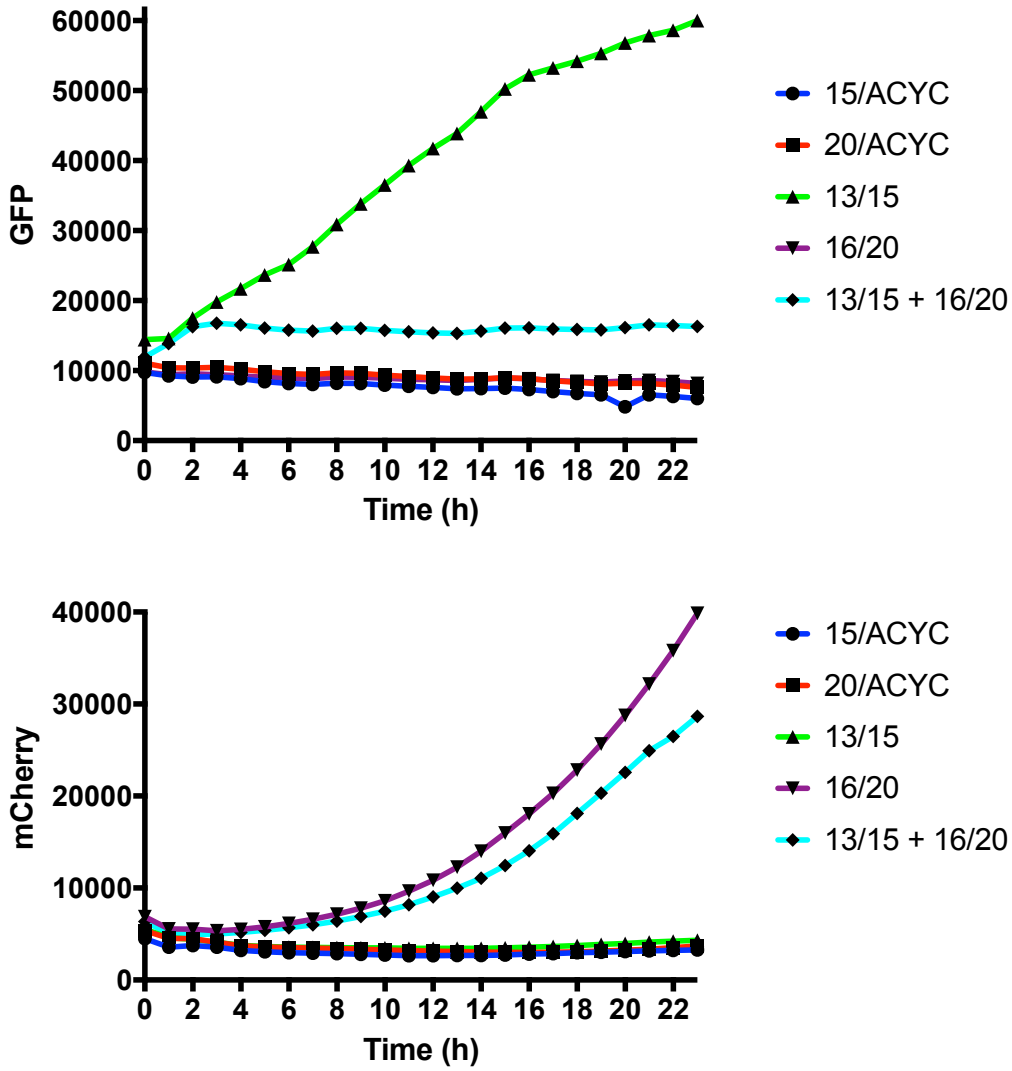

Figure 3-7: Dual fluorescence reporter assay was carried out using Tecan to monitor the competition between ColE9 labelled with GFP (pSBM 13/15) and ColE7 labelled with mcherry (pSBM 16/20) strains in real-time over 24 h.

A). The GFP value for the competing mixture (pSBM13/15 + pSBM16/20) initially increases at the similar rate to that of control (pSBM13/15) but reaches a plateau after 2 hours indicating no further growth of ColE9 cells due to the antagonistic action of ColE7 producers.

B). mCherry values for the competing mixture (pSBM13/15 + pSBM16/20) increases consistently over time to ~ 30,000 closely following the control (pSBM16/20) which indicates that ColE7 producing cells outcompete ColE9 cells.

3.4 The roles of ColE9 and ColE7 lysis genes in the outcome of competition experiment

The results have suggested that synthesis and release of ColE9 requires MMC induction and ColE9 operon undergoes limited cell lysis on MMC induction compared to that of ColE7. Therefore, to investigate the roles of lysis genes of ColE9 and ColE7 in conferring the selective advantage of ColE9 producing cells over ColE7 producers in an unstructured well-mixed environment, their lysis genes were switched.

