The Evolutionary Ecology of the PlcR-PapR Quorum Sensing System in *Bacillus thuringiensis*

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Abstract

Bacteria can engage in multicellular behaviour via a process called quorum sensing (QS), whereby bacteria monitor population density through the secretion of small, diffusible signal molecules. However, the evolutionary forces that maintain QS have rarely been investigated in naturally co-evolved host-pathogen systems. This thesis investigated the evolutionary ecology of the PlcR-PapR QS system in *Bacillus thuringiensis*, in which the PlcR regulon controls the production of various extracellular proteins, often involved in virulence, in response to an autoinduced heptapeptide signal PapR.

The thesis tested the hypothesis that both signal production and PlcR regulated gene expression are social traits, and measured the invasion of isogenic mutants at varying pathogen doses and mutant frequencies. Productivity in host and infection success was positively correlated with the abundance of wild type in inocula. However, mutants could not outcompete wild type bacteria *in vivo*. Experiments with homogenized insects indicate that mutants can outcompete wild type bacteria in homogeneous environment. Microscopic observation of insect sections with fluorescent QS cells showed that, in the midgut, bacteria population was founded on isolated patches of 1 to 3 individual cells 24 hours post infection. However, a mixed population consisted of wild type and mutants was evident 48 hours post infection. The results suggested that spatial heterogeneity and population bottleneck imposed by the midgut barriers limited invasion of QS mutants.

A polymorphism comprising four distinct communication groups (pherotypes) of the PlcR-PapR QS system was found in the *B. cereus* group. Strains from the same pherotype exchange information efficiently while strains from different pherotypes communicate far less effectively. Recent theory has suggested that social interactions may account for the within-species diversity of QS systems. Pairwise competition between pherotypes of the studied QS system *in vivo* revealed fitness

to be positively frequency dependent. However, when challenged with a competitive gut bacterium, *Enterobacter cloacae*, each pherotype exhibited different fitness benefits when competing with other pherotypes in the presence of competitors. The results suggested that environmental dependent fitness has the potential to maintain a stable polymorphism.

The results of this thesis confirm that clinical interventions to prevent QS might be beneficial, as diverse virulence factors are often regulated by QS. If QS regulated virulence factors are required for an essential part of infection, inhibiting these behaviours can be helpful.

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I must acknowledge Dad and Mum for all their support and help.

Declaration of Authorship

I, Liqin Zhou, hereby declare that this thesis and the work presented in it is entirely my own. Where I have consulted the work of others, this is always clearly stated.

Data shown in chapter 3, 4 and 5 were presented as a poster, “The evolutionary ecology of the peptide-based quorum sensing system in *Bacillus thuringiensis*”, at the Spring Conference 2011 (SGM), at Harrogate 2011; and as a poster at the 5th Congress of European Microbiologists (FEMS), at Leipzig, Germany, 2013. Data in chapter 3 and 4 were written and submitted as a manuscript to Science, “The social biology of quorum sensing in a naturalistic host pathogen system”, in April 2014.

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CHAPTER 1 GENERAL INTRODUCTION

1.1 Aims

Bacteria can engage in multicellular behaviour via a process called quorum sensing (QS), whereby bacteria monitor population density through the secretion of small, diffusible signal molecules. There is a wealth of theoretical models to explain the evolution of QS and an increasing body of empirical evidence suggests that QS is common in bacteria (Brown and Johnstone, 2001, Diggle et al., 2007, Hense et al., 2007, Miller and Bassler, 2001, Redfield, 2002, Waters and Bassler, 2005, West et al., 2006). The present thesis is the first *in vivo* study of QS in a naturally co-evolved host-pathogen system. The aim of this thesis is to study the evolutionary ecology of the peptide-based PlcR-PapR QS system in *Bacillus thuringiensis*. In this chapter, I will start by generally introducing the problem of cooperation and quorum sensing in bacteria, particularly focusing in Gram-positive bacteria. Later, I will discuss the theoretical models that have developed to explain the evolution of QS. Finally, I will deal with the life cycle of the model organism, *B. thuringiensis* in its natural host, the diamondback moth *Plutella xylostella*.

1.2 The problem and evolution of cooperation

Cooperation is defined as behaviour that increases the fitness (the relative reproductive success) of the recipients, having either positive (mutually beneficial) or negative (altruistic) effect on the actor (Hamilton, 1964). Cooperation is commonly observed in nature, such as the sterile female workers in eusocial insects (Andersson, 1984, Nowak et al., 2010, Seger, 1991), communal nursing in Florida scrub jays (Woolfenden and Fitzpatrick, 1978, Woolfenden and Fitzpatrick, 1984), warning calls in ground squirrels (Sherman, 1977) and the stalk cells in social amoeba (Strassmann et al., 2000). The question is why would an individual cooperate if cooperation reduces its fitness? The apparent lack of selfishness in

many species has presented a long-standing challenge to Darwin's theory of evolution. Darwin (Darwin, 1859) considered it as "one special difficulty" and attended to the problem carefully:

> I…will confine myself to one special difficulty, which at first appeared to me insuperable, and actually fatal to my whole theory. I allude to the neuters or sterile females in insect communities: for these neuters often differ widely in instinct and in structure from both the male and fertile females, and yet from being sterile they cannot propagate their kind.

At the time when Darwin published his notable book *On the Origin of Species*, the neuters were referred to as "somatic tissues" thereby repudiating the apparent opposition of cooperation to his theory of natural selection.

Before the 1960s, cooperation was not regarded as a problem because of the naive "good of the species" thinking. In the early 1960s, Wynne-Edwards (Wynne-Edwards, 1962, Wynne-Edwards, 1986) argued that group selection could explain cooperation. Over-exploitation of nature resources has a detrimental effect on the survival of species and it was believed that it would be swiftly suppressed by natural selection. Groups consisting of cooperative individuals would survive better than groups of selfish individuals. Hence, by natural selection, groups of cooperative individuals would out-compete and eradicate groups of selfish individuals. However, Wynne-Edwards' idea was criticized vigorously by evolutionists (Williams, 1966, Maynard Smith, 1976). Particularly, Maynard Smith (Maynard Smith, 1976, Maynard Smith, 1993) argued that group selection was a weak force and it cannot stop cheating, in which selfish individuals who do not cooperate gain fitness benefits from cooperative individuals. The difference in survival between groups is usually small and frequent movement between groups permits invasion of selfish individuals into cooperative groups. To date, numerous theoretical models have been developed to explain the evolution of cooperation (Bowles et al., 2003,

Fletcher and Zwick, 2007, Grafen, 1984, Hamilton, 1964, Lehmann and Keller, 2006, Taylor, 1992). Among these models, Hamilton's rule or kin selection has gained popularity since its proposal in 1964 and many current theoretical models have developed from Hamilton's insight of indirect fitness.

According to kin selection (Hamilton, 1964), cooperation is favoured when $rb - c > 0$, where r is the genetic relatedness of the actor to the recipient, relative to the average relatedness of all individuals in the population, c is the fitness cost to the actor, and b is the fitness benefit to the recipient. Therefore, by helping a close relative to reproduce, an individual still gains an indirect fitness benefit from aiding the survival of non-descendant kin. In his papers, Hamilton identified two mechanisms that help directing cooperative aid towards relatives: 1) limited dispersal (population viscosity) and 2) kin recognition.

The first mechanism, limited dispersal of individuals from the natal group keeps relatives together, increasing the relatedness among interacting individuals. Therefore, cooperative aid can be directed indiscriminately to all surrounding neighbours (Hamilton, 1964). However, limited dispersal also increases competition between relatives for resources, reducing selection for cooperation (Wilson et al., 1992). Interaction between kin favours cooperation, but competition between kin hinders the evolution of cooperation. In consideration of competition between kin, a modified version of Hamilton's rule has been introduced. Grafen suggested that $r_{xy}b - c - r_{xe}d > 0$, where r_{xy} is the relatedness of actor to the recipient, and r_{xe} is the relatedness of actor to individuals suffering from increased competition (Grafen, 1984). Depending on the scale of competition, selection for cooperation between relatives can be reduced or completely removed (Wilson et al., 1992). On the other hand, cooperation can be favoured if r_{xe} and d are relatively small (Frank, 1986, Gandon and Michalakis, 1999, Taylor, 1988). For example, when starting a new breeding nest, birds tend to disperse long distance from their natal nest (Greenwood

et al., 1978, Greenwood, 1980). Hence, r_{xe} is as small as 0. Competition between relatives is negligible. In such condition, cooperation occurs before dispersal but competition occurs after dispersal.

Apart from limited dispersal, kin recognition or kin discrimination is also believed to contribute to kin selection. In kin discrimination, individuals are able to distinguish relatives from non-relatives, using either environmental cues or genetic cues (Hamilton, 1964). As a result cooperative aid is directed towards relatives selectively. There is a vast amount of studies of kin discrimination in cooperatively breeding vertebrates (Hanken and Sherman, 1981, Holmes and Sherman, 1982, Leclaire et al., 2013). Quantitative comparison across species of these studies described a prevalent theme of kin discrimination in cooperatively breeding vertebrates. The level of helping is positively correlated with the relatedness and the fitness benefit gaining from helping. When there are greater fitness benefits from helping, more help is directed towards closer relatives (Griffin and West, 2003).

Hamilton's rule or kin selection is theoretically interesting and possibly the most important mechanism for the evolution of cooperation in related individuals. However, cooperation between unrelated individuals have also been discovered in many species including the hunting alliance in African lions (Packer et al., 1991), parental care in starlings (Yom-Tov, 1980), spawning in black hamlet fish (Fischer, 1980) and regurgitation of blood by vampire bats (Wilkinson, 1984). Several mechanisms have been proposed to account for the cooperation among unrelated individuals. Mutualism in which individual survival and fitness may be greater from cooperation has been suggested to be important in hunting coalition between unrelated African lions (Caraco and Wolf, 1975, Stander, 1992). Manipulation in which one individual is manipulated by another to behave altruistically may be responsible for the apparent cooperative parental care of unrelated eggs in starlings (Dawkins, 1982). Reciprocity where the benefit of an altruistic act of the actor is

paid back by the recipient at some point later is a possible mechanism for the evolution of cooperative spawning in black hamlet fish and regurgitation of blood by vampire bats (Trivers, 1971).

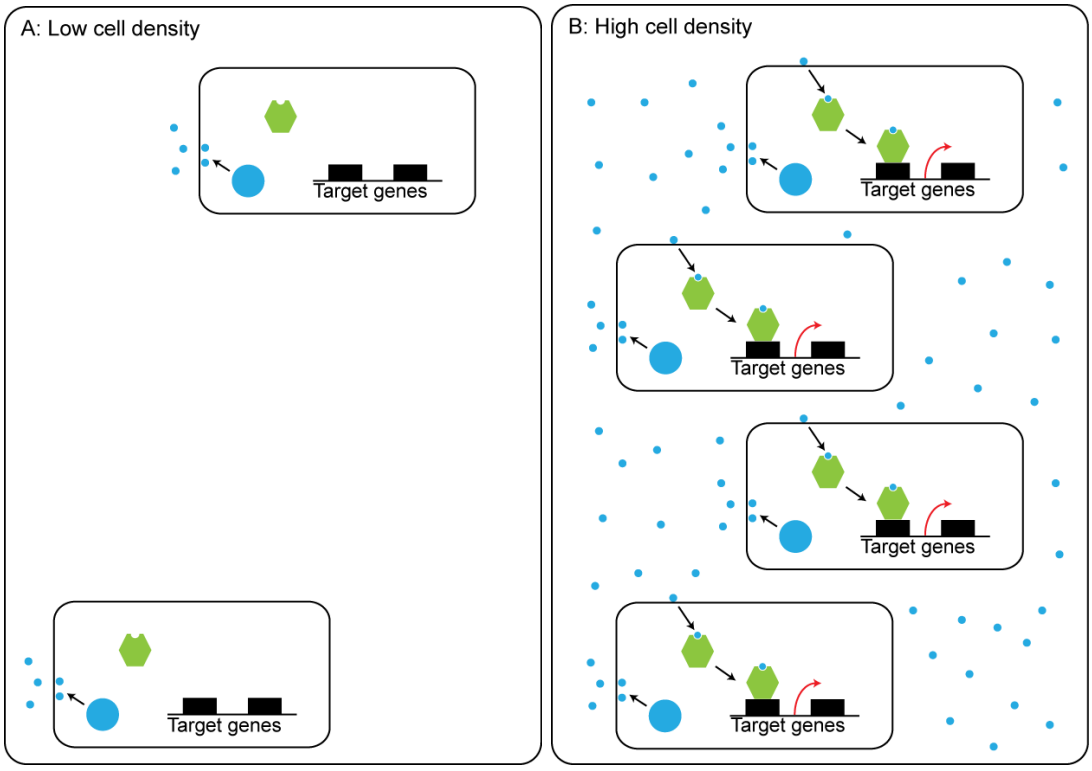
1.3 Quorum sensing in microorganisms

Since the proposal of kin selection, there has been intensive research into cooperation in animal kingdom. Little attention was paid to microorganisms, which were once thought to behaved solitarily even in a group of bacterial cells, because bacteria lacked the ability to communicate and therefore could not cooperate at the population level. However, an increasing body of work has shown that bacteria can engage in multicellular or coordinated behaviours through a process called quorum sensing (Kleerebezem et al., 1997, Miller and Bassler, 2001, Waters and Bassler, 2005, Whitehead et al., 2001). As shown in Fig 1.1, in quorum sensing, bacteria secrete small, diffusible, signal molecules to the extracellular environment. The concentration of the signaling molecules increases along with the population density until it reaches a threshold, thereby notifying the bacteria that the population has reached a critical level, a quorum. The bacteria then begin expressing sets of quorum-dependent genes, which have diverse functions, such as, in nutrient acquisition, virulence, gene transfer, antibiotic resistance and biofilm formation (Kleerebezem et al., 1997, Miller and Bassler, 2001, Waters and Bassler, 2005, Whitehead et al., 2001).

1.3.1 Quorum sensing in Gram-negative bacteria

Quorum sensing was first discovered in a Gram-negative bacterium, *Vibrio fischeri* (Nealson and Hastings, 1979). *V. fischeri* lives free in the ocean but is also found in high density in the light-emitting organs of Hawaiian bobtail squids, *Euprymna scolopes*, where it synthesizes a light-generating enzyme, luciferase (Engebrecht and Silverman, 1984). Luciferase is a product of the *lux A* and *lux B* genes in a

Fig 1.1 A schematic diagram giving an overview of quorum sensing in bacteria. Small signalling molecules (little circles) are secreted to the extracellular environment. (**A)** At low cell density, the signalling molecules are diffused into the extracellular environment. The concentration of the signaling molecules increases along with the population density until it reaches a threshold. (**B**) At high cell density, the signalling molecules are reimported into the cells and bound to their receptor proteins, activating quorum-dependent genes.



bacterial operon called the *lux* operon (Engebrecht et al., 1983). A region in the *lux* operon promoter binds a transcription factor, LuxR, which is only active when it has bound to a small, uncharged molecule called an *N*-acyl-homoserine lactone (AHL, also called autoinducer). AHLs are made by an enzyme called an AHL synthase that is encoded by the gene *luxI*, which is part of the *lux* operon (Engebrecht et al., 1983, Engebrecht and Silverman, 1984, Schaefer et al., 1996, Stevens et al., 1994, Stevens et al., 1999, Stevens and Greenberg, 1997, Hanzelka and Greenberg, 1995). In free-living *V. fischeri* the *lux* operon is transcribed at a low level (Engebrecht and Silverman, 1984). Small amount of AHLs are made, which immediately leak out of the cell into the open sea without binding to LuxR. When the bacterium is concentrated in the squid's light organs, AHLs can diffuse back to the bacterium and bind to LuxR, increasing transcription of the *lux* operon (Engebrecht et al., 1983). This makes more luciferase as well as more AHL synthase. The concentration of AHL therefore rises, so transcription of the *lux* operon increases further. This positive feedback loop is sometimes called auto-induction. The end result is the emission of light from the light-emitting organs of Hawaiian bobtail squids.

The intense studies of *Vibrio fischeri* revealed a common molecule that regulates quorum sensing in Gram-negative bacteria. A large number of Gram-negative bacteria possesses the LuxI/LuxR-type quorum sensing systems, including the synthesis and release of virulence factors by the opportunistic human pathogen *Pseudomonas aeruginosa* (LasI/LasR-RhlI/RhlR virulence system); conjugal transfer of genetic material by the plant pathogen *Agrobacterium tumefaciens* (TraI/TraR virulence system); and antibiotic production by plant pathogen *Erwinia carotovora* (ExpI/ExpR-CarI/CarR virulence/antibiotic system) (Manefield and Turner, 2002). These QS systems exhibit great specificity between the LuxR-type proteins and their cognate AHL signals. Specificity can be demonstrated as the inability of one strain to utilize highly specific molecules produced by another strains. While the homoserine lactone backbone of the AHL molecules is relatively conserved, a

diverse collection of fatty acyl side chains of varying length and side substitution has been documented (Fuqua and Eberhard, 1999). Together with the specific acyl-binding pockets found on the LuxR-type proteins that fit a particular side-chain moiety of AHLs, AHL signals show high fidelity to their cognate LuxR-type proteins (Gould et al., 2004, Vannini et al., 2002, Watson et al., 2002, Zhang et al., 2002). Such specificity of the LuxI/LuxR-type QS systems allows bacteria in a mixed-species environment respond only to their own signals. More importantly, Gram-negative bacteria communicate with multiple QS systems. Expression of one or two QS systems simultaneously is feasible with each AHL binding to its own cognate LuxR-type protein (Waters and Bassler, 2005).