3.4.1 Switching of the lysis genes of ColE9 and ColE7

ColE9/Im9 genes were transferred to the ColE7 background and vice versa using restriction endonuclease digestions and DNA cloning (Figure 3-8). Therefore, site directed mutagenesis was used to insert *Pst* I sites upstream of ColE9 and ColE7 structural gene and *Bam*H I sites downstream of immunity genes to allow the switching of their lysis genes. The plasmids encoding ColE9 (pSBM15) and ColE7 (pSBM20) with *Pst* I and *Bam*H I sites inserted were labelled pYC12 and pYC13 respectively. Double digestion of pYC12 and pYC 13 with restriction endonucleases *Pst* I and *Bam*H I, produced two fragments of expected sizes (around 2 kb for colicin structural and immunity genes and >5kb for vector backbone containing the lysis genes) respectively (Figure 3-9) which was subsequently ligated and transformed to produce pYC14 (E9VE7I) and pYC15 (E7VE9I). The DNA sequence of pYC14 and pYC15 was confirmed by sequencing.

The recombinant plasmid pYC14 consists of ColE7 structural genes and Im7 genes followed by ColE9 lysis gene, ColE5 immunity and lysis genes while pYC15 contain ColE9 structural genes and Im9 genes followed by ColE7 lysis gene.

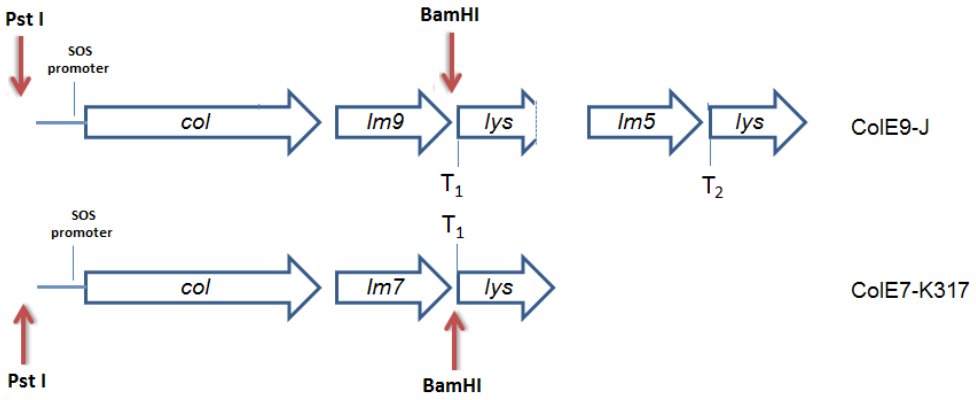

Figure 3-8: The diagrammatic representation of colicin E9 and colicin E7 gene clusters.

Col is the colicin structure gene, Im is the immunity gene, and lys is the lysis gene. ColE9 lysis gene is truncated as shown above. T1 and T2 are the transcription terminators 1 and 2. *Pst* I and *Bam*H I sites are inserted at the above sites to allow switching of their lysis genes.

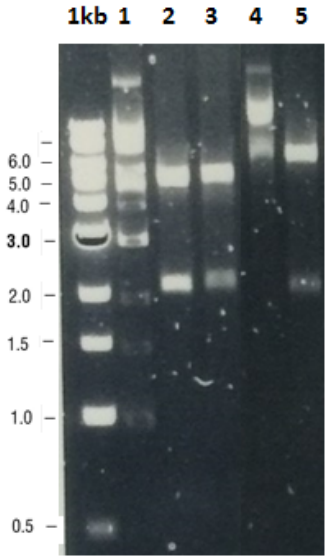

Figure 3-9: Agarose gel electrophoresis of pYC12 and pYC13 following digestion with *Pst* I and *Bam*H I restriction endonucleases. Lane 1 contains an undigested pSBM20 (around 7.5kb) which may exist as nicked, supercoiled and circular. Lanes 2 and 3 contain pYC13 double digested with *Pst* I and *Bam*H I which produce two fragments of about 5.5kb (E7V) and 2kb (E7I) respectively. Lane 4 contains an undigested pSBM15 undigested (around 9kb). Lane 5 contains pYC12 double digested which produces two fragments of about 7kb (E9V) and 2kb (E9I) respectively.

3.4.2 ColE7 producers win against ColE9 producers in a well-mixed environment after switching their lysis genes

In this study, the competition experiment between ColE9 and ColE7 producing cells in a well-mixed environment have shown the selective advantage of ColE9 producing cells over ColE7 producers. MMC sensitivity assay and spot tests have suggested the ColE7 lysis gene may be responsible for the observed outcome. Therefore, new competition experiment between *E. coli* JM83 cells containing pYC14 (E9VE7I) labelled with GFP and pYC15 (E7VE9I) labelled with mCherry were carried out using the methods as previously described.

Interestingly, following 48 h of incubation, ColE7 producing cells (pYC14) containing E9 lysis gene (green) appear to be greater than ColE9 producing cells (red) indicating that the previously observed outcome of competition between ColE9 and ColE7 have been reversed after swapping their lysis genes (Figure 3-10). This supports the hypothesis that the observed competitive advantage may be due to their lysis genes. It is possible that ColE9 producers (pYC15) may undergo more efficient cell lysis on induction due to its ColE7 lysis gene whereas ColE7 producers (pYC14) may only have limited lysis due to the ColE9 lysis genes.

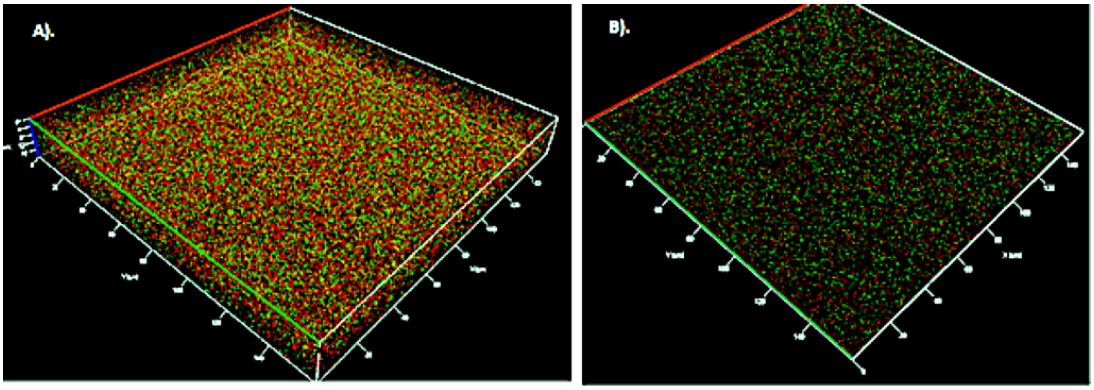

Figure 3-10: Confocal microscopy images showing the result of competition between ColE9 and ColE7 producing cells with their lysis genes switched.

A). at time = 0 h (at the time of mixing both cultures for competition in well mixed environment), there is approximately equal ratio of ColE7 producers with ColE9 lysis genes labelled green (E9VE7I) and ColE9 producing cells with ColE7 lysis genes labelled red (E7VE9I)

B). After 48 h, the image appears to be greener. This may be due to increased level of GFP indicating the selective growth advantage of the ColE7 producing cells (pYC14 or E9VE7I).

3.4.3 Both pYC14 (E9VE7I) and pYC15 (E7VE9I) plasmids cause some degree of cell lysis upon MMC induction

Previous MMC sensitivity assays have demonstrated a decline in culture turbidity in *E. coli* JM83 cells carrying pSBM20 whereas there was no decline in *E. coli* JM83 cells carrying pSBM15 (Figure 3-3). This indicates that ColE7 lysis gene may be causing significant lysis on MMC induction whereas ColE9 lysis gene does not. MMC sensitivity assay was performed with *E. coli* JM83 cells carrying pYC14 (E9VE7I) and pYC15 (E7VE9I) as previously described (Figure 3-11).

Surprisingly, *E. coli* cells carrying pYC14 and pYC15 start with similar starting OD_{600} of ~ 0.3 and then follow similar growth pattern over 7 h; cell density initially increases up to 2 h post MMC induction followed by a slight decline in turbidity of cell cultures in both. The OD_{600} values for cells carrying ColE7 lysis gene (E7VE9I) are lower throughout 7 h of MMC induction

compared to those with the ColE9 lysis genes (E9VE7I). Direct comparison of OD_{600} values of cells with pSBM15 and pSBM20 are difficult as the starting OD_{600} were different to those of pYC14 and pYC15.

The results suggest that whilst ColE7 lysis gene can cause significant cell lysis, ColE9 producing cells containing ColE7 lysis genes may have slightly higher level of lysis on MMC induction compared to ColE7 producing cells containing ColE9 lysis genes. This suggests that the expression of lysis genes may also be dependent on its co-expression of their structural genes. It is possible that ColE7 operon has stronger SOS promoter than ColE9 operon and therefore undergo greater level of expression on MMC induction. This may explain why ColE7 producing cells (ColE7 operon + ColE9 lysis genes) show similar amount of lysis to ColE9 producing cells (ColE9 operon + ColE7 lysis genes) upon MMC induction.