In addition to the positive feedback loop, down regulation mechanisms for the LuxI/LuxR-type QS system has also been reported (Pearson et al., 1999, Zhu and Winans, 1999, Zhu and Winans, 2001). In *P. aeruginosa*, active export of the AHL signalling molecule, namely N-butyryl homoserine lactone (C4-HSL), prevents premature activation of the RhlI/RhlR QS system in the cytoplasm (Pearson et al., 1999). In *A. tumefaciens*, the life span of TraR proteins is significantly shorter in the absence of its cognate AHL signals, N-3-oxooctanoyl-L-homoserine lactone (AAI). TraR is only stabilized when AAI has accumulated to a threshold concentration both inside and outside the cells (Zhu and Winans, 1999, Zhu and Winans, 2001).

1.3.2 Quorum sensing in Gram-positive bacteria

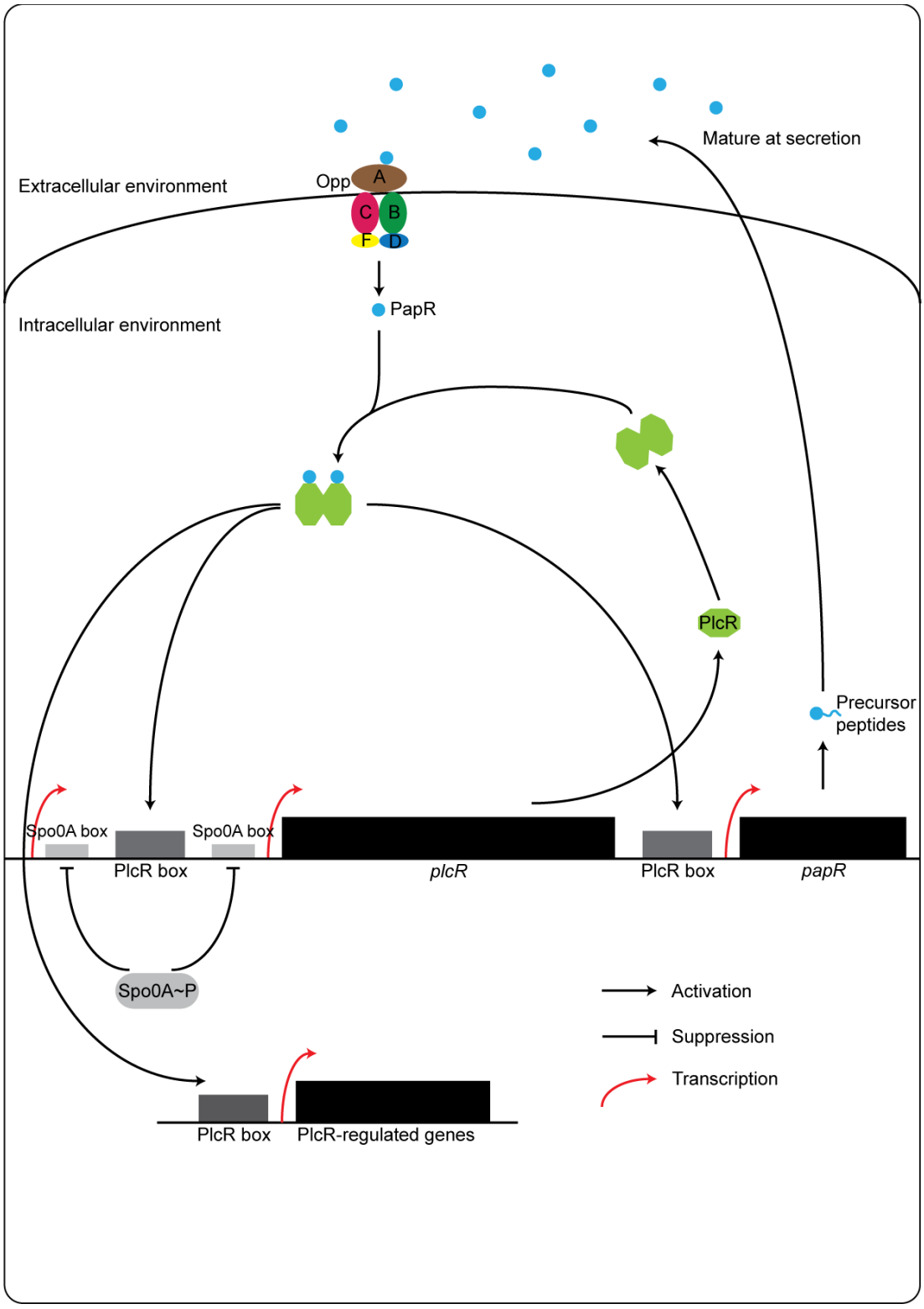
Quorum sensing has also been observed in Gram-positive bacteria. While gram-negative bacteria use AHL signaling molecules as autoinducers, Gram-positive bacteria employ secreted modified peptides for quorum sensing through a phosphorylation/dephosphorylation cascade known as two-component signaling. Some well-studied examples are mating in *Enterococcus faecalis*, competence induction in *Streptococcus pneumoniae*, sporulation by *Bacillus subtilis*, toxin and virulence factor production by *Straphylococcus aureus* (Sturme et al., 2002). As

shown in Fig 1.2, in *Bacillus cereus*, the gene *papR*, located downstream from the gene *plcR*, is transcribed and translated into a precursor peptide, which is exported out of the cell (Slamti and Lereclus, 2002). The precursor peptide is processed into a heptapeptide either during export or in the intercellular environment (Slamti and Lereclus, 2002, Bouillaut et al., 2008). The active heptapeptide is recaptured into the cell through the oligopeptide permease systems OppABCDF (Gominet et al., 2001), which can then bind to and activate the transcriptional regulator PlcR (phospholipase C Regulator) (Slamti and Lereclus, 2002). The PapR-PlcR complex (the PlcR regulon) later binds to the promoter region, namely the "PlcR box", of PlcR-regulated genes, initiating the transcription of several genes, including the *papR* and *plcR* genes, genes for extracellular virulence factors and biofilm formation and the phosphatidylinositol-specific phospholipase C gene (Agaisse et al., 1999, Gohar et al., 2008, Hsueh et al., 2006, Lereclus et al., 1996, Okstad et al., 1999, Salamitou et al., 2000). Similar to the LuxI/LuxR QS system in *V. fischeri*, *B. cereus* can monitor cell density via the PapR peptides. As the cell density increases, the concentration of PapR increases. More PapR peptides are recruited back to the cells and bind with PlcR, producing more PlcR-regulated proteins, including PapR and PlcR, which in turn enhances the binding of PapR and PlcR. This again forms the positive feedback loop similar to the autoinduction of LuxI/LuxR-type QS system in Gram-negative bacteria. The PlcR-PapR QS system well conserved in *B. cereus* and *Bacillus thuringiensis* strains (Lereclus et al., 2000)

The peptide based QS systems in Gram-positive bacteria have been noted for their specificity and polymorphism (Kleerebezem et al., 1997). Specific and polymorphic signalling systems have been previously described in the competence QS system in *B. subtilis* and in *Streptococcus* species (Pozzi et al., 1996, Tortosa et al., 2001, Whatmore et al., 1999) as well as in the virulence QS system in *Staphylococcus* species (Dufour et al., 2002, Ji et al., 1997). Genetic studies have suggested the polymorphism observed in the various QS systems evolved via accumulation of point mutations or recombinational changes in an element of the QS systems

Fig 1.2 A model for the regulation of the expression of the PlcR regulon.

Details of the mechanism are given in the text.



followed by positive selection of compensatory mutations in the second element of the same QS systems (Ansaldi and Dubnau, 2003, Dufour et al., 2002, Havarstein et al., 1997, Ichihara et al., 2006, Tortosa et al., 2001). A polymorphism comprising four distinct communication groups (pherotypes) was also found in the *B. cereus* group (Slamti and Lereclus, 2005). Strains from the same pherotype exchange information efficiently while strains from different pherotypes communicate far less effectively (Bouillaut et al., 2008). Apart from specificity in communication, signalling peptides from one pherotype of *Staphylococcus aureus* have also been found to inhibit QS activation of other pherotypes by competitive binding to the non-cognate AgrC receptors (Lyon et al., 2002). This cross-inhibition of QS system, namely quorum quenching, could affect the growth of other pherotypes while promoting the growth of own pherotype. In support to the idea, the virulence of a group II *S. aureus* in a mouse infection model was greatly reduced when supplemented with a group II signalling peptides (Mayville et al., 1999).

1.4 Evolution of quorum sensing

Despite a good understanding of the mechanism of quorum sensing, the evolutionary forces that have selected and maintained quorum sensing have been subject to debates. The signalling molecules and the extracellular products regulated by QS are examples of public goods, which are resources costly for the individual to produce but benefit all members in the local group or population (Diggle et al., 2007, Frank, 1998, Keller and Surette, 2006). In the case of the iron-scavenging molecule – siderophore production in many bacteria, siderophores are public goods that are costly to produce but are beneficial to all neighbouring cells (Ratledge and Dover, 2000, West and Buckling, 2003). As demonstrated by experiments with the pathogenic bacteria *P. aeruginosa*, in iron rich environment, wild type strains capable of producing siderophores grow slower than mutant strains that do not engage in siderophore production, while in iron-limiting environment, wild types grow faster than mutants (Griffin et al., 2004). Consequently, in a mixed population where both wild type and mutant *P. aeruginosa* are present, mutants can

exploit the siderophores produced by wild types and out-compete wild types. The question is if quorum sensing benefits all members in the local group, what stops the invasion of cheats that do not engage in communication and do not produce public goods? How can quorum sensing be maintained?

First, quorum sensing can provide a direct fitness benefit to individuals performing the cooperative behaviour. When the direct fitness benefit outweighs the cost of engaging in QS, the cooperative behaviour is mutually beneficial. Individuals have a shared interest in communicating with each other and producing public goods. Second, QS can decrease the direct fitness of the individuals that performs them. In this instance, QS is altruistic and can only be explained via an indirect fitness benefit gained by helping individuals who share the cooperative genes. The complication here is that QS can provide both direct and indirect fitness benefits. In the example of siderophore production, the iron-chelator provides a benefit to both the individual that produces them and to nearby relatives. In this case, whether the cooperative behaviour is mutually beneficial or altruistic will depend on the relative importance of the direct and indirect fitness benefits.

Kin selection in the form of limited dispersal has been suggested to be important for the evolution and maintenance of QS in bacteria (Brown, 1999, Smith, 2001, West and Buckling, 2003). Limited dispersal of public goods confines the benefit of public goods sharing to neighouring bacteria that tend to be identical clones as a result of asexual reproduction. In this case, in the local group, the relatedness of neighbouring bacteria is 1, Hamilton's rule suggests that the production of public goods is likely to evolve (Hamilton, 1964). Empirical support for the idea that limited dispersal favours the production of public goods can be found in the production of the iron-scavenging molecules, siderophores, in *P. aeruginosa* (Griffin et al., 2004). Relatively high relatedness in siderophore-producing *P. aeruginosa* (derived from a single clone) prevent the invasion of cheats who do not produce siderophores, but

at relatively low relatedness of cooperators (derived from two clones), cheats can exploit and out-compete cooperators (Griffin et al., 2004). At high relatedness, cooperation of public goods production is evolutionary stable, while at low relatedness, cooperation is subject to cheating from individuals who do not produce public goods (West and Buckling, 2003). However, in the dome-shape function formulated by Brown and Johnstone (2001), a slightly different prediction of signalling effort against relatedness emerged. At low relatedness, the model suggests that little is to be gained from the production of public goods and signaling is deselected for. At intermediate relatedness, manipulating other local cells into greater production of public goods while minimizing self-production of public goods is favoured. At intermediate relatedness individuals are selected to invest in signaling. At high relatedness, all local cells are highly related and there is a shared interest in signaling. Signaling effort decreases and a cheap signaling molecule is selected for (Brown and Johnstone, 2001).

Kin selection in the form of kin discrimination could also be important in the evolution and maintenance of QS in bacteria. In microorganisms, kin discrimination could be possible through specificity and diversity. As previously discussed, specific and polymorphic QS systems have been found in both Gram-negative and Gram-positive bacteria (Kleerebezem et al., 1997, Manefield and Turner, 2002). Kin selection via kin discrimination would favour the evolution of highly specific molecules other strains could not utilize. Sequence data of the pyoverdine region of siderophore of *P. aeruginosa* suggest that the genes were under diversifying selection for novelty and specificity (Smith et al., 2005). Similarly, in *Bacillus*, the sequence data of the regulatory components ComQ and ComX of the competence QS system suggest that the genes were also under diversifying selection (Ansaldi and Dubnau, 2003). However, conflicting selection on a universal signal molecule to manipulate other individuals into cooperation could possibly counteract the selection on signals for novelty and specificity (Dawkins and Krebs, 1978). The metabolic cost of signalling peptides from gram-positive bacteria has been

suggested to be higher than the AHL molecules from gram-negative bacteria (Keller and Surette, 2006). The possible benefit gain from manipulation and the cost of signal production in QS at this current stage are still ambiguous. The evolution of QS is further complicated by spiteful behaviour, namely the production of antagonistic peptides (bacteriocins), a behavior found in all major bacteria lineages (Riley and Wertz, 2002). Bacteriocins are antimicrobial compounds that target members of the same species or genus suggesting a role in competition within species (Riley et al., 2003). Bacteriocin production has been reported to be regulated by QS in *S. pneumoniae* (Suntharalingam and Cvitkovitch, 2005). Theoretical modeling on how relatedness and competition influence bacteriocin production found a peak of bacteriocin production at intermediate relatedness and a minimized production at low and high relatedness, suggesting a facultative adjustment of bacteriocin production in response to kin discrimination (Gardner et al., 2004). The spiteful behaviour can evolve as an altruistic behaviour when the local group size is small and movement between groups is minimal (Lehmann et al., 2006). The indirect fitness benefit gain from reduced competition on close relatives could possibly out-weight the cost of bacteriocin production and favours the evolution of bacteriocin production (Lehmann et al., 2006)

Apart from kin selection, direct fitness benefits gained from cooperation could also possibly select for the evolution of QS (West et al., 2006). For example, during the human dental plaque formation, complex biofilm formed from different cooperating species allowed colonization of human teeth (Egland et al., 2004, Kolenbrander et al., 2002). Neither of the initial colonizers *Streptococcus oralis* and *Actinomyces naeslundii* were able to survive alone without the presence of the other strains, suggesting a mutually beneficial cooperation between *S. oralis* and *A. naeslundii* (Palmer et al., 2001). Host selection on cooperative bacteria could also favour the evolution of QS in bacteria that colonize the host. For instance, the luminescent bacteria *V. fischeri* is supported by the host possibly by oxygen supply. Mutants defected in luciferase synthesis were unable to colonize the bobtail squid (Visick et

al., 2000). All in all, the evolutionary forces for the evolution and maintenance of QS are complex. No unified theory has been developed to explain the evolution of QS.

In addition to the idea that bacteria communicate via QS and QS is likely to be a cooperative behaviour, Redfield (2002) argued that the release of small molecules is not to signal other surrounding cells or to sense the population density, but to determine the extent of diffusion and mixing in a cell's immediate environment, namely diffusion sensing (Redfield, 2002). If there was too much diffusion and mixing, it would not be in an individual's interest to engage in the production of public goods, since they would diffuse away immediately and be rendered effectiveness because of dilution (Redfield, 2002). Diffusion sensing is suggested to evolve because of individual fitness benefits (Redfield, 2002). Another theory, efficiency sensing has been developed to unify quorum and diffusion sensing. It was argued that autoinducer signalling molecules can be used to measure cell density, mass-transfer properties or bacterial spatial distribution in different ecological contexts (Hense et al., 2007). As different signalling molecules vary in structure and size, the efficiency of producing different signalling molecules in the extracellular environment would be different. Through testing the efficiency, cells are able to gather information about their surrounding environment (Hense et al., 2007).

1.5 Life cycles of diamondback moth, *P. xylostella*

The diamondback moth, *P. xylostella,* originated in the Mediterranean area, has now spread worldwide and is one of the most destructive pests of cruciferous crops. Control failure of the diamondback moth is major concern in agriculture, as the moth has developed resistance to every synthetic insecticide used in the field as well as the biological insecticide known as the *Bt* toxin (Talekar and Shelton, 1993).

P. xylostella has a short live cycle of approximately14 days at 25°C. Most diamondback moth adult emergence occurs during the first 8 h of photophase (Pivnick et al., 1990). Adults become active at dusk, feeding on water drops and mate at the same day of emergence. Soon after mating, female moths start to lay eggs, and the oviposition period lasts 4 days. A significantly number of factors can affect oviposition, such as light during night hours, plant secondary chemicals, temperature, waxes on leaf surfaces, *etc.* (Tabashnik and Mau, 1986). Depending on temperature, it takes about 5 to 6 days for the eggs to hatch, after which neonate larvae start feeding on leaves. The larvae undergo four instars, which last about 4 to 5 days again conditional on temperature and humidity (Sarnthoy et al., 1989). Upon completion of feeding at 4th instar, the larvae spend about 2 days constructing a loose silk cocoon on the leaf surface where it fed. The duration of the pupal period varies from 4 to 15 days depending on temperature (Lu and Lee, 1984, Abraham and Padmanaban, 1968).