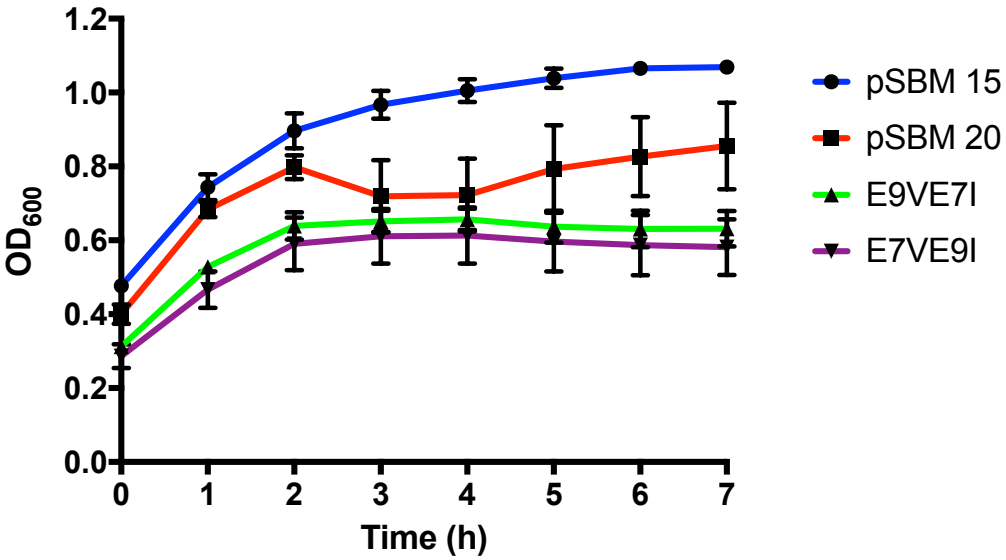

Figure 3-11: MMC sensitivity assay showing changes in average cell density

(OD measured in triplet) of *E. coli* JM83 cells carrying pSBM15, pSBM20, pYC14 (E9VE7I) and pYC15 (E7VE9I). The MMC was added on time = 0 h and the final concentration of MMC was 0.5 µg ml^{-1}. *E. coli* cells carrying pYC14 and pYC15 start with similar starting OD_{600} of ~ 0.3 and then follow similar growth pattern over 7 h. The OD_{600} values for cells carrying ColE7 lysis gene (E7VE9I) are slightly lower throughout 7 h of MMC induction compared to those with the ColE9 lysis genes (E9VE7I). This suggests both strains (E9VE7I and E7VE9I) have similar level of lysis.

3.4.4 ColE7 lysis protein promotes synthesis and release on MMC induction

The spot test was performed to compare the amount of intracellular and extracellular ColE7 and ColE9 from cells carrying pYC14 (E9VE7I) and pYC15 (E7VE9I) on induction with sub-MIC concentration of MMC as previously described.

After 1 h of MMC induction, pYC14 (E9VE7I) was found to produce zones of inhibition for intracellular ColE7 up to the 10^{-3} dilution but there was extracellular ColE7 in the undiluted sample only (Figure 3-12A). The similar amount of intracellular and extracellular ColE7 was also observed in the un-induced sample indicating that ColE7 are produced regardless of the MMC induction. On the other hand, pYC15 (E7VE9I) produced hazy zones of clearance for both intracellular and extracellular ColE9 on MMC induction which was significantly greater than un-induced samples. Interestingly, there was greater amount of extracellular ColE9 (Ext E7VE9I) as the zone was seen up to the 10^{-1} dilution compared to amount of extracellular ColE7 (Ext E9VE7I) in which the small zone was only seen in undiluted samples. This may be due to expression of ColE7 lysis genes causing significant cell lysis releasing of ColE9 into the external medium. Un-induced sample showed similar pattern with greater amount of extracellular ColE9.

After 4 h of MMC induction, the intracellular ColE7 (Int E9VE7I) killing was observed up to the 10^{-3} dilution and extracellular ColE7 killing was observed up to the 10^{-2} dilution (Figure 3-12B). Uninduced sample had only very small and faint zone of inhibition on extracellular ColE7 indicating the lysis genes in ColE9 operon requires MMC induction to cause significant cell lysis to release its intracellular ColE7. The zones of clearance produced by both intracellular and extracellular ColE9 (E7VE9I) were similar in both induced and un-induced samples indicating that ColE7 lysis gene does not require MMC induction to release ColE9.

These data show that the ColE7 is being produced faster which may suggests that the promoter activity of ColE7 operon may be more efficient than that of ColE9. The results also suggest that ColE7 lysis genes may be more efficient than ColE9 lysis genes to cause cell lysis and releasing the colicins into the external medium.

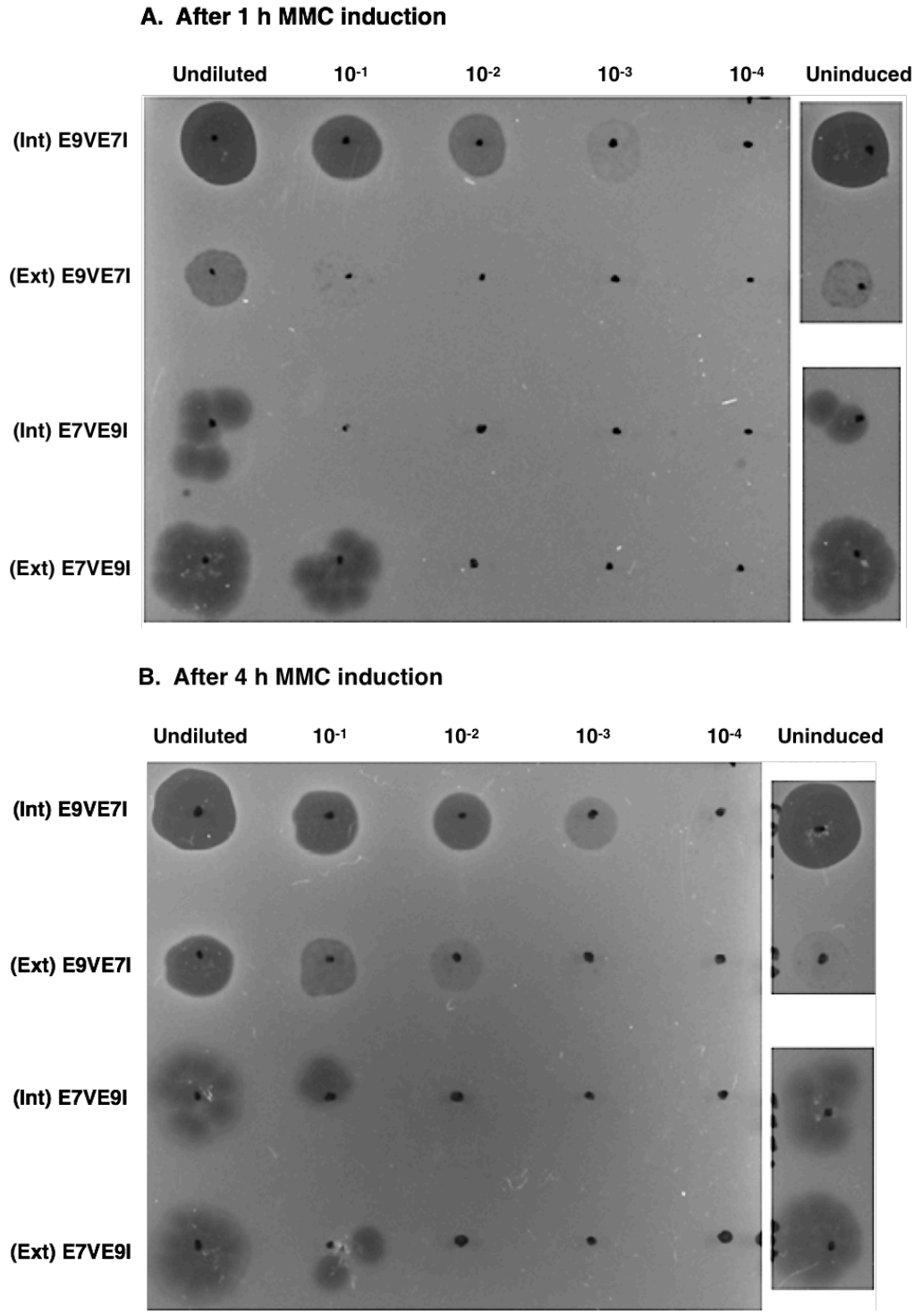

Figure 3-12: Biological activity assay of internal and external secreted colicin against JM83 *E. coli* cells

A. 1 h of MMC induction. pYC14 (E9VE7I) produces zones of inhibition for intracellular ColE7 up to the 10^{-3} dilution and extracellular ColE7 in the undiluted sample. The similar amount of intracellular and extracellular ColE7 was also observed in the un-induced sample indicating that ColE7 are produced regardless of the MMC induction. On the other hand, pYC15 (E7VE9I) produced hazy zones of clearance for both intracellular and extracellular

ColE9 on MMC induction which was significantly greater than un-induced samples. There was greater amount of extracellular ColE9 (Ext E7VE9I) than extracellular ColE7 (Ext E9VE7I). This suggests greater expression of ColE7 lysis genes.

B. 4 h of MMC induction. The intracellular ColE7 (Int E9VE7I) was observed up to the 10^{-3} dilution and extracellular ColE7 was seen up to the 10^{-2} dilution. Uninduced sample had only very small and faint zone of inhibition on extracellular ColE7 indicating the lysis genes in ColE9 operon requires MMC induction. The zones of clearance produced by both intracellular and extracellular ColE9 (E7VE9I) were similar in both induced and un-induced samples indicating that ColE7 lysis gene does not require MMC induction to release ColE9.

3.4.5 ColE7 producing strains outcompete ColE9 producers in early phase of competition despite the lysis genes swap

The previous real-time dual fluorescence reporter assay to monitor competition between ColE9 and ColE7 producers have indicated that ColE7 producers may have selective advantage in the first 24 h of the competition in stationary mixture (Figure 3-7). Given the reversed outcome in competition assay after switching of lysis genes (Figure 3-10), the real-time dual fluorescence reporter assay was carried out to investigate the competition between ColE7 producing strains labelled with GFP (E9VE7I + pSBM13) and ColE9 producing strains labelled with mCherry (E7VE9I + pSBM16) in real-time (Figure 3-13).