1.6 The model organism *B. thuringiensis* and significant events in the infection process of *B. thuringiensis*

B. thuringiensis is a gram-positive, spore-forming bacterium commonly found in soil (Raymond et al., 2010, Martin and Travers, 1989), but it also occurs naturally in other environmental niches, including phylloplane (Smith and Couche, 1991, Damgaard et al., 1998, Hansen et al., 1998), insect (Damgaard et al., 1997, Hansen et al., 1998) and stored-product environments (Meadows et al., 1992, Kaelin et al., 1994). *B. thuringiensis* is renowned for its entomopathogenic property owing to the specific insecticidal crystal proteins (Cry toxins and Cyt toxins) produced by the bacteria (Schnepf et al., 1998). However, spores of *B. thuringiensis* have also been reported to play a part in the overall virulence of the bacteria (Li et al., 1987, Liu et al., 1998). It is generally proposed that the crystal proteins display toxic effects through an interaction with the larval mid gut epithelium cells causing a disruption in membrane integrity and creating favorable conditions for the germination of spores and ultimately leading to insect death (Bravo et al., 2007). The virulence factors

regulated by the PlcR regulon of *B. thuringiensis* allow the bacteria to overcome host barriers and defences and proliferate in host (Lereclus et al., 2000). In the following paragraphs, I will discuss the significantly events in the infection process of *B. thuringiensis* and the host barriers and defences the bacteria must overcome in order to be infectious and proliferate in host.

1.6.1 Mode of Action of B. thuringiensis Cry toxins

B. thuringiensis has been used as a biopesticide agent against insect pests in agriculture and forestry for more than 50 years (Bravo et al., 2011, Sanchis, 2011). *B. thuringiensis* is closely related to *Bacillus anthracis*, which causes the acute fatal disease anthrax, and to *Bacillus cereus*, an opportunistic human pathogen that is a common source of food poisoning (Helgason et al., 2000). The feature that distinguishes *B. thuringiensis* from *B. cereus* is the ability of *B. thuringiensis* to produce large crystal protein inclusions during sporulation (Baumann et al., 1984). These crystal protein inclusions are predominantly comprised of spores and one or more crystal (Cry) and cytolitic (Cyt) toxins (also named δ-endotoxins), which are often encoded by plasmid carried genes (Kronstad et al., 1983, Gonzalez and Carlton, 1984). The δ-endotoxins account for up to 25% of the dry weight of the sporulated cells (Agaisse and Lereclus, 1995) and are responsible for the pathogenicity of *B. thuringiensis*. Specifically, Cry toxins target insect larvae from *Lepidoptera*, *Diptera*, *Coleoptera* and other invertebrates like nematodes, while Cyt toxins are mostly active against *Diptera* (van Frankenhuyzen, 2009). To date, 717 Cry toxins and 38 Cyt toxins have been reported (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/intro.html). In addition, various *B. thuringiensis* strains are also known to produce vegetative insecticidal proteins (Vip toxins) during vegetative stage of growth (Estruch et al., 1996, Yu et al., 2011). Although the expression and mode of action of the Vip toxins are not as well documented as the Cry toxins, studies have suggested that the Vip toxins show insecticidal activity through lysing insect midgut epithelium cells (Yu et al., 1997, Selvapandiyan et al., 2001, Lee et al., 2003).

In contrast to the Vip toxins, the mode of action of the Cry toxin has been extensively studied for many insect species, especially lepidopteran insects. In nature, the insect larvae ingest *B. thuringiensis* spores along with the Cry toxin orally. In addition to oral infection, insect larvae are also infected accidentally by wounding through the cuticle (Nielsen-LeRoux et al., 2012). Upon ingestion, the crystal inclusions dissolve in the alkaline environment of the gut and the solubilized protoxins are activated by midgut proteases. The digestive enzymes remove 25 to 58 amino acids from the N-terminal of the protoxins, and in the case of long Cry protoxins (for example Cry 1A and Cry 4B), the proteases cleave approximately half of the remaining proteins from the C-terminal of the protoxins, yielding 60-70 kDa protease resistant proteins (Bravo et al., 2007). The activated Cry toxins are then able to cross the peritrophic matrix (protective chitin rich web surrounding the midgut) and bind to specific receptors on the microvillar membranes of the midgut epithelial cells, leading to insertions into the microvillar membranes (de Maagd et al., 2003, Bravo et al., 2004). Toxin insertions result in the formation of lytic pores in the apical microvillar membranes, causing osmotic lysis of midgut epithelial cells in their target insects (Knowles and Ellar, 1987, Aronson and Shai, 2001). Subsequently cells lyse and the cell contents are released into the midgut, providing spores with a germinating medium (de Maagd et al., 2003).

In addition to the pore-forming action, the Cry toxins have also been found embedded in the spore wall of *B. thuringiensis ssp. kurstaki* HD-73 (Du and Nickerson, 1996). *B. thuringiensis ssp. kurstaki* HD-73 with Cry1Ac embedded in its spore wall germinated under alkaline condition, while the isogenic mutant *B. thuringiensis ssp. kurstaki* HD-73 cry$^-$ could not germinate under the same condition. It is also suggested that the embedded Cry1Ac toxins stimulated the binding of spores to the microvillar membrane, creating favourable conditions for spore germination (Du and Nickerson, 1996). This phenomenon may explain one of the possible evolutionary advantages of possessing the Cry toxins, which might

encourage the germination of *B. thuringiensis* spore and the bacteria thus have a greater chance to proliferate. Meanwhile the Cry toxins have also been reported to bind to the petritrophic matrix (Rees et al., 2009) and interact with the commensal gut bacteria *Enterococcus* in tobacco hornworm *Manduca sexta* (Johnston and Crickmore, 2009) and *P. xylostella* (Raymond et al., 2009). The presence of *Enterococcus* species and other midgut bacteria in *M. sexta* and *P. xylostella* can reduce the toxicity of Cry1Ac toxins and confers some protection against *B. thuringiensis* infection (Jarosz, 1979, Johnston and Crickmore, 2009, Raymond et al., 2009)

1.6.2 Germination of B. thuringiensis spores

To proliferate and dominate the host successfully, *B. thuringiensis* spores need to germinate and return to vegetative growth. The germination process of *B. thuringiensis* is poorly studied. However, based on various studies of *B. cereus* spore-germination, we understand the germination of *Bacillus* spores is induced by various nutrient germinants, including amino acids, purine nucleosides, sugar, ions and combinations of these (Setlow, 2003, Moir, 2006). Specific nutrient germinants bind to specific receptor proteins located in the inner membrane of the spore (Behravan et al., 2000, Clements and Moir, 1998, Southworth et al., 2001, Thackray et al., 2001, Warren and Gould, 1968). The receptor proteins are encoded by the *gerA* family operons (Moir and Smith, 1990, Ross and Abel-Santos, 2010). The binding of nutrient germinants to the receptors triggers the release of cations, including H^+, K^+ and Na^+, from the spore core into the environment (Swerdlow et al., 1981), followed by release of Ca^{2+} and dipicolinic acid (DPA) and replacement of these cations and DPA by water. These events trigger the hydrolysis of the spore cortex (thick layer of peptidoglycan surrounding the spore core wall) by either of the two crucial cortex lytic enzymes, namely CwlJ and SleB (Moir, 2006). Upon completion of cortex hydrolysis, the spore core rehydrates and expands, following resumption of metabolism and macromolecular synthesis.

In addition to nutrient germinants, non-nutrient agents can also trigger spore germination. For example, Ca^{2+} and DPA released from one spore are believed to prompt the germination of neighbouring spores (Riemann and Ordal, 1961). Exposure to high pressure (Gould and Sale, 1970) or alkylamines (Rode and Foster, 1961) can also induce germination. Although these non-nutrient agents are not physiological, they use several components of the nutrient germination pathway. For instance, exposure to high pressure (600MPa) (Wuytack et al., 2000, Black et al., 2005) or dodecylamine (Setlow et al., 2003) resulted in the release of Ca^{2+} and DPA from spore without the initial binding of nutrient germinants to receptors.

1.6.3 Vegetative growth of B. thuringiensis in host

The newly geminated *B. thuringiensis* cells face a cascade of immune response from the host. Before entering the larval body cavity (hemocoel), in order to survive and grow in the gut, *B. thuringiensis* need to compete with natural gut bacteria (Jarosz, 1979, Raymond et al., 2009) and overcome host humoral immune responses such as antimicrobial peptides and lysozyme (Jiang et al., 2010). *B. thuringiensis* has been found to produce the bacteriocin entomocin 110 to inhibit the growth of gram-positive bacteria (Cherif et al., 2008). The vegetative cell-type peptidoglycan structure of *B. thuringiensis* was also suggested to provide resistant to host lysozyme (Atrih and Foster, 1999). Moreover, *B. thuringiensis* need to digest the peritrophic matrix and midgut epithelial cells to gain access to the insect hemocoel. The genes regulated by the PlcR regulon were thought to involve in gut pathogenesis. The PlcR regulon controls the expression of 45 genes encoding secreted proteins including bacteriocins to kill competitors, the pore forming enterotoxins complexes (Hbl and Nhe), hemolysins known for cytotoxic and hemolytic activities and degradative enzymes to destroy host antimicrobial peptides (Gohar et al., 2008). Experiments with *Galleria mellonella* larvae have shown that deletion of the transcriptional activator, plcR, reduced bacterial virulence

significantly in orally infected insects (Salamitou et al., 2000, Fedhila et al., 2003). However, the virulence of *B. thuringiensis* plcR negative was not affect when injecting the bacteria directly into the insect hemocoel, indicating a key role in pathogenicity of the PlcR regulon in the gut rather than the hemocoel (Salamitou et al., 2000, Fedhila et al., 2003).

Once *B. thuringiensis* succeed in entering the insect hemocoel, the bacteria have to face the insect immune system consisting of several defensive mechanisms including the humoral antimicrobial peptides, circulating hemocytes and sequestering iron sources to limit bacteria growth (Jiang et al., 2010, Cherayil, 2011). *B. thuringiensis* battle against humoral antimicrobial peptides by neutralizing the negative charge of the teichoic acids found on the surface of gram-positive bacteria and thus prevent the binding of cationic antimicrobial peptides (Khattar et al., 2009). *B. thuringiensis* also produce a metalloprotease, InhA1, localized in *Bacillus* exosporium, to destroy host antibacterial proteins (Dalhammar and Steiner, 1984). Hemocytes produced by the host cellular immune response eliminate invading bacteria through encapsulation, nodulation and phagocytosis. Apoptosis of insect hemocytes is provoked by a pore-forming toxin, Haemolysin II (HlyII), produced by *B. thuringiensis* (Tran et al., 2011). The InhA1 metalloprotease also allows spores of *B. thuringiensis* to escape from murine macrophages, but the role of InhA1 in insect hemocytes remains unclear (Ramarao and Lereclus, 2005). The insect hosts also withhold ion to combat bacterial infection. Due to ion deprivation in the host, *B. thuringiensis* is found to express a leucine-rich surface protein, IlsA, localized in peptidoglycan, to uptake iron from ferritin and heme (Fedhila et al., 2006). The aforementioned toxin, HlyII also lysis cells known to sequester iron and thus releasing iron into the extracellular environment. Genes regulated by the transcriptional activator, Fur, were also found to modulate iron uptake (Harvie et al., 2005).

Apart from the aforementioned secreted proteins to overcome host immune response, *B. thuringiensis* also need to digest the host tissue and acquire nutrients for growth. The PlcR regulon also regulate the expression of enzymes involved in food supply, including phospholipase and proteases (Gohar et al., 2008), suggesting that the genes regulated by the PlcR regulon may be expressed at various stages during the *B. thuringiensis* life cycle. Another quorum sensing system in *B. thuringiensis*, namely NprR-NprRB, is activated after the death of the insect. The quorum sensor NprR regulates at least 41 genes encoding degradative enzymes, chitinases and a lipopeptide allowing *B. thuringiensis* to survive and eventually sporulate in the insect cadaver (Perchat et al., 2011, Dubois et al., 2012). The NprR-regulated chitinases could also be involved in the degradation of the insect cadaver cuticle and hence contribute to the dispersion of *B. thuringiensis* spore and crystal protein inclusions into the environment (Raymond et al., 2010).

1.6.4 Sporulation in B. thuringiensis

Eventually, the nutrients within the host cadaver are depleted as *B. thuringiensis* cells grow to a maximum capacity. *B. thuringiensis* need to cope with this condition and produce resistant spores and the crystal protein inclusions to disseminate in the environment to start a new cycle of infection in another insect. Based on various studies in *B. subtilis* sporulation, the sporulation of *B. thuringiensis* is believed to be controlled by the activation of the master transcriptional regulator Spo0A, which is activated via a multicomponent phosphorelay. A phosphoryl group is added to Spo0F, then passed on to Spo0B, and finally to Spo0A through the histidine kinases KinA, KinB and KinC respectively (Burbulys et al., 1991, Hoch, 1993, Sonenshein, 2000). The phosphorylated regulator Spo0A (Spo0A~P) then activates the transcription of more than 120 genes by binding to their promoters, leading to a global change in gene expression which further altering the transcription profile of over 500 genes and eventually leading to sporulation (Fawcett et al., 2000, Liu et al., 2003, Molle et al., 2003). At the onset of sporulation, the cell undergoes asymmetric division, giving rise to a small forespore compartment and a larger mother cell,

which engulfs the immature spore in a second membrane and nurtures the developing spore. A cortex is laid down in the space between the two membranes and then protein coats are formed around the cortex. Upon the completion of sporulation, the resilient spore is discharged by lysis of the mother cell.

As in all, in nature, the infection process of *B. thuringiensis* is a constant battle against the host immunity. Interruption in the key steps of the infection process could drastically reduce the virulence of *B. thuringiensis*.

CHAPTER 2 METHOD AND ASSUMPTION VALIDATION

2.1 Introduction

This chapter will focus on a number of experiments on sporulation, germination, *in vitro* vegetative growth and *in vitro* competition assays of *B. thuringiensis* in search for an improved methodology for subsequent experiments. The pathogenicity of the Cry1Ac toxin in the diamondback moth *P. xylostella* has also been investigated in search for an effective lethal dose for the following *in vivo* experiments.

First of all, the amount of time required for the QS strains of interest to reach maximal sporulation is tested in order to develop a method that allows us to accurately estimate the bacterial population size in succeeding experiments. Typically, in nature, microorganisms reproduce by processes such as budding or binary fission. Very often, before the daughter cell buds off from the mother cell, it has started a new cycle of production, resulting in a chain of bacteria connected to each other. This poses a problem when population size is obtained by plating, as the information whether a colony is formed from a single bacterium or a chain of bacteria could not be acquired via plating. Counts of colonies formed from a single bacterium would lead to an accurate estimate of bacterial population size, but counts of colonies formed from a chain of bacteria would underestimate the bacterial population size. Therefore, it is essential to ensure that the studied bacterial population have reached maximal sporulation before plating since spores are single cells. The information of how many cells in the population have sporulated can be acquired via comparing the colony forming unit (CFU) counts before and after pasteurization. Due to the widely known property of *Bacillus* spores surviving extreme environments (Nicholson et al., 2000), heating at 65℃ for 20 minutes (pasteurization) would kill the vegetative cells but not the spores in the

population. Therefore, before pasteurization, CFU counts on plates estimate the total number of cells in the bacterial population, while CFU counts after pasteurization estimate the number of spores in the bacterial population. The amount of time required for the QS strains of interest to reach maximal sporulation can then be obtained for later experiments.

In addition to ensuring the bacteria have sporulated before plating, the differences in germination ability between the PlcR-PapR QS wild type and its isogenic QS null mutants also affect experiments that rely on plating. To infect the host successfully, *B. thuringiensis* need to return from spores to vegetative growth. Hence, differences in the ability to germinate from spore could also hugely affect the virulence between the PlcR-PapR QS wild type and QS null mutants. In addition, as discussed above, *B. thuringiensis* spores are used for infecting diamondback moth larvae and also after infection, the PlcR-PapR QS strains are allowed to sporulate in the moth cadavers for an accurate estimate of bacterial population size via plating. Hence, plating involves spreading spore dilutions on LB agar plates and allowing the spores to germinate on LB agar plates. This presents a problem if the PlcR-PapR QS wild type and its isogenic QS null mutants differ in their ability to germinate from spores on LB agar plates. As in the following experiments, bacterial growth in host and relative fitness of mutants were measured via plating spores on appropriate LB plates. Any differences in spore germination rates between the PlcR-PapR QS wild type and QS null mutant strains have the potential to bias calculations of relative fitness. Therefore, it is critical to assess the germination ability of the PlcR-PapR QS wild type and its isogenic QS null mutants. The study is conducted via comparing the direct counts of spores using a counting chamber under microscope with the CFU counts on LB plates.