As shown in Figure 3-13, the GFP values for competing mixture (E9VE7I/13 and E7VE9I/16) increases at a constant rate and reaches to similar level of GFP value of the negative control after 24 h which suggests that ColE7 producers (with ColE9 lysis gene) can grow well in presence of ColE9 producing cells. On the other hand, mCherry values for the competing mixture remain at constant value compared to the non-competing cells (E7VE9I/16) which increases continuously over time indicating that ColE9 producing cells (with ColE7 lysis gene) are unable to grow. This suggests that in the competing environment of a stationary mixture, ColE7 producing cells (regardless of their lysis genes) seem to inhibit the growth of ColE9 producing cells due to the antagonistic actions of the colicins. This agrees with results from previous biological assays (Figure 3-4 and Figure 3-12) where the ColE7 is produced

faster in larger quantities and will be released by lysis on induction of its SOS system.

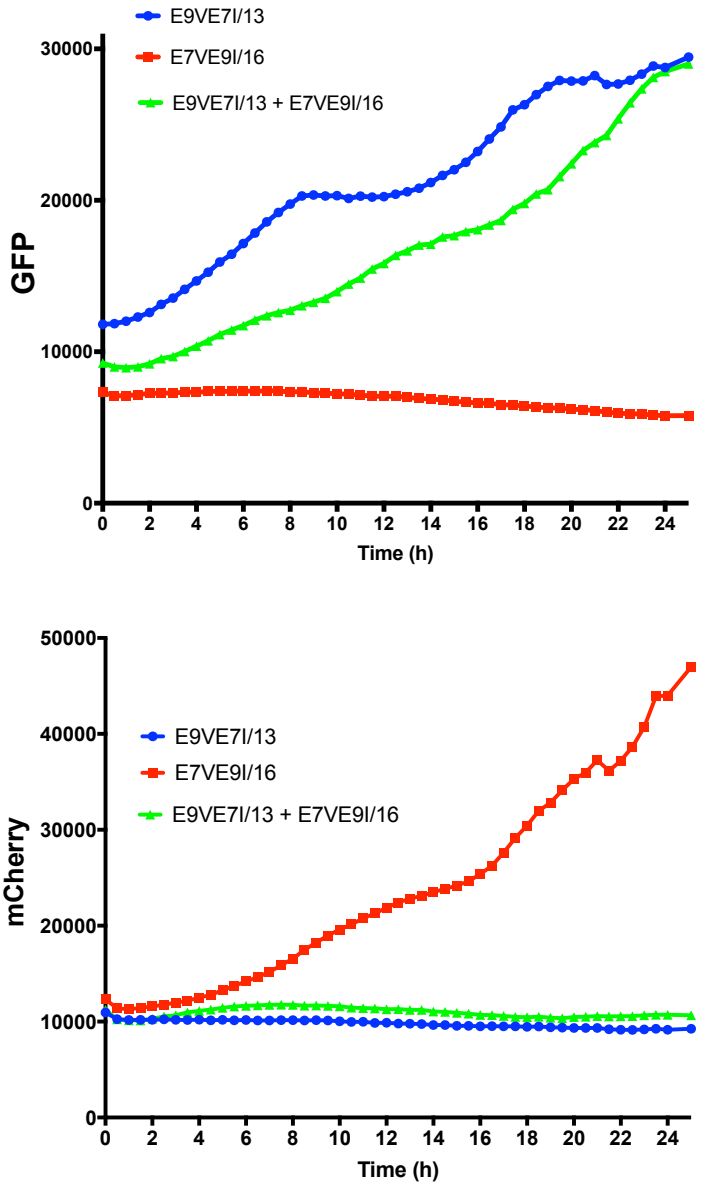

Figure 3-13: Dual fluorescence reporter assay was carried out as previously described. The competition between ColE7 producing strains labelled with GFP (E9VE7I + pSBM13) and ColE9 producing strains labelled with mCherry (E7VE9I + pSBM16) were monitored in real-time over 24 h.

A). The GFP values for competing mixture (E9VE7I/13 + E7VE9I/16) increases at a constant rate and reaches to similar level of GFP value of the negative control after 24 h indicating that ColE7 producers can grow well in the presence of ColE9.

B). The mCherry values for the competing mixture do not increase over 24h which suggest that the growth of ColE9 producers are inhibited in the presence of ColE7 strains.

4 Discussion and Concluding remarks

4.1 Discussion

Colicins play an important role in promoting the diversity of microbial communities and provide selective advantage to the producers both as an offensive and defensive weapon. It may help producers either to invade into new microbial community or inhibit invasion into their niche by other strains (Kerr et al. 2002). E colicins produced by *E. coli* have been used as a model system to provide insight into the competition between *E. coli* strains producing different types of colicin. It has been shown previously that producers of ColE2 and ColE9 have competitive advantage against ColE7 producers (Majeed et al. 2013; Bano 2010). These findings were unexpected as ColE7 is known to have faster cell entry and killing and therefore was expected to kill competing strains more effectively (Walker et al. 2007). This work set out to elucidate a better understanding of the evolution and diversity of the colicin productions within microbial community where there are more than one colicin producing strains. Firstly, the factors that may be responsible for the selective advantages shown by ColE9 producers over ColE7 cells were investigated. Secondly, the roles of lysis genes of ColE9 and ColE7 during the competition were also studied.

The dual fluorescent reporter assay using confocal microscopy have confirmed that ColE9 producing strains were the dominant strain and had clearly outcompeted ColE7 producing strains after 48 h. Difference in the level of cell lysis of ColE9 and ColE7 operons were an important factor in the observed competitive advantage of ColE9 producers. Therefore, MMC sensitivity assay was performed which demonstrated that ColE7 producing cells were subjected to decline in cell density on MMC induction whilst ColE9 producing cells continued to grow freely. These results strongly suggest that there is induction of ColE7 operon by MMC leading to the expression of the distal ColE7 lysis gene. Therefore, this causes cell lysis of ColE7 strains whereas there may be limited induction of ColE9 operon by MMC. Previous studies have suggested that the truncated, proximal ColE9 lysis gene is less effective at promoting the ColE9 release from the producing cells than the distal ColE5 gene (Chak & James 1986). The presence of two terminators before the ColE5 lysis gene is

also thought to play a role in the limited lysis of ColE9 producers (Figure 3-8). However, studies using deletion mutants of each of ColE9 and ColE5 lysis genes have shown that both ColE9 and ColE5 lysis genes are functional and required for release of ColE9 produced by *E. coli* (Bano 2010). Similar growth curve assay using externally added colicins have demonstrated that ColE7 has faster speed of cell entry and killing than ColE9. This agreed with the previous studies where the half-life killing of *E. coli* BW25113 was 1 min for ColE7 and 16 min for ColE9, whilst the half-life for colicin-induced DNA damage using the *lux* reporter strain *E. coli* DPD1718 was 7 min for ColE7 and 13 min for ColE9 (Walker et al. 2007; Bano 2010; Vankemmelbeke et al. 2005). However, it was shown that despite slower action of ColE9, it causes significant decline in cell turbidity of ColE7 cells. This suggests that ColE7 cells undergo significant cell lysis due to strong induction of ColE7 operon by externally added ColE9 which agrees with results from MMC sensitivity assay. Biological activity assay using spot tests have shown that ColE7 is produced quicker and in greater amounts and released more efficiently than ColE9 which accounts for the shorter half-life killing of *E. coli* cells by ColE7. ColE9 producers required MMC induction for release of extracellular ColE9 which suggests that the expression of ColE9 operon is limited. This could explain the observed upper-hand of ColE7 producing cells over ColE9 cells during the early phase of competition in a stationary mixture as shown in (Figure 3-7).

It was previously shown that Im9 was found to offer slightly higher protection against ColE7 than Im7 against ColE9 in fluorescence reporter assay (Bano 2010). Moreover, it was shown that Im9 had a higher affinity of K_d (3.8×10^{-8} M) against ColE7 than to Im7 against ColE9 had a (K_d of 10^{-4} M) (Li et al. 2004). The conclusion from the study was that an E colicin immunity protein with a K_d of $> 10^{-6}$ M for binding to the DNase domain of an E colicin provide no protection against that colicin, whilst an immunity protein with a K_d of $< 10^{-10}$ M provide complete biological protection (Li et al. 2004). Therefore, it may be expected that Im9 offer at least partial protection against ColE7. The results from *lux* assays have suggested that both Im9 and Im7 provide some protection against non-cognate colicins and that Im9 may have higher protection than Im7 at higher concentration of non-cognate colicins. However,

the average percentage protection values at colicin concentrations of 0.4 nM had high standard error values which made it difficult to compare the percentage protections of Im 9 and Im7 against ColE7 and ColE9 respectively. Therefore, it will be very informative to repeat the experiments with several concentrations of colicins between 0.4 nM and 4 nM to improve the accuracy and reliability of the *lux* assay.

In the later part of this project, ColE9/Im9 genes were transferred in to the ColE7 background (containing ColE7 lysis gene) and vice versa to allow switching of the lysis genes of ColE9 and ColE7. New competition experiment using confocal microscopy following the switching of their lysis genes have shown that the ColE7 producing cells (containing ColE9 lysis genes) had competitive advantage over ColE9 producing cells (containing ColE7 lysis gene) after 48 h. This reversed outcome strongly suggests that differences in the level of lysis gene expression may play an important role during the competition between ColE9 and ColE7 producing cells. Further experiments using biological activity assay and the real-time dual fluorescence reporter assay have suggested that ColE7 is produced faster in larger quantities likely to be due to the stronger promoter activity of ColE7 which may also account for greater expression of ColE7 lysis genes. ColE7 also has faster folding and cell killing than ColE9. It is also possible that ColE7 lysis genes are more efficient (sensitive) than ColE9 lysis genes on the same level of induction to cause greater level of cell lysis.