Apart from sporulation and germination, before investigating the evolutionary ecology of the peptide-based PlcR-PapR QS system in *B. thuringiensis*, it is crucial

to establish whether the PlcR-PapR QS system encodes a cooperative trait. If the PlcR-PapR QS system regulates cooperative behaviours, QS null mutants (cheats) could out-compete wild type bacteria in mixed infections by taking advantage of the signals and secreted proteins regulated by the PlcR regulon of wild type and also by avoiding the metabolic cost of production of the signals and secreted proteins. However, the above reasoning is based on the assumption that the production of the signals and/or secreted proteins is metabolically costly. It is therefore important to validate the assumption in relatively less a sophisticated laboratory system before examining the PlcR-PapR QS system *in vivo*. To validate the assumption that the production of the signals and secreted proteins is metabolic costly, the PlcR-PapR QS wild type and its isogenic QS null mutants are allowed to growth in LB broth. The PlcR-PapR QS null mutants are also allowed to compete with the QS wild type at various frequencies in LB broth. The LB broth (L-Broth) is a nutrient medium first described by Bertani to promote bacterial growth (Bertani, 1951). *B. thuringiensis* has been reported to activate the PlcR regulon in this medium at early stationary phase (Lereclus et al., 1996), but the expression of the secreted proteins regulated by the PlcR regulon is not required for bacteria growth. Therefore differences in growth rate during exponential phase between the PlcR-PapR QS wild type and its isogenic QS null mutants is a result of the metabolic cost in the production of the signals and secreted proteins regulated by the PlcR regulon. As the PlcR regulon controls the expression of at least 45 genes (Gohar et al., 2008), it is expected that it will be metabolically expensive to produce the proteins in this regulon. The PlcR regulon also control the expression of its own activator, the PapR peptide. Consequently, the PlcR-PapR QS null mutants could gain growth benefits by avoiding the production of signals or secreted proteins regulated by the PlcR regulon.

As previously described, to date 717 Cry toxins and 38 Cyt toxins have been reported (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/intro.html), all of which disagreeing in genetic coding, mode of action and toxicity in different insect

(Schnepf et al., 1998). In particularly, diamondback moths (*P. xylostella*) have been reported to resistance to the toxin Cry1Ac in the field (Sayyed et al., 2005). It is therefore necessary to test the pathogenicity of Cry1Ac in diamondback moth larvae in a laboratory environment in search for an effective lethal dose for the proceeding *in vivo* experiments. To search for an effective lethal dose, diamondback moth larvae were inoculated with a range of toxin dose and the percentage of larvae surviving infection is monitored over time.

All in all, in this chapter, a number of tests were carried out in search for improved protocols for subsequent experiments. The tests include sporulation of *B. thuringiensis in vitro*, germination of *B. thuringiensis* spores *in vitro*, vegetative growth of *B. thuringiensis in vitro*, *in vitro* competition assay and toxin bioassay.

2.2 Materials and methods

2.2.1 Bacterial strains and growth condition

The acrystalliferous strain *B. thuringiensis* 407 Cry⁻ belonging to the *B. thuringiensis* subspecies (serotype 1) (Lereclus et al., 1989) was used as QS wild type. *B. thuringiensis* 407 Cry⁻ A'Z Δ*papR* and Δ*plcR* mutant strains have been described previously (Salamitou et al., 2000, Slamti and Lereclus, 2002) and were used as isogenic QS signal null and signal blind mutants respectively. For succeeding experiments, spores of the above-stated QS strains were acquired by culturing cells separately on *B. cereus* selective agar plates for 5 to 7 days at 37℃. The *B. cereus* selective agar plates contained 4% w/v Bacillus cereus Selective Agar Base (Oxoid), 100IU ml^{-1} of polymyxin B (Oxoid) and 4% v/v egg yolk (Oxoid) for the isolation and enumeration of *B. thuringiensis* cells (Donovan, 1958, Holbrook and Anderson, 1980, Mossel et al., 1967, Willis and Hobbs, 1959). After 5 to 7 days, the QS cells were harvested with a flame-sterilized spatula scraping across the agar plates and were then put into 1.5 ml micro-centrifuge tubes. Subsequently, the QS cells were washed with 0.75 ml sterile saline solution containing 0.85% w/v NaCl (Fisher Scientific). The cell suspensions were then centrifuged at 6000 g min^{-1} for 3 minutes and the supernatants were discarded. After three washes, the QS cells were resuspended in 1 ml 0.85% NaCl solution. The cell resuspensions were aliquoted and heat-treated (pasteurized) at 65℃ for 20 minutes. At the end of the cell preparation, all the aliquots were stored at – 20℃. Due to the widely known property of *Bacillus* spores surviving extreme temperatures (Nicholson et al., 2000), the above treatments left only *B. thuringiensis* spores in the aliquots.

One aliquot for every aforementioned QS strain were taken out from – 20℃ and defrosted. The spore concentrations of the three defrosted aliquots were counted before bioassays by spreading 100 µl of serial dilutions on 2% LB agar plates. The 2% LB agar plates contained 2% w/v LB broth (Fisher Scientific) and 2% w/v

bacteriological agar (Oxoid). Within the countable dilution (30 – 200 colonies), 6 plates were counted to estimate the spore concentration in each aliquot. The defrosted aliquots were placed at 4℃ and were discarded after one month. Fresh aliquots for each QS strains were taken out from – 20℃ and the same method was used to estimate the spore concentrations in the fresh aliquots. The antibiotic used for strain selection was kanamycin (200 μg ml^{-1}) for QS null mutants. The QS wild type was identified on LB agar plates containing 4% v/v egg yolk (Oxoid). The primary diagnostic feature is the colony appearance. The PlcR regulon regulates the production of the extracellular protein, phosphatidylinositol-specific phospholipase C (Lereclus et al., 1996), which hydrolyses the lecithin found in egg yolk. The QS wild type is able to produce the phosphatidylinositol-specific phospholipase C but the isogenic QS null mutants are incapable of manufacturing this extracellular protein (Salamitou et al., 2000, Slamti and Lereclus, 2002). Therefore, precipitation of hydrolyzed lecithin produced a "white halo" around the colonies of the QS wild type, while the inability of the QS null mutants to utilize lecithin resulted in colonies without haloes.

2.2.2 Insects and growth condition

Diamondback moths (*P. xylostella*) were used for *in vivo* infections. *P. xylostella* larvae (Geneva population) were acquired from CEH Oxford and reared on a semi-synthetic diet at 23°C. Adopted from Hoffman's tobacco hornworm diet (Smith, 1966), the semi-synthetic diet was made from 45 g wheat germ, 20 g Casein from bovine milk (Sigma), 18 g sugar, 11 g Agar Bacteriological (Oxoid), 9 g dried brewers yeast, 6 g Wessons salts, 1 g Sorbic acid (Sigma), 0.6 g Cholesterol (Sigma), 0.6 g Methyl-4-hydroxybenzoate (Sigma) and distilled water making up a total volume of 500 ml. The semi-synthetic diet was autoclaved. To make antibiotic diet pots, 0.4% w/v Vanderzant vitamin mixture for insects (Sigma), 0.36% w/v L-Ascorbic acid (Sigma), 0.012% w/v Chlortetracycline hydrochloride (Sigma) and 0.012% w/v Streptomycin sulfate salt (Sigma) were added to the autoclaved semi-synthetic diet after it had cooled to 60℃. Similarly, to make vitamin diet pots, 0.4%

w/v Vanderzant vitamin mixture for insects (Sigma) and 0.36% w/v L-Ascorbic acid (Sigma) were added to the autoclaved semi-synthetic diet after cooling to 60℃. The semi-synthetic diet with antibiotics and vitamins was poured into 55 × 14 mm Petri dishes.

To maintain the stock, *P. xylostella* eggs were allowed to hatch at 23°C in a clean box accommodating eight antibiotic diet pots. The larvae were culled once when they reached late 3rd or early 4th instar and placed onto fresh antibiotic diet pots. The larvae were allowed to pupate in the box. Before adults emerged from the pupal cocoons, a damp cotton bud was placed into the box as well as two cabbage strips made from Parafilm (Alcan). The Parafilm was cut into strips measuring a size of 50.8 mm × 152.4 mm. The strips were soaked in cabbage soup made from boiling organic cabbage in distilled water. After soaking the strips for roughly 5 seconds, the cabbage strips were left to dry in a laminar flow cabinet for 20 min and stored at 4℃. As *P. xylostella* adults were attracted to the savour of cabbage, most of the eggs were laid onto the cabbage strips. *P. xylostella* adults also drank water from the damp cotton bud prior to mating. The eggs were collected every 24 h and the box was resupplied with fresh cabbage strips. A cabbage strip with *P. xylostella* eggs on it was put into a clean box accommodating eight antibiotic diet pots and eggs were allowed to hatch as before. For *in vivo* experiments, *P. xylostella* eggs were allowed to hatch at 23℃ onto vitamin diet pots and 3rd larvae were used for all *in vivo* infections.

2.2.3 Sporulation of B. thuringiensis in vitro

A sloppy semi-synthetic diet was prepared using 15 g wheat germ, 6 g Casein from bovine milk (Sigma), 6 g sugar, 3g Agar Bacteriological (Oxoid), 3 g dried brewers yeast, 2 g Wessons salts, 0.3 g Sorbic acid (Sigma), 0.2 g Cholesterol (Sigma), 0.2 g Methyl-4-hydroxybenzoate (Sigma) and distilled water making up a total volume of 200 ml. As mentioned before, 0.4% w/v Vanderzant vitamin mixture for insects

(Sigma) and 0.36% w/v L-Ascorbic acid (Sigma) were added to the autoclaved sloppy diet after it had cooled to 60℃. Sixty microliters of the sloppy diet were pipeptted quickly into each well of a 96-well plate and allowed to dry in laminar flow cabinet for 10 min.

A inoculum was prepared at a final spore concentration of 5 x 10^4 spores μl^{-1} with 51% QS wild type and 49% QS signal blind mutant, and a Cry1Ac toxin concentration of 1.25 µg ml^{-1}. The Cry1Ac toxin was purified from an *E. coli* strain *E. coli* JM109, which contains a plasmid pGEM1Ac that carries the *Cry1Ac* gene (kindly supplied by Dr Neil Crickmore, University of Sussex, United Kingdom). Four microliters of the inoculum were pipetted into each well of the sloppy diet plate and allowed to dry in the laminar flow cabinet for 10 min. After that, 3rd instar larvae of *P. xylostella* were introduced to the diet plate, with one well accommodating only one larva. The diet plate was overlaid with flattened tissue to fill up the space between the diet plate and its cover to prevent larvae from moving between wells. The plate was then sealed with cling film and the larvae were allowed to eat the diet for 48 h at 23℃. After 2 days, the cadavers of the infected larvae were picked up using sterile toothpicks and incubated in 1.5 ml micro-centrifuge tubes containing10 µl sterile distilled water at 30℃. After 6 days incubation, 490 µl 0.85% NaCl solution were added to 5 cadavers. The cadavers were then ground with sterile pellet pestles (Fisher Scientific) connected to a cordless motor (Fisher Scientific). For each cadaver, the homogenate was aliquoted into two halves. One half of the cadaver homogenate was serial diluted and 20 µl of the serial dilutions were pipetted onto 2% LB agar plates to obtain CFU counts. For each serial dilution, a total of 120 µl were pipetted onto the 2% LB agar plates, with 20 µl forming 6 dots on the 2% LB agar plates. Another half of the same cadaver homogenate was pasteurized at 65℃ for 20 min. The pasteurized cadaver homogenate was also serial diluted and the serial dilutions were plated as the unpasteurized cadaver homogenate. After 9 days incubation, another 5 cadavers were sampled in the same procedure. After 13 days incubation, another 2 cadavers were sampled as before.

2.2.4 Germination of B. thuringiensis spores in vitro

To test the germination rate of *B. thuringiensis* spore *in vitro*, microscopic counts of *B. thuringiensis* spores were compared with their CFU counts. Fresh aliquots of the prepared spores of each strain were taken out from – 20℃. The aliquots were serial diluted twice independently to obtain spore counts using a Zeiss Compound microscope. To count the spores under the microscope, 9 µl of the serial dilutions were pipetted into a counting chamber of a FastRead 102 disposable counting slide (Immune Systems, UK). Dilutions with spore count exceeding 200 were discarded as described in the user manual. Within each countable dilution, spores in 2 complete 4 x 4 grids were counted. The spore concentrations of each strain in the aliquots were calculated by the total counts of spore divided by 4 (the number of complete 4 x 4 grids counted) and then multiplied by 10 and sample dilution (if any) as described in the user manual. Fifty microliters of each independent serial dilutions for the aliquots were also spread onto two 2% LB agar plates to obtain CFU counts. The study was repeated 4 times, i.e. four different aliquots for each strain were examined.

2.2.5 Vegetative growth of B. thuringiensis in vitro

To monitor the vegetative growth of *B. thuringiensis in vitro*, QS wild type and its isogenic QS null mutants were allowed to grow in 200 µl 2% LB broth in the wells of a 96-well plate (Greiner Bio-One, UK) for 24 hours at 30℃. The absorbances of each well of the 96-well plate were measured every 5 minutes for 24 hours at 600nm using the SpectraMax® 190 Absorbance Microplate Reader (Molecular Devices, US). To prepare the growth cultures, QS wild type and its isogenic QS null mutants were grown in 5 ml 2% LB broth overnight with vigorous shaking (200 rpm) at 30℃. For the QS null mutants, kanamycin was added to the overnight cultures, to a final concentration of 200 µg ml^{-1}. After overnight growth, the growth cultures for the three strains were started from the overnight cultures. The absorbances of the overnight cultures were first measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). The growth cultures were adjusted

to an absorbance of 0.05 at 600nm for each strain via adding appropriate quantities of the overnight cultures to fresh 5 ml 2% LB broth. Two hundred microliters of the growth cultures for the three strains were added to the wells of the 96-well plate randomly. Thirty-six wells of the 96-well plate contained the growth cultures for the above-stated QS strains, 12 wells for each strain. The rest of the wells of the 96-well plate were filled with 200 µl 2% LB broth. After 24 h growth, growth curves for the cultures in each well were produced by the software SoftMax®Pro 5 (Molecular Devices, US). The experiment was replicated twice.

2.2.6 In vitro competition assay

To establish whether QS null mutants had a growth benefit in 2% LB broth compared to the QS wild type, QS null mutants were mixed with QS wild type at various frequencies and the mixed cultures were allowed to grow for 24 h at 30℃. To prepare the mixed cultures, QS wild type and QS null mutants were allowed to grow overnight in 10 ml 2% LB broth with vigorous shaking (200 rpm) at 30℃. For the QS null mutants, kanamycin was added to the overnight cultures at a final concentration of 200 µg ml^{-1}. After overnight growth, the absorbances of the overnight cultures were first measured at 600 nm. New cultures for each QS strains were adjusted to an absorbance of 0.05 at 600 nm via adding appropriate quantities of the overnight cultures to fresh 10 ml 2% LB broth. The new cultures were incubated at 30℃ with vigorous shaking at 200 rpm. The absorbance of the new cultures were measured every 30 min. According to the absorbance of the new cultures measured between 0.5 ~ 1 at 600 nm, QS null mutants were mixed with QS wild type at various frequencies by adding appropriate quantities of the new cultures to fresh 10 ml 2% LB broth, adjusting the mixed cultures to an absorbance of 0.005 at 600 nm. The mixed cultures were incubated for 24 h at 30℃ with vigorous shaking at 200 rpm. The initial frequencies and final frequencies of QS null mutants in the mixed cultures were obtained by serial dilution and plating 50 µl of the serial dilutions on two 2% LB agar plates containing 4% v/v egg yolk (Oxoid). The experiment was replicated twice.

2.2.7 Toxin bioassay

The activity of the Cry1Ac toxin was assessed in search for an inoculation dose for proceeding *in vivo* experiments. Inocula were prepared with toxin concentrations of 1.31 µg ml^{-1}, 0.262 µg ml^{-1}, 0.0524 µg ml^{-1}, 0.01048 µg ml^{-1} and 0.002096 µg ml^{-1}. Another set of inocula was also prepared with the listed toxin concentrations and a QS wild type spore concentration of 5×10^4 cfu μl^{-1}. *P. xylostella* larvae were also tested with the 0.85% NaCl solution.

Vitamin diet pots were prepared as previously described, measuring 10 ml of the vitamin semi-synthetic diet per 55×14 mm Petri dish. The diet pots were cut into quarters with a flame-sterilized spatula, leaving only a quarter of the diet per pot. A hundred microliters of the inocula were pipetted on the surface of the quarter diet and left to dry at laminar flow cabinet for 20 min. After that, ten 3rd instar *P. xylostella* larvae were placed onto the inoculated quarter diet pots. For each inoculum, forty larvae were infected in the same procedure.