A model has been proposed that summarizes the factors involved in the competition between ColE9 and ColE7 producing *E. coli* cells in an unstructured well- mixed environment (Figure 4-1). ColE7 is produced and released quicker from ColE7 producing cells than ColE9 which enters the ColE9 producing cells. The entry of ColE7 into ColE9 producing cells may be causing limited DNA damage due to higher affinity of Im9 for the ColE7 DNase domain. It is also thought that the export and the lysis of ColE9 from the producing cells could be delayed or limited because of extended nature of the ColE9 operon, where lysis gene is farther away from the SOS inducible

ColE9 promoter as well as due to less efficient cell lysis by the truncated, proximal ColE9 lysis gene. The presence of two terminators upstream of the functional ColE5 lysis gene may also contribute to reduced cell lysis. On the other hand, despite slower production and release of ColE9, ColE9 enters the ColE7 producing cells and cause significant DNA damage possibly due to low affinity of Im7 for the ColE9 DNase domain. This may induce SOS promoters and thus increase expression of the ColE7 operon, resulting in significant cell lysis due to the action of ColE7 lysis gene. This may explain the increase in the number of ColE9 producing cells at expense of ColE7 producing cells as seen in confocal microscopy.

However, this model does not explain why ColE7 producers have selective advantage over ColE9 producers in the early phase in non-mixed environment such as in microtitre wells. As it is unlikely that ColE9 producers will have produced and released ColE9 during the early phase, this selective advantage of ColE7 producers are most likely due to the fact the large amount of ColE7 present in external medium, kill ColE9 producing cells and inhibit their growth. It is still possible that once enough ColE9 is produced and released by ColE9 strains over longer course of time, ColE9 producers may have the upper-hand.

This study can be repeated by using continuous or frequent shaking for competition assay under Tecan to make sure the competing mixtures are well-mixed and by carrying out the real-time competition experiments up to 48 h by adopting similar methods used for confocal microscopy. This study suggested that ColE7 promoter may have higher activity than that of ColE9. Therefore, further studies could be done to investigate the roles of the promoters in ColE9 and ColE7 operons by switching their promoters and repeating the experiments.

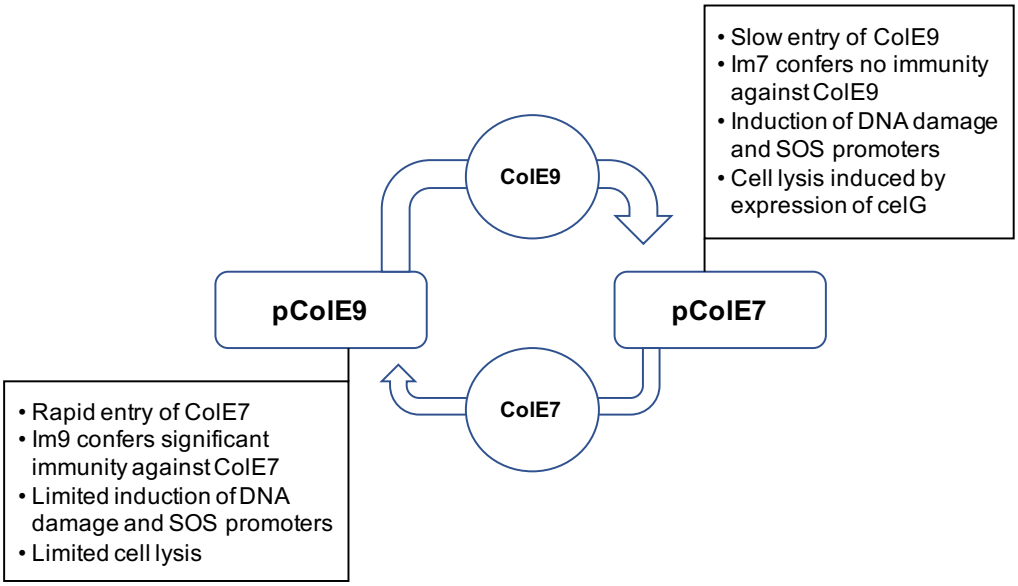

Figure 4-1: A proposed model for competition between ColE9 and ColE7 producing *E. coli* cells.

The rapid entry of ColE7 into ColE9 producing cells cause limited induction of DNA damage due to higher affinity of Im9 against ColE7 and limited induction of SOS promoter in ColE9 operon due to extended nature of ColE9 operon with less effective proximal ColE9 lysis gene and presence of two terminators upstream of functional ColE5 lysis gene. All these factors may contribute in limited cell lysis of ColE9 producing cells. On the other hand, slower entry of ColE9 into ColE7 producing cells cause significant induction of DNA damage due to lower affinity of Im7 against ColE7 and resulting in expression of ColE7 lysis genes causing significant cells lysis of ColE7 producing cells. This results in the competitive advantage of ColE9 producers over ColE7 producers in an unstructured well-mixed environment.

4.2 Concluding remarks

In this project, the observed selective advantage of ColE9 producers over ColE7 producers in an unstructured well-mixed environment after 48 h was confirmed. This study has also identified important factors that influence the outcome of their competition such as limited expression of ColE9 lysis genes, slower production and release of ColE9 likely due to less efficient promoter in ColE9 operon. It was also shown that lysis genes play an important role determining the outcome of the competition between ColE9 and ColE7 producing cells

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