2.2.8 Data analysis

Data analysis was carried out in R v 2.15.2 (http://www.r-project.org/). For the sporulation of *B. thuringiensis* 407 Cry^- *in vitro,* sporulation rate was computed as the CFU counts after pasteurization divided by the CFU counts before pasteurization. The sporulation rate data was first analysed with a generalized linear model with binomial errors. However, in the binomial errors GLM model, the overdispersion factor was 222.02, suggesting that the errors were not binomial as assumed but larger than this by a factor of 222.02. The sporulation rate data was then analysed with quasibinomial errors to account for the overdispersion. For germination of *B. thuringiensis* 407 Cry^- spores *in vitro*, germination rates of QS mutants and wild type spores *in vitro* were derived from the CFU counts divided by microscopic spore counts. The germination rate data were analyzed using a mixed-effects model to account for the fixed effect (difference in spore germination rates

between strains) and random effect (variation in different aliquots of the same strain). The germination rate data were also analyzed using the GLM model with binomial errors. Comparison of AIC between the two models suggested that the mixed-effect model provided a better fit of the germination data. For *in vitro* growth of *B. thuringiensis* 407 Cry^- in LB, vegetative growth data obtained from the edges of the 96-well plates were excluded from analysis to minimize edge effects. VMax was calculated by the software SoftMax®Pro 5 (Molecular Devices, US), which was instructed to use 11 VMax points in each well to estimate VMax. To calculate VMax, a line was drawn between the 1st reading and the 12th reading. The slope of the line was calculated by mOD/min, where mOD and min were the difference in absorbance and time respectively between the two data points. The second and subsequent slopes were calculated beginning at the 2nd data point and ending at a total number of readings equal to the VMax points. VMax was reported as the steepest positive slope. VMax was analyzed with a GLM model. For *in vitro* competition assay, the relative fitness of mutants is calculated by $v = x_2(1 - x_1)/x_1(1 - x_2)$, where x_1 is the initial frequency of mutants in the population and x_2 is the final frequency of the mutants (Ross-Gillespie et al., 2007). The relative fitness of mutant is the estimated growth rate of mutant in relation to that of the wild type. The computed relative fitness were analysed with a generalized linear model. For toxin bioassay, the data were analysed with a Cox proportional hazard model. All model assumptions were checked with graphical analyses of error distribution and normality.

2.3 Results

2.3.1 Sporulation of B. thuringiensis in vitro

The sporulation of *B. thuringiensis* 407 Cry^- *in vitro* was examined through plating homogenized insect cadavers on 2% LB agar plate before pasteurization (grey boxes) and after pasteurization (black boxes) (Fig 2.1). As shown in Fig 2.1, after 13 days of incubation in 10 µl sterilized distilled water at 30℃, the tested QS strains had a significantly higher sporulation rate in 3rd instar *P. xylostella* larval cadavers (t = 3.099, d.f. = 9, p = 0.0128). At 13 days of incubation 91% of the bacteria had sporulated.

2.3.2 Germination of B. thuringiensis spores in vitro

The germination rates of QS null mutants and QS wild type spores *in vitro* were assessed. Microscopic spore counts (grey boxes) were used to estimate the actual spores that were present in the aliquots, while CFU counts (black boxes) were the counts of spores in the aliquots that had germinated on 2% LB agar plate (Fig 2.2). As shown in Fig 2.2, QS null mutants and QS wild type differed significantly in their ability to germinate from spores ($F_{2,6}$ = 12.0, p = 0.008, n = 12). Particularly, signal blind mutant had significantly higher number of spores germinated compared to the wild type (t = 4.84, d.f. = 6, p = 0.0029) (Fig 2.2).

2.3.3 Vegetative growth of B. thuringiensis in vitro

The growth of the QS wild type and QS null mutants in 200 µl 2% LB broth at 30℃ was monitored via measuring the absorbance of the growth cultures every 5 minutes for 24 hours. The absorbances starting at the beginning (time point zero) were extracted at 2 hours intervals to plot the growth curves for the QS wild type (black solid line, black circles), signal blind mutant (blue dotted line, blue triangles)

and signal null mutant (blue dashed line, red squares) (Fig 2.3). The growth data in experiment block 1 and 2 were plotted in Fig 2.3 A and Fig 2.3 B respectively as the lag phases of the growth curves for each strains were slightly different in the two experimental blocks. In block 1, there were 7, 9 and 9 replicates for QS wild type, signal blind mutant and signal null mutant respectively. In block 2, there were 8, 7 and 9 replicates for QS wild type, signal blind mutant and signal null mutant respectively. The replicates of each strain in block 1 were sampled from one overnight culture of each strain. Block 2 was carried out using the same procedure. The sampling units are 15, 16 and 18 for QS wild type, signal blind mutant and signal null mutant respectively, as the absorbances of different cells were measured in each sampling unit. However, block effect was observed between experimental block 1 and 2. Data analysis has taken account of this block effect and did not find any significance. As shown in Fig 2.3 A and Fig 2.3 B, although the lag phases in the two experimental blocks varied, both signal blind mutant and signal null mutant had steeper exponential phase than the QS wild type in both experimental blocks. Both signal blind mutant (t = 3.370, d.f. = 46, p = 0.00153) and signal null mutant (t = 7.954, d.f. = 46, p < 0.0001) had significantly higher VMax than the QS wild type. QS wild type and its isogenic mutants differed significantly in exponential growth phase ($F_{2,46}$ = 32.134, p < 0.0001, n = 49) (Fig 2.3 C). However, although signal null mutant grew faster during exponential phase compared to the QS wild type, signal null mutants reached stationary phase earlier and started to decline soon after stationary phase (Fig 2.2 A and Fig 2.2 B). In addition, a variation in the growth curves of signal blind mutant was observed in the two experimental blocks. In experimental block 1, as the signal null mutant, signal blind mutant ceased growth earlier than the wild type, but in experimental block 2, signal blind mutant reached a higher population density compared to the QS wild type at the end of the growth curve.

2.3.4 In vitro competition assay

QS null mutants were mixed with QS wild type at various frequencies and mixed cultures were allowed to grow in 2% LB broth at 30℃ for 24 h. After 24 h growth, the relative fitness of QS null mutants were computed and plotted for signal null mutant (solid circles) and signal blind mutant (open circles) (Fig 2.4). As shown in Fig 2.4, the relative fitness of signal null mutant decreased significantly with increased initial frequency of signal null mutant in 2% LB broth (Fig 2.4, dashed line, $F_{1,10} = 17.174$, $p < 0.00200$, n = 12). At all frequencies, the relative fitness of signal blind mutant was bigger than one. As the relative fitness of mutant measures the growth rate of mutant in relation to that of the wild type, the relative fitness data of signal blind mutant suggested that signal blind mutant grew faster than the QS wild type in 2% LB broth.

2.3.5 Toxin bioassay

The pathogenicity of the toxin Cry1Ac in 3rd instar *P. xylostella* larvae was investigated in search for an effective lethal dose for later experiments. Fig 2.5 shows the survivorship over a 6-day period. Survivorship is the proportion of insect larvae in the quarter diet pot surviving after inoculated with a range of toxin concentrations (Fig 2.5 A) and also the listed toxin doses with a wild type *B. thuringiensis* 407 Cry⁻ spore concentration of 5 x 10^4 spores μl^{-1} (Fig 2.5 B). Overall, the administration of the Cry1Ac and wild type *B. thuringiensis* 407 Cry⁻ spores had a significant effect on larval survival (d.f. = 10, $X^2 = 303.3$, $P < 0.0001$, n = 435) (Fig 2.5). However, as shown in Fig 2.5 A, inocula with toxin concentration of 0.002096 $\mu g\ ml^{-1}$ (black dashed line, z = 0.054, d.f. = 10, $p = 0.957$) and 0.01048 $\mu g\ ml^{-1}$ (black dotted line, z = 1.580, d.f. = 10, $p = 0.114$) did not have a significant influence on larva survival. However, the addition of wild type *B. thuringiensis* 407 Cry⁻ spores had a significant improvement in larval death for the toxin concentration of 0.002096 $\mu g\ ml^{-1}$ (red dashed line, z = 1.779, d.f. = 10, $p = 0.0753$) and 0.01048 $\mu g\ ml^{-1}$ (red dotted line, z = 2.940, d.f. = 10, $p = 0.00328$) (Fig 2.5 B), corroborating with previous experiments (Li et al., 1987). With *B. thuringiensis* 407 Cry⁻ spores,

inocula with all the listed toxin concentrations showed a significant effect on larval survival. Particularly, with QS wild type spore concentration of 5×10^4 spores μl^{-1} and toxin concentration of 1.31 µg ml^{-1} (Fig 2.5 B, red two-dash line), all larvae died within two days.

Fig 2.1 At least 13 days were required for the QS strains to reach 91% sporulation. A inoculum was prepared at a spore concentration of 5×10^4 cfu μl^{-1} with 51% QS wild type and 49% QS signal blind mutant, and a Cry1Ac toxin concentration of 1.25 μg ml^{-1}. A total of 12 insect cadavers were sampled. For each insect cadaver, cell densities were obtained from CFU counts on 2% LB plates before pasteurization (grey boxes) and after pasteurization (black boxes). The bars represent the mean cell densities of 5 cadavers in Day 6, 5 cadavers in Day 9 and 2 cadavers in Day 13. Error bars represent one standard error about the mean.

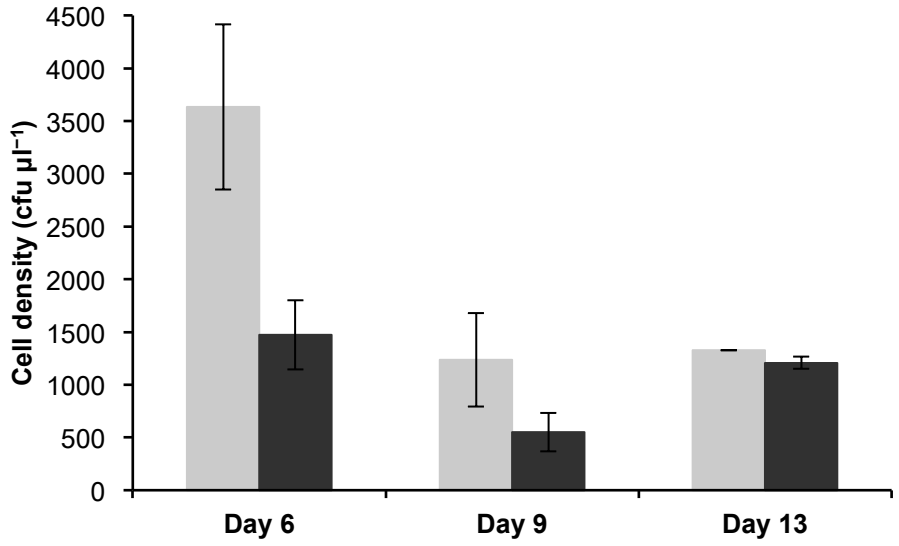


Fig 2.2 QS mutants and wild type differed in their ability to germinate from spore. Spore density obtained from microscopic spore counts (grey boxes) and CFU counts on 2% LB agar plates (black boxes) were plotted for QS wild type and its isogenic QS null mutants. The bars represent the mean spore densities of 4 aliquots for each QS strains. Error bars represent one standard error about the mean.

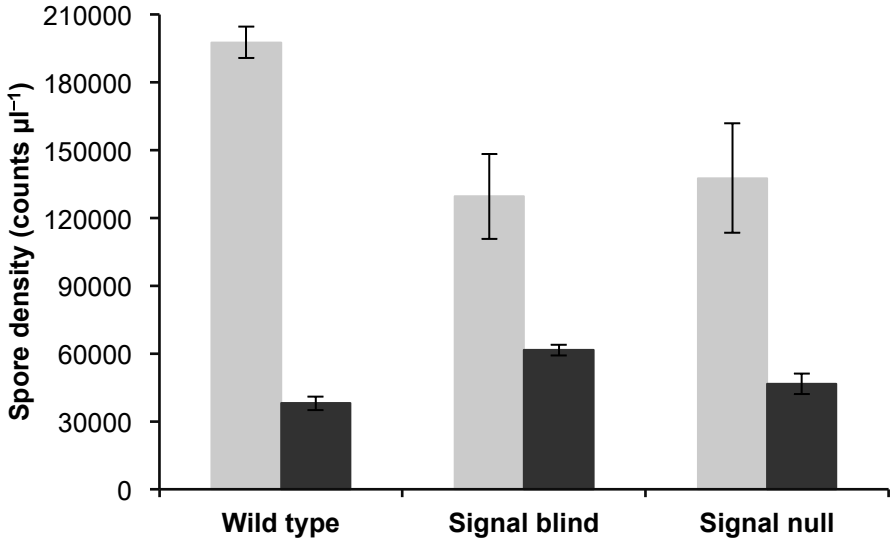


Fig 2.3 QS mutants grew significantly faster than wild type in LB. (**A, B**) The absorbances were plotted for QS wild type (solid line, black circles), signal blind mutant (dotted line, blue triangles) and signal null mutant (dashed line, red squares). The data points represent the mean absorbance for each QS strains at 2 hours interval at 30℃. **A** shows data in experiment block 1 (25 replicates) and **B** shows block 2 data (24 replicates). (**C**) VMax (absorbance/min) was plotted for QS wild type and QS null mutants. The bars represent the mean VMax of the growth curves for each strain that was allowed to grow in 200 µl 2% LB broth at 30℃. Error bars represent one standard error about the mean.

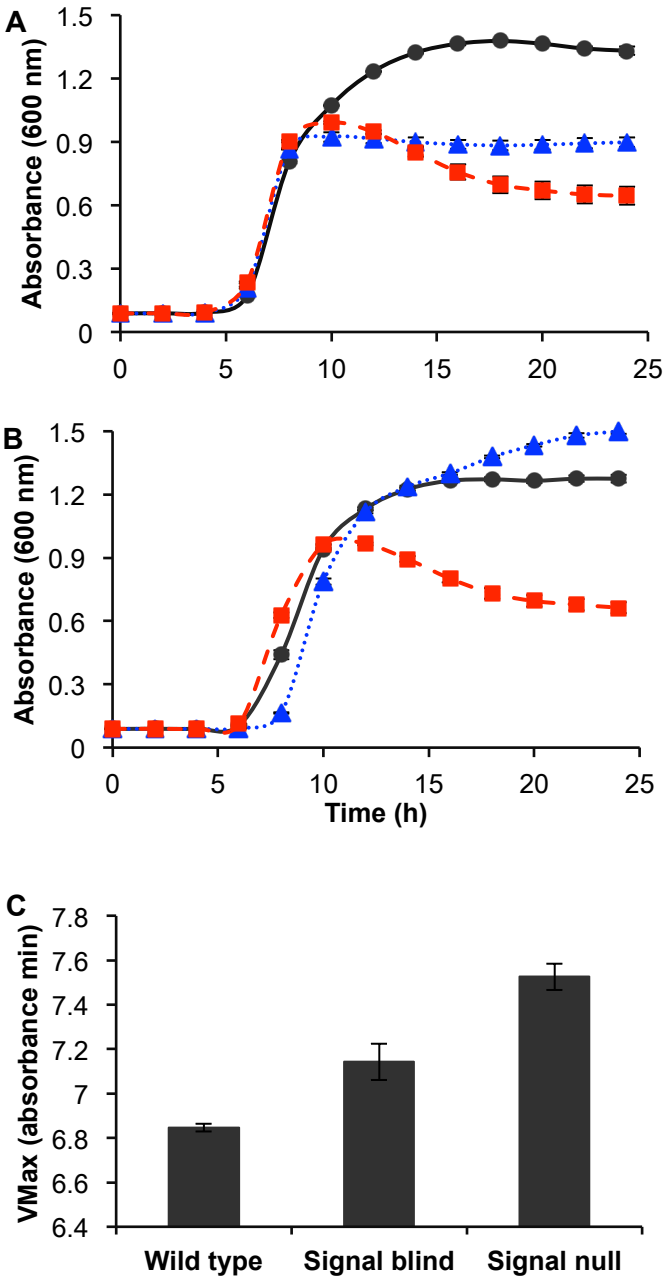


Fig 2.4 The relative fitness of signal null mutant was negative frequency dependent and signal blind mutant grew significantly faster than the QS wild type. The relative fitness of QS null mutants was plotted for signal blind mutant (open circles) and signal null mutant (solid circles) at various initial frequencies. Relative fitness of signal null mutant decreased significantly with increased initial frequency (dashed line, $F_{1,10} = 17.174$, $p < 0.00200$, n = 12).

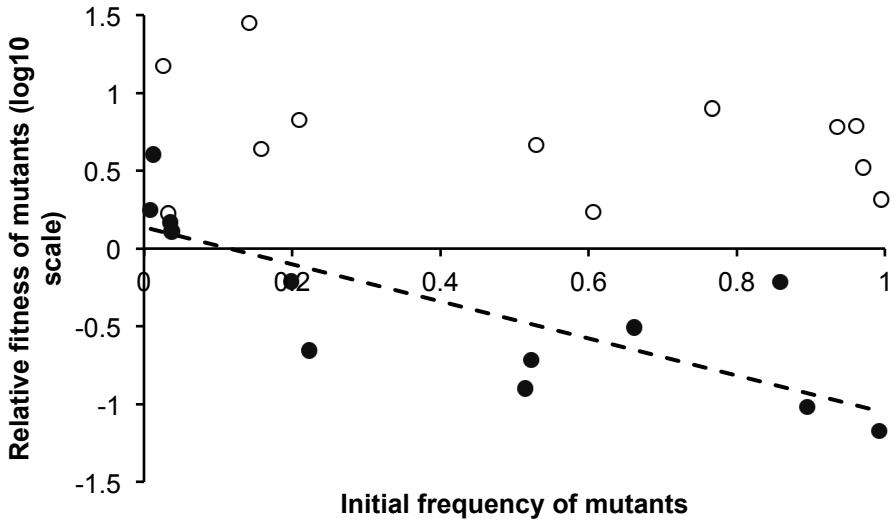
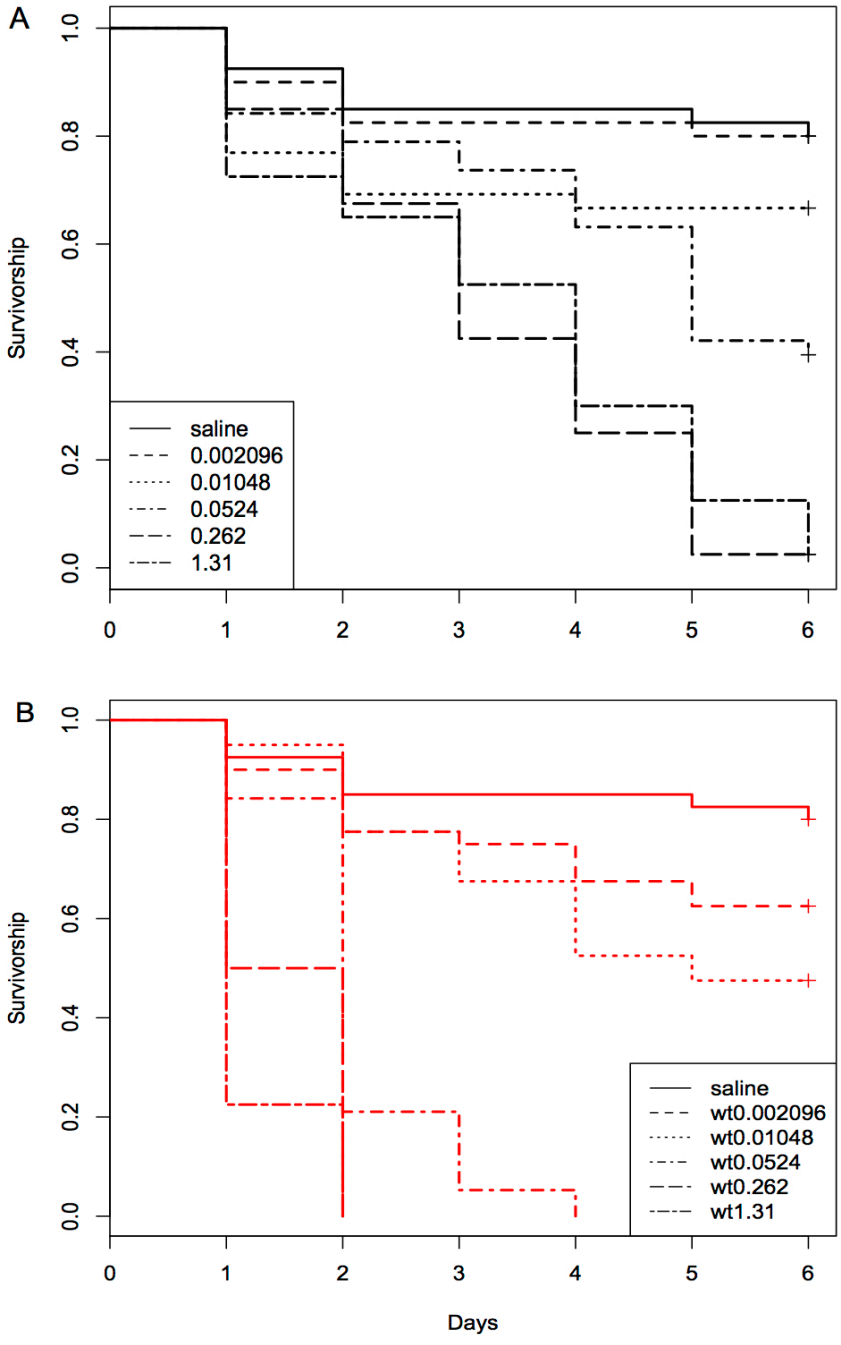


Fig 2.5 The Cry1Ac toxin and *B. thuringiensis* spores significantly affected larval survival. Insect larvae were inoculated with toxin concentrations of 1.31 µg ml^{-1} (two-dash line), 0.262 µg ml^{-1} (long-dash line), 0.0524 µg ml^{-1} (dot-dash line), 0.01048 µg ml^{-1} (dotted line), 0.002096 µg ml^{-1} (dashed line) and 0.85% NaCl saline control (solid line). The percentage of larvae surviving after inoculation (survivorship) was monitored every 24 h for 6 days. **A** shows toxin only infections (black lines), while **B** shows toxin plus spore infections (red lines). Spore concentration used was 5 x 10^4 spores $µl^{-1}$.



2.4 Discussion

Resistance of *Bacillus* spores to heat, freeze-drying, radiation and oxidizing agents has been well established (Nicholson et al., 2000). It is therefore possible to kill *Bacillus* vegetative cells through means of heat, freeze-drying, radiation and oxidizing agents. However, the use of freeze-drying, radiation and oxidizing agents have been reported to cause DNA damage in surviving spores (Setlow, 1995), while studies have suggested that heat treatment targeted the enzymes in the vegetative cells rather than DNA (Warth, 1980). Enzymes in *B. cereus* vegetative cells such as glucose 6-phosphate dehydrogenase and NADH oxidase were inactivated after heating the cells in Tris buffer for 10 to 20 min from 47℃ to 70℃, whereas enzymes in *B. cereus* spores remained stable under the same heat treatment (Warth, 1980). Therefore, heating the cells for 20 min at 65℃ was used to kill *B. thuringiensis* vegetative cells and select for *B. thuringiensis* spores. As shown in Fig 2.1, after 13 days of incubation post infection, *B. thuringiensis* 407 Cry$^-$ reached 91% sporulation in insect cadavers. However, the insect larvae were inoculated with both wild type and signal blind mutants. Whether the QS wild type and signal blind mutant sporulated at the same time is not known. The NprR-NprX quorum sensing system has been described to regulate sporulation in *B. cereus* group (Perchat et al., 2011). The PlcR regulon has also been reported to regulate the expression of an S-layer protein in *B. cereus*, which has been described to form crystalline surface layers overlying the peptidoglycan of some *B. cereus* strains (Gohar et al., 2008, Sidhu and Olsen, 1997). The PlcR regulon could potentially play a role in sporulation in *B. cereus*. However, the studied strain, *B. thuringiensis* 407, has been found to possess the S-layer gene but is devoid of an S-layer (Mignot et al., 2001). It is unlikely that *B. thuringiensis* 407 Cry$^-$ wild type and its isogenic mutants would differ in their ability to sporulate. This assumption was justified by the results of imaging studies in a later chapter. For later experiments, to ensure near-complete sporulation, at least 13 days incubation of insect cadavers at 30℃ after insect death was used.

In the germination study, a counting chamber was employed to estimate the actual spore concentration for each of the QS strains. Using a counting chamber is easy, inexpensive and relatively quick; it also gives information about the size and morphology of spores. It is also possible to count spore numbers using techniques like spectrophotometry and flow cytometry. However, studies on *B. cereus* spores have found bright and dark spores in a population and the optical densities of the bright and dark spores were different (Knaysi, 1959). The existence of bright spore and dark spores would hugely affect spectrophotometric and flow cytometric measurements. Quantitative real-time PCR (qPCR) has also shown promise for rapid and specific enumeration of spores, but the release of nucleic acid from spores is difficult and this could greatly limit the accuracy and precision of qPCR (Hospodsky et al., 2010). Besides, the detection of DNA does not necessarily tie in with the presence of viable spores. Therefore, germination of *B. thuringiensis* 407 Cry^- *in vitro* were examined through counting the spores under the microscope using a counting chamber and plating the spores on LB agar plates. As shown in Fig 2.2, signal blind mutant exhibited significantly higher germination rate compared to the QS wild type ($t = 4.84$, d.f. = 6, $p = 0.0029$). The discrepancy observed in germination results between the PlcR-PapR QS wild type *B. thuringiensis* 407 Cry^- and its isogenic signal null and signal blind mutants was unexpected. Studies on *B. subtilis* spores have shown that Ca^{2+} and DPA released from one spore triggered the germination of neighbouring spores (Riemann and Ordal, 1961). With increasing exogenous Ca^{2+} and DPA, more *B. subtilis* spores became germinated (Paidhungat and Setlow, 2000), suggesting that germination in *B. subtilis* could be regulated by a quorum sensing mechanism. However, in *B. thuringiensis*, the role of the PlcR regulon in germination has not been reported. The results pose a potential problem that extremely dormant spores might be present in lab cultures and there were more dormant wild type spores than mutant spores. Although the results from the above studies presented new insight into spore germination, these data suggest that culture based assessments of fitness will, if anything, overestimate the fitness of QS null mutants relative to the QS wild type strain. Having a lower germination rate could underestimate the number of the PlcR-PapR QS wild type in the population. It could, on the other hand, potentially explain why

the QS null mutants could be advantageous compared to the QS wild type, as their spores germinated faster than wild type *in vitro* and hence would be able to seize nutrients and grow faster. However, in insect gut environment, whether wild type and its isogenic mutants would differ in their ability to germinate is yet to be investigated. Also the role of the PlcR regulon in germination needs further investigation.

Before investigating the evolutionary ecology of the peptide-based PlcR-PapR QS system in *B. thuringiensis* in a naturally co-evolved host-pathogen system, it is crucial to validate the assumption that the production of signals and secreted proteins regulated by the PlcR regulon is metabolically costly. Previous studies has shown that signal production could be metabolically costly (Keller and Surette, 2006) and QS null mutant could gain a growth benefit by exploiting the signals and secreted proteins produced by the QS wild type and by avoiding the cost of production of signals and secreted proteins (Diggle et al., 2007, Sandoz et al., 2007). Corroborating previous studies, the vegetative growth data and *in vitro* competition assay of the PlcR-PapR QS wild type and QS null mutants also suggested that the QS null mutants have a growth advantage compared to the QS wild type in LB broth. As the signals and proteins regulated by the PlcR regulon are expressed in LB broth but are not required for growth (Lereclus et al., 1996), PlcR-PapR QS null mutants gained a growth benefit via avoiding the cost of production of the signals and proteins regulated by the PlcR regulon. This study validated the assumption that the production of signals and secreted proteins regulated by the PlcR regulon is metabolically costly. It is then possible to further establish whether the PlcR-PapR QS system encodes cooperative traits and to further investigate the evolutionary forces that maintain the PlcR-PapR QS system in a naturally co-evolved host-pathogen system. However, as shown in Fig 2.3, signal null mutant ceased growth earlier than the signal blind mutant and the wild type. Soon after stationary phase, signal null mutants declined in number, suggesting that the signalling peptide, PapR, was required for late exponential growth and subsequent stationary phase. *In vitro*

competition assay in LB also suggested that the PapR peptide was possibly involved in another negative frequency dependent selection for reasons that we do not fully understand at this stage (Fig 4). One possible process is the NprR-NprX QS system in *B. thuringiensis*, which is activated in late stationary phase and regulates the expression of degradative enzymes and a lipopeptide involved in swarming and biofilm formation (Dubois et al., 2012). The peptides, PapR, have been reported to activate NprR (Dubois et al., 2012). Whether the PapR peptide is involved in the NprR-NprX QS system and the role of the PapR peptide in the life cycle of *B. thuringiensis* need further investigation.

The pathogenicity of Cry1Ac toxin in 3rd instar larvae of *P. xylostella* was tested by inoculating the insect larvae with a range of toxin concentrations. As shown in Fig 2.5, with the addition of *B. thuringiensis* 407 Cry$^-$ spores, the biopesticide activity of Cry1Ac was greatly enhanced and the dose required to kill half of the *P. xylostella* larvae of a tested population (LD50) was 0.01048 µg ml^{-1}, while on its own, LD50 of Cry1Ac was 0.0524 µg ml^{-1}. However, as seen in Fig 2.5, at different toxin concentrations, although eventually more than half of the *P. xylostella* larvae died at the sixth day of infection, there was a disparity in the time of death of *P. xylostella* larvae between different toxin concentrations. This could introduce a variation in later *in vivo* bioassay. Therefore, for later *in vivo* bioassay, a toxin dose of 1.31 µg ml^{-1} was suggested, as at this concentration all *P. xylostella* larvae died at the second day of infection (Fig 2.5 B).

In conclusion, this chapter reported experiments on sporulation, germination, *in vitro* vegetative growth and *in vitro* competition assay of *B. thuringiensis* 407 Cry$^-$. The pathogenicity of the Cry1Ac toxin in 3rd instar larvae of *P. xylostella* has also been investigated. For later experiments, a toxin dose of 1.31 µg ml^{-1} was used as a standard inoculation dose. After inoculation, following insect death, cadavers were incubating for at least 13 days incubation at 30°C. The PlcR-PapR QS wild type and

its isogenic QS null mutants differed in their ability to germinate on 2% LB agar plates and this should be considered carefully in later experiments. The PlcR-PapR QS null mutants had a growth advantage compared to the QS wild type in LB broth via avoiding the cost of signals and proteins production regulated by the PlcR regulon.

CHAPTER 3 EVOLUTIONARY ECOLOGY OF THE PLCR-PAPR QUORUM SENSING SYSTEM IN *B. THURINGIENSIS*

3.1 Introduction

Once thought to be solitary, an increasing body of work suggests that bacteria routinely engage in multicellular behaviour via a process called quorum sensing (QS), whereby bacteria communicate and monitor population density through the secretion of small, diffusible signal molecules (Miller and Bassler, 2001, Waters and Bassler, 2005). Several theoretical models have been developed to explain the evolution of QS (Diggle et al., 2007a, Hense et al., 2007, Redfield, 2002, West et al., 2006). QS has been demonstrated to be a cooperative trait in Gram-negative bacteria *P. aeruginosa* (Diggle et al., 2007b) as well as in Gram-positive bacteria *S. aureus* (Pollitt et al., 2013). The necessity of export of signal molecules and extracellular proteins in QS is good *prima facie* evidence that these products are cooperative: they benefit local groups, but are costly to produce and can be exploited by selfish individual cells (Frank, 1998, Hamilton, 1964, Keller and Surette, 2006). However, the evolutionary forces that maintain QS in a naturally co-evolved host-pathogen system have rarely been investigated. Experimental validation of the ecological function of QS genes is lacking. The assertion that signalling and density dependent responses in QS are maintained by cooperation is still controversial for some microbiologists (Hense et al., 2007, Redfield, 2002). In this thesis, I will explore the evolutionary ecology of the peptide based PlcR-PapR QS system in *B. thuringiensis* using a natural host of the bacteria, the diamondback moth *P. xylostella*. I will test social evolution theory of QS in diamondback moth larvae by investigating the standard predictions of cheat invasion, density and frequency dependence.

To understand the evolution of the peptide based PlcR-PapR QS system in *B. thuringiensis*, it is important to establish first whether the PlcR-PapR QS system is a cooperative trait. In an attempt to answer the question, I design an *in vivo* competition assay, in which the isogenic signal blind and signal null mutants are mixed with the PlcR-PapR QS wild type under different densities and frequencies and investigate how the frequency and density of mutants affects total production of infectious spores in cadavers. The PlcR-PapR QS wild type strain, *B. thuringiensis* 407 Cry⁻, can produce the PapR peptide and PlcR protein, allowing the bacteria to express QS regulated genes (Lereclus et al., 1989). The signal blind mutant (*B. thuringiensis* 407 Cry⁻ *ΔplcR*) is an isogenic strain of QS wild type that carries the *aphA3* gene conferring kanamycin resistance at the *plcR* gene site (Salamitou et al., 2000). The signal blind mutant therefore is unable to produce the PlcR protein and hence PlcR-PapR complex could not be gathered in the cells, devoid of the expression of proteins regulated by the PlcR regulon. The signal null mutant (*B. thuringiensis* 407 Cry⁻ A'Z *ΔpapR*) is also an isogenic strain of QS wild type. Signal null mutants have an insertion of the *aphA3* gene at the *papR* gene site (Slamti and Lereclus, 2002). Thus the signal null mutant is incapable of producing the PapR peptides and cannot express genes regulated by the PlcR regulon unless supplemented with PapR peptides from the PlcR-PapR QS wild type. The signalling peptides and extracellular proteins regulated by the PlcR regulon, representing "public goods" or cooperative traits, are costly to produce but can potentially benefit the local group surrounding the producers (Frank, 1998, Keller and Surette, 2006). The logic in the *in vivo* competition assay is that an increased concentration of extracellular proteins regulated by the PlcR regulon would enable the pathogenic bacteria to exploit their host more efficiently. I hypothesize that the productivity of *B. thuringiensis* 407 Cry⁻ in host increases with the concentration of QS wild type that have functional *plcR* and *papR* genes. However, as social evolution theory predicted, cooperative behaviours are subject to invasion of selfish individuals who do not cooperate (Diggle et al., 2007a, Hamilton, 1964, West et al., 2006). In mixed infections, population of QS wild type can be exploited by QS mutants who do not invest in public good production. The fitness benefits of cheating would be greater when cheats are rare as there would be more public goods for the cheats to exploit

(Ross-Gillespie et al., 2007). And the same logic applies to density dependence. I hypothesize a negative frequency dependent relationship between the initial frequency of QS mutants and the relative fitness of QS mutants, which measure the grow rate of QS mutants in relationship to that of QS wild type (Ross-Gillespie et al., 2007).

The hypothesis that spatial heterogeneity or structure in host limits the invasion of the QS mutants is also tested. Spatial heterogeneity can be seen as spatial pattern formed by the segregation of bacteria in space. Studies have suggested that cells from the same lineage form patches readily in space under a wild range of condition (Nadell et al., 2010). The importance of such spatial heterogeneity in the evolution of cooperation within and between species has been revealed in several theoretical models (Doebeli and Knowlton, 1998, Foster and Wenseleers, 2006, Yamamura et al., 2004). Experiments on the growth of antibiotic-producing *Streptomyces* species on surface media showed a elevated antibiotic production in spatial clustered cells, suggesting the role of spatial structure in maintaining the cooperative behaviour (Wiener, 2000). Intermediate level of spatial structure also prevented the invasion of non cooperative cheats into the biofilm producing *Pseudomonas fluorescens* (Brockhurst et al., 2007). Despite the importance of spatial structure, many empirical studies of the evolution of quorum sensing in microorganisms have mainly focused on the power of relatedness, assuming a well mixed interacting population in shaken liquid media (Diggle et al., 2007b, Griffin et al., 2004, Pollitt et al., 2013). However, the assumption of a well-mixed homogeneous population is probably ecologically unrealistic for *Bt* (Raymond et al 2012). The present chapter will provide the first empirical support for the effect of spatial heterogeneity in the evolution of QS. The study is conducted via disrupting spatial structure at early stage of infection or via examining fitness of QS mutant in a control environment with reduced level of spatial structure. In shaken conditions, spatial structure is interrupted and the QS mutants are able to interact with QS wild type freely, while in unshaken condition, QS wild type is able to segregate in space. Segregation of QS

wild type can potentially prevent QS mutants from utilizing the public goods produced by the wild type. In addition, the role of biofilm in the studied QS system is also investigated. Biofilms are formed from densely packed microbial cells that grow on biological or non-biological surfaces and surround themselves with secreted polymers. Biofilms are ubiquitous in nature and many bacterial species form biofilms (Hall-Stoodley et al., 2004). Biofilm formation has been shown to reduce virulence of *Staphylococcus* species (Xu et al., 2006) while increasing antibiotic resistance of *P. aeruginosa* (Popat et al., 2012). Previous work with *B. cereus* (Hsueh et al., 2006) reported an elevated biofilm production in the PlcR-PapR QS signal blind mutant of *B. cereus*. The role of biofilm on the evolution of QS is open to question and there are contrasting reports on the effects of biofilm on virulence (Kong et al., 2006). I will examine the effect of biofilm on fitness of QS mutants via disrupting biofilm formation using solid-glass beads under shaken condition.

Finally, the hypothesis that there is a direct relation between the concentration of the signalling peptides and the total growth of bacteria is explored by measuring the productivity of signal null mutant in host when supplemented with a synthetic peptide, PapRI. The strain, *B. thuringiensis* 407, belonging to group I pherotype, produced a signalling peptide, PapRI, with the sequence, ADLPFE (Bouillaut et al., 2008). Signal null mutants have functional *plcR* gene and are able to engage in public good production when supplemented with PapRI (Bouillaut et al., 2008, Slamti and Lereclus, 2002). The hypothesis is that public goods production increases as the concentration of PapRI increases, and that these public goods have benefits for bacterial replication *in vivo*. If the genes regulated by the PlcR regulon were cooperative as tested in the *in vivo* competition assay, the productivity of signal null mutant should increase the availability of public goods, thus the productivity of signal null mutant is hypothesized to increase with the concentration of the PapRI peptides. The peptides used in the experiment were synthesized using the published sequence ADLPFE. The quality of the synthetic peptides was

assured and checked by high-performance liquid chromatography (HLPC) and mass spectrometry (MS) by the manufacturer.

All in all, in this chapter, a range of experiments will be carried out in an attempt to understand the evolutionary ecology of the peptide based PlcR-PapR quorum sensing system in *B. thuringiensis* using the diamondback moth larvae as an *in vivo* system. I will test standard predictions of social evolution theory in the *in vivo* system. The predictions include cheat invasion of cooperative population, density dependence of productivity in host and frequency dependence of QS mutant fitness. The experiments include *in vivo* competition assay, competition assays in homogenized insects after or before being inoculated with a mixed culture of QS strains as well as a signal peptide assay.

3.2 Materials and methods

3.2.1 In vivo competition assay

Bacterial strains, insects and their growth conditions have been described in chapter two. To establish if the signalling peptides and extracellular proteins regulated by the PlcR regulon are cooperative traits, QS wild type were mixed with either signal null or signal blind mutants at various densities and frequencies. Aliquots of spore preparation of QS wild type, signal null and signal blind mutant were taken out from – 20℃ and defrosted. The spore concentrations of the three defrosted aliquots were counted via serial dilution and plating 100 µl of all serial dilutions on 2% LB agar plates as detailed in chapter one. According to the spore concentrations of the defrosted aliquots, twenty-seven inocula were prepared, namely wild type, 90% signal null, 90% signal blind, 50% signal null, 50% signal blind, 10% signal null, 10% signal blind, signal null and signal blind. Each of the above inocula was prepared through diluting appropriate quantities of the defrosted aliquots in 0.85% NaCl solution for final spore densities of 5×10^4 cfu μl^{-1}, 1×10^4 cfu μl^{-1}, and 2×10^3 cfu μl^{-1}. Wild type, signal null and signal blind were single strain inocula. 90% signal null, 90% signal blind, 50% signal null, 50% signal blind, 10% signal null, 10% signal blind were mixed inocula with 90%, 50% and 10% signal null and signal blind mutants respectively. All strains lack the ability to make the crystal toxin for infecting *P. xylostella*. Therefore the crystal toxin Cry1Ac purified from an *E. coli* JM109 strain that carries the *Cry1Ac* gene on the pGEM1Ac plasmid was added to all the inocula at a final concentration of 1.25 µg ml^{-1}. The toxin Cry1Ac and the *E. coli* JM109 strain were kindly provided by Dr Neil Crickmore, University of Sussex, United Kingdom. To determine the exact starting frequency of mutants in the inocula, all inocula were serial diluted and 100 µl of the serial dilutions were plated out on 2% LB agar plates containing 4% v/v egg yolk (Oxoid) and 2% LB agar plates containing 200 µg ml^{-1} kanamycin. The plates were incubated overnight (16h) at 26℃. At the suitable dilution (with 30 – 200 colonies per plate), 6 egg yolk LB agar

plates and 6 kanamycin LB agar plates were counted to estimate the spore density and initial frequency of mutant in the inocula.

To infect the larvae, sloppy semi-synthetic diet was prepared as described in chapter 2 with 0.4% w/v Vanderzant vitamin mixture for insects (Sigma) and 0.36% w/v L-Ascorbic acid (Sigma). Sixty µl of the sloppy diet were pipetted into the wells of 96-well plates and allowed to dry in a laminar flow cabinet for 10 min. After the sloppy diet had become dry and solidified, 4 µl of the inocula were pipetted into each well of the sloppy diet plate and allowed to dry in the laminar flow cabinet for 10 min. One 96-well plate was used for one inoculum. After that, 3rd instar larvae of *P. xylostella* were introduced to the sloppy diet plates, with one larva per well. Sterile saline (0.85% w/v NaCl solution) with the same concentration of toxin was used as a negative control. The plates were then covered with dry tissue and sealed tightly to minimize the movement of larvae from one well to another. After that, all the 96-well plates were incubated at 23°C. Forty-eight hours post infection, *P. xylostella* cadavers were picked up using sterile toothpicks and put into collection microtubes (Qiagen) containing 10 µl sterilized distilled water. The collection microtubes were then incubated for 2 weeks at 30°C to make sure that the bacteria within the cadavers were fully sporulated. After that 490 µl of saline (as above) and a 4 mm sterile stainless steel ball were added to each microtube, which were then pasteurized for 20 minutes at 65°C to remove any remaining vegetative cells. The cadavers were then homogenized using TisseLyser II (Qiagen) at 22.5 Hz for 4 minutes. The homogenized cadavers were serial diluted and 10 µl of the serial dilutions were plated out on 2% LB agar plates containing 4% v/v egg yolk (Oxoid) as well as 2% LB agar plates containing 200 µg ml^{-1} kanamycin. All the agar plates were incubated overnight (16h) at 26℃. The final frequency of mutants could be calculated via dividing the number of mutants by the total number of mutant and wild type in the homogenized cadavers. A total of 698 third instar *P. xylostella* larvae were infected with the above-stated single strain and mixed strain inocula.

The experiment was repeated once with inocula that contained 90% signal null, 90% signal blind, 50% signal null, 50% signal blind, 10% signal null and 10% signal blind mutant at the spore density of 5×10^4 cfu μl^{-1}. A total of 332 third instar *P. xylostella* larvae were inoculated. The experiment was also replicated a second time with inocula that contained 1% signal null and 1% signal blind mutant at the spore density of 5×10^4 cfu μl^{-1}. A total of 96 third instar *P. xylostella* larvae were infected.

3.2.2 Competition assay in homogenized cadavers

To examine whether spatial heterogeneity in the insect cadavers could be one of the factors that restricted QS mutants from invading the QS wild type population, 3rd instar *P. xylostella* larvae were inoculated with mixed inocula that contained 17% signal null mutant or 14% signal blind mutant with 1.25 μg ml^{-1} Cry1Ac toxin at the spore density of 5×10^4 cfu μl^{-1}. Sterile 0.85% NaCl solution with the same concentration of toxin was used as a negative control. The insect larvae were infected using the aformentioned sloppy diet plate method. After feeding on the diet for 48 h, *P. xylostella* cadavers were picked up using sterile toothpicks and put into collection microtubes (Qiagen) containing 10 μl sterilized distilled water. The collection microtubes were then incubated for 48 h at 30°C, after which 490 μl of 0.85% NaCl solution and a 4 mm sterile stainless steel ball were added to each microtube. The collection microtubes were then pasteurized for 20 minutes at 65°C to eliminate vegetative cells. The cadavers were then homogenized using TisseLyser II (Qiagen) at 22.5 Hz for 4 minutes. The homogenized cadavers were then serial diluted. The frequencies of the QS mutants and the spore densities of the bacterial population in each homogenized cadavers were counted by pipetting 10 μl of the serial dilutions on 2% LB agar plates containing 4% v/v egg yolk (Oxoid) as well as 2% LB agar plates with kanamycin at a final concentration of 200 μg ml^{-1}. All the plates were incubated overnight (16 h) at 26℃. The homogenized cadavers were incubated again for 7 days at 30°C. After 7 days incubation, the homogenized cadavers were pasteurized for 20 minutes at 65°C. The frequencies of the QS mutants and the spore densities of the bacterial population in each homogenized

cadavers were estimated by pipetting 10 µl of the serial dilutions on egg yolk LB agar plates and kanamycin LB agar plates. All the plates were incubated overnight (16 h) at 26℃. Fifty-six 3rd instar *P. xylostella* larvae were infected with inoculum that contained 14% signal blind mutant while 46 insect larvae were inoculated with 17% signal null mutant.

3.2.3 Competition assay in homogenized insect larvae

To investigate whether spatial heterogeneity in insect larvae could be one of the factors that limit the invasion of mutants, I conducted an experiment in which spatial heterogeneity was minimized through homogenizing the insect larvae and disrupting the biofilm formation of bacteria with 5 mm solid-glass beads (Sigma) placed in the homogenized insects shaking at a frequency of 200 rpm. A factorial experiment was conducted in which a spatial structure treatment (shaking and non-shaking) was crossed with a competition treatment (signal null or signal blind mixtures). In the shaking treatment each well of the 24-well plates (Corning® Costar® cell culture plates, Sigma) contained a sterile 5 mm solid-glass bead (Sigma) to provide additional disturbance to biofilm formation and the 24-well plates were cultured on a shaker at 200 rpm, for 24 h at 37℃, while plates in the non-shaking treatment were cultured without either glass beads or shaking in the same incubator. QS wild type was competed with 10% signal null and 11% signal blind mutants respectively. In each case insect homogenates were inoculated with 10 µl of standard spore stocks containing 5×10^4 cfu μl^{-1}. The initial frequencies of QS mutants in the inocula were confirmed by serial dilution and plating on 2% LB egg yolk agar plates and 2% LB kanamycin agar plates as mentioned before. For each treatment, there were 16 insect homogenates in one 24-well plate and the rest of the wells of the 24-well plate were filled with 0.85% NaCl. After 24h incubation at 37℃, the homogenates were pipetted out to collection microtubes (Qiagen). The homogenates were serial diluted and 10 µl of each dilution was plated out on 2% LB plates containing 4% v/v egg yolk (Oxoid) as well as 2% LB plates with kanamycin at a final concentration of

200 μg ml^{-1}. All the plates were incubated overnight (16 h) at 26℃. The final frequencies of QS mutants were measured as mentioned above.

Biofilm structure was measured by staining the wells with 0.5% crystal violet solution. After the homogenates in the 24-well plates were pipetted out, the wells were rinsed with 1 ml phosphate buffered saline (PBS) once. After that, the wells were stained with 1 ml 0.5% crystal violet solution for 20 minutes at room temperature. The wells were then washed with 1 ml PBS three times. The dye was solubilized with 20% acetone 80% ethanol, and absorbances measured using SpectraMax® Plus384 Absorbance Microplate Reader (Molecular Devices, UK) at 595 nm. In the experiment, background staining was corrected by subtracting the absorbances of the wells incubated with 0.85% NaCl.

3.2.4 Signalling peptide assay

To study whether the cost of responding to peptide signals increase with the concentration of the signalling peptide, 5 inocula were prepared with standard spore stocks containing 5×10^4 cfu μl^{-1} signal null mutant and a synthetic heptapeptide PapRI (ADLPFEF) (Cambridge Peptides) at concentrations of 60 μM, 20 μM, 6.7 μM, 2.2 μM and 0.74 μM. The synthetic heptapeptide was an inoculum prepared with 5×10^4 cfu μl^{-1} signal null mutant but without the heptapeptide PapRI was used as the control. Two insect larvae were homogenized with 1 ml sterile distilled water using sterile collection microtubes (Qiagen) and the weight of the insect larvae was noted. The homogenized insect larvae were pipetted into the wells of 24-well plates. A total of 168 insect homogenates were prepared, resulting in seven 24-well plates. The insect homogenates were then inoculated with 10 μl of the above inocula, 28 insect homogenates for each inoculum. Therefore, in the insect homogenates, the concentrations of the heptapeptide PapRI (ADLPFEF) were 60 nM, 20 nM, 6.7 nM, 2.2 nM and 0.74 nM respectively. The 24-well plates were then incubated at 37℃ for 4 days. After 4 days growth, the insect homogenates were pipetted into sterile

the collection microtubes (Qiagen) and pasteurized at 65℃ for 20 min. The productivity of signal null mutant in the insect homogenates were confirmed by serial dilution and plating on 2% LB agar plates.

3.2.5 Data analysis

Data analysis was carried out in R v 2.15.2 (http://www.r-project.org/) using generalized linear models. For *in vivo* competition assay data, the final spore densities of signal strain infections were transformed with the logarithm to the base 10 and were grouped into intervals of 0.5. To accommodate the zeros, the first interval started at –0.25 and the final interval ended at 5.25. Successful infections were defined as infections resulting in more than 5×10^2 spores per cadaver. For infection success data, the proportion of successful infections were analysed with a generalized linear model with binomial errors. For all assay, the relative fitness of QS mutants is calculated by $v = x_2(1 - x_1)/x_1(1 - x_2)$, where x_1 is the initial frequency of mutants in the population and x_2 is the final frequency of the mutants (Ross-Gillespie et al., 2007). The relative fitness of mutant is the estimated growth rate of mutant in relation to that of the wild type. The relative fitness of mutant was transformed with the logarithm to base 10. Therefore, a positive relative fitness of mutant indicated that QS mutants grew faster than the QS wild type, while a negative fitness of mutant marked a slower growth rate than the wild type. All model assumptions were checked with graphical analyses of error distribution and normality.

3.3 Results

3.3.1 In vivo competition assay

To understand the evolution and ecology of the PlcR-PapR QS system in diamondback moth larvae, I first of all investigated the productivity of single infection of QS will type and QS mutants in host. As shown in Fig 3.1 A, infections with QS wild type (circles, solid line) were significantly more productive than infections with QS signal blind (triangles, dotted line) and signal null mutants (squares, dashed line) ($F_{2,228}$ = 5.08, p < 0.0069, n = 231). The productivity of QS wild type and QS mutants in cadaver increased with the initial spore density in the inoculum ($F_{1,227}$ = 4.93, p < 0.027, n = 231) (Fig 3.1 A). However, there was a strong bimodal distribution of final spore density in cadaver for all three single infections after 2 weeks incubation at 30℃ (Fig 3.1 B, C & D). Productivity data were clustered near zero counts and also around 5×10^5 cfu per cadaver (spore density in cadaver of 3 log10 cfu μl^{-1}).

Near zero counts, larvae died as a result of Cry toxin on gut paralysis, but the bacteria have not colonized the cadavers successfully. Productivity data suggested the imporant role of QS regulated virulence factors in establishing successful infection. To investigate whether infection success was dependent on QS regulated virulence factors, I examined the relationship between infection success and initial frequency of QS mutants as well as the relationship between infection success and initial wild type density (Fig 3.2). For QS signal blind mutant, the proportion of successful infections increased with inoculum density (solid lines, triangles, 5×10^4 cfu μl^{-1} ; dashed line, circles, 1×10^4 cfu μl^{-1}; dotted line, diamonds 2×10^3 cfu μl^{-1}) ($F_{1,12}$ = 16.19, p < 0.001, n = 15) and decreased with the initial frequency of signal blind mutants ($F_{1,13}$ = 24.14, p < 0.001, n = 15) (Fig 3.2 A). Similarly, signal null mutants established more successful infections with higher inoculum density (3 stars, triangles, 5×10^4 cfu μl^{-1} ; 2 stars, circles, 1×10^4 cfu μl^{-1}; one star, diamonds 2×

10^3 cfu μl^{-1}) ($F_{1,12}$ = 24.69, p < 0.001, n = 15) (Fig 3.2 B). However, for signal null mutants, there was a sharp decline in infection success when the initial frequency of signal null mutant was bigger than zero. In order to account for this phenomenon, I grouped the initial frequency of signal null mutant into 2 categories, which were zero and greater than zero. As shown in Fig 3.2 B, there was a stepwise decline in infection success when signal null mutants increased in frequency. When the frequency of signal null mutants were greater than zero, the infection success decline significantly ($F_{1,13}$ = 19.61, p < 0.001) (Fig 3.2 B). Alternatively, if the virulence factors reguated by QS were important for establishing a successful infection, the infection success should increase with initial wild type density. As shown in Fig 3.2 C, the proportion of successful infections increased significantly with increasing initial wild type density in the inoculum (Fig 3.2 C, $F_{1,25}$ = 7.64, p = 0.11, n = 27), suggesting that the QS regulated virulence factors were required for establishing a successful infection.

To investigate whether QS mutant can out compete QS wild type in hosts, I calculated the relative fitness of QS mutants using the initial frequency of QS mutants in inocula and the final frequency of QS mutants in host. Relative fitness of QS mutants measures the growth rate of mutants in relationship to that of the wild type. As the relative fitness of mutant was transformed with the logarithm to base 10, a positive relative fitness of mutant indicated that QS mutants grew faster than the QS wild type, while a negative fitness of mutant marked a slower growth rate than the wild type. As shown in Fig 3.3 A, relative fitness of signal null mutant increased significantly with initial wild type density (solid lines, $F_{1,136}$ = 8.49, p < 0.004, n = 138). However, initial wild type density did not impact on the relative fitness of signal blind mutant ($F_{1,134}$ = 0.093, p = 0.76, n = 136) (Fig 3.3 B). Increased initial wild type density accelerated the growth of signal null mutants in cadavers but not the growth of signal blind mutants. The experiment was replicated once at 5×10^4 cfu μl^{-1} initial spore density, relative fitness of signal null mutant (solid line, $F_{1,151}$ = 65.09, p < 0.001, n = 153) and signal blind mutant (dashed line, $F_{1,141}$ =

35.67, $p < 0.001$, n = 143) decreased significantly with increased initial frequency of mutant (Fig 3.4). However, the overall negative relative fitness of QS mutants suggested that the QS mutants did not grow faster than the QS wild type. The only exception is at 7.5% of signal null mutants with 5×10^4 cfu μl^{-1} total spores, where the signal null mutant had a faster growth rate in relationship to the QS wild type. Since I observed a positive relative fitness of signal null mutant with 5×10^4 cfu μl^{-1} total spores at 7.5% initial frequency, suggesting a potential elevated growth rate of QS mutant at high dose and at initial frequencies below 10%. The expriment was replicated again at 5×10^4 cfu μl^{-1} total spores with 1.5% signal blind mutant and 2.2% signal null mutant. However, the relative fitness of QS mutants did not increase further with a lower frequency of QS mutant. Instead, the relative fitness of signal null mutant dropped to –0.67±0.081. For signal blind mutant, the relative fitness was –0.62±0.093.

3.3.2 Competition assay in homogenized cadavers

To test whether the spatial heterogeneity in cadavers restricted QS mutants from exploiting QS wild type, I disrupted spatial structure in the early stage of infection to see if this increased the fitness of QS mutant. The relative fitness of QS mutants were correlated with a simple logarithmic function of the initial frequency of mutant in homogenized insect cadavers. Similar to previous *in vivo* competition assays, the initial frequency of mutant had a significantly negative influence on the relative fitness of mutant ($F_{1,101} = 34.48$, $p < 0.001$, n = 103). The relative fitness of QS mutants became positive and increased rapidly when the initial frequency of QS mutant dropped below 6.4%. Mutants had higher relative fitness when they were rare.

3.3.3 Competition assay in homogenized insect larvae

To test whether the spatial heterogeneity in insect larvae restricted QS mutants from exploiting QS wild type, I compared relative fitness of QS mutants in a controlled

environment with reduced spatial structure. QS mutants had a significantly higher the relative fitness in relation to the wild type in shaken insect homogenates ($F_{1,62}$ = 9.85, p < 0.003, n = 64) (Fig 3.6 A). Mutants invaded a wild type population in shaken insect homogenates. Biofilm production, measured as absorbance at 595nm, was significantly higher in unshaken insect homogenates ($F_{1,62}$ = 24.58, p < 0.001, n = 64) (Fig 3.6 B).

3.3.4 Signalling peptide assay

To investigate the whether signalling effort increased with the concentration of PapRI, I measured the productivity of signal null mutants when supplemented with its cognate peptide. The productivity of signal null mutant in homogenized insect larvae did not increase with the concentration of the signalling peptide PapRI. However, at 0.74 μM peptide concentration (indicated by star), the productivity of signal null mutant was significantly lower to that of treated with no peptides (t = –1.819, d.f. = 162, p = 0.0708) (Fig 3.7). Conversely, the productivity of signal null mutant was significantly higher at 20 μM (indicated by diamond) compared to all other treatment (t = 1.735, d.f. = 162, p = 0.0845).

Fig 3.1 Determinants of total bacterial reproduction *in vivo*. (**A**) Spore density in cadaver after 2 weeks incubation at 30℃ was plotted against initial spore density in single infections of QS wild type (circles, solid line), signal blind mutant (triangles, dotted line) and signal null mutant (squares, dashed line). Infections with QS wild type were significantly more productive than infections with QS mutants ($F_{2,228}$ = 5.08, p < 0.0069, n = 231). Data were transformed using logarithm with base 10. **B**, **C**, and **D** show the distribution of spore density in cadaver. Data were grouped into an interval of 0.5. To accommodate the zeros, breaks started at –0.25 and ended at 5.25. There was a strong bimodal distribution of spore density in cadaver for all three single infections.

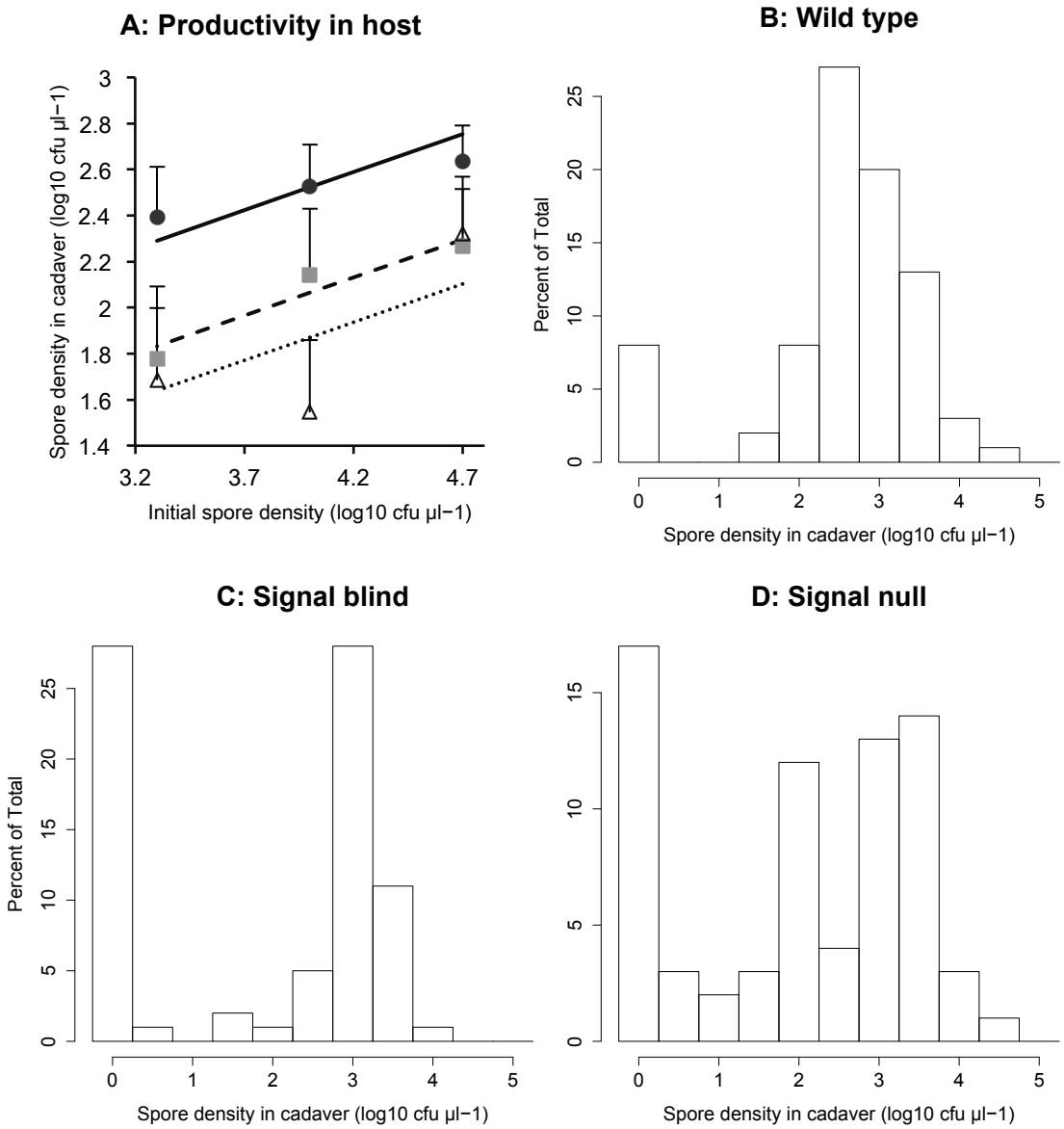


Fig 3.2 Infection success was dependent on proportion of mutants and inoculum concentration. Proportion of successful infections was plotted against initial frequency of mutants. The proportion of successful infections increased with inoculum concentration (solid lines or 3 stars, triangles, 5×10^4 cfu μl^{-1} ; dashed line or 2 stars, circles, 1×10^4 cfu μl^{-1}; dotted line or one star, diamonds, 2×10^3 cfu μl^{-1}) and decreased with the initial frequency of (**A**) signal blind mutants and (**B**) signal null mutants. **C** shows a significantly positive relationship between the successful infections and initial wild type density. Successful infections were defined as those resulting in more than $5x10^2$ spores per cadaver.

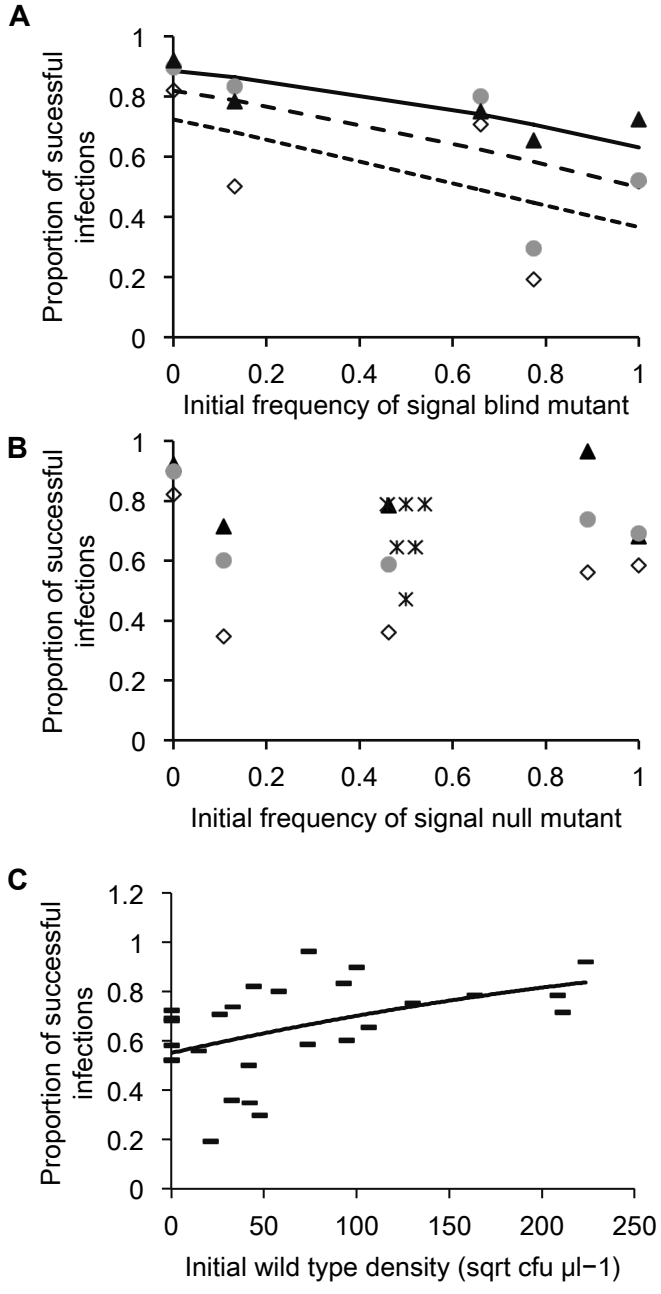


Fig 3.3 The relative fitness of QS mutants increased with abundance of public goods. Signal blind mutant (triangles) and signal null mutant (circles, solid line) were mixed with wild type at various frequencies and densities. Relative fitness of signal null mutants (**A**) and signal blind mutant (**B**) is plotted against initial wild type density in the inoculum. **A** reveals a significantly positive relationship between the relative fitness of signal null mutants and initial wild type density (solid lines, $F_{(1,136)}$ = 8.49, P < 0.004, n = 138). Increased initial wild type density accelerated the growth of signal null mutants in cadavers. Error bars represent one standard error about the mean.

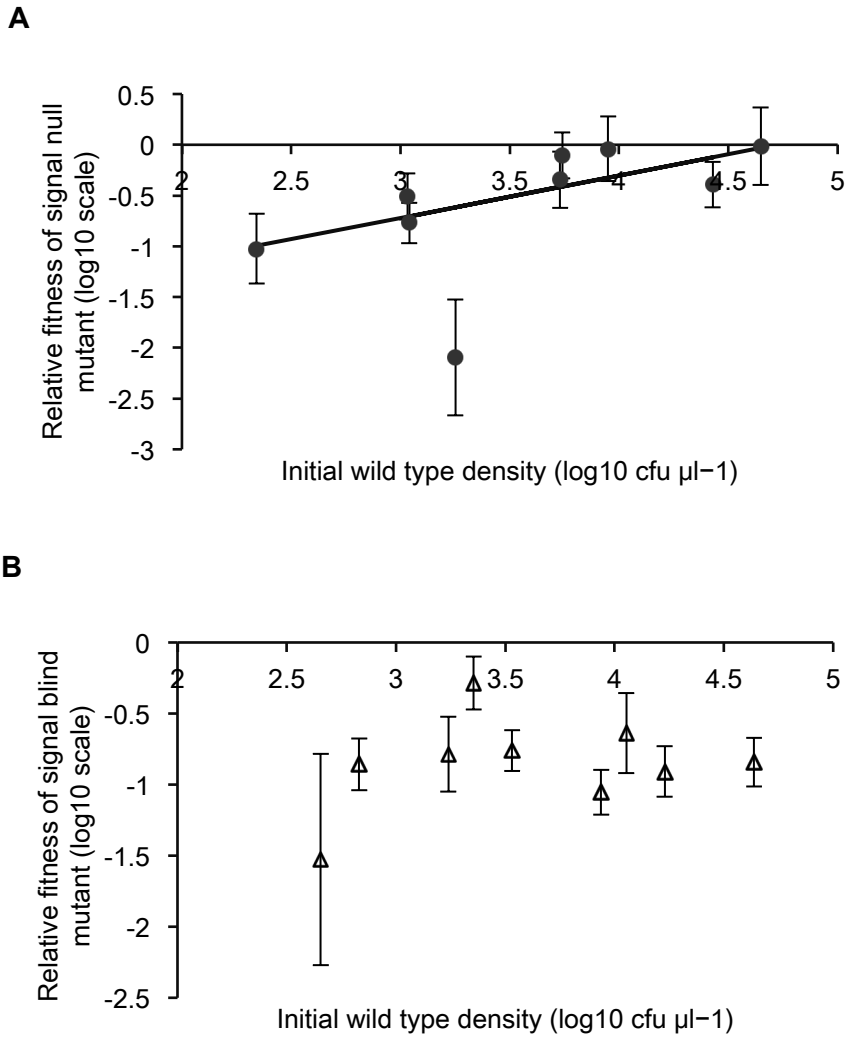


Fig 3.4 The relative fitness of QS mutants decreased with initial frequency of mutants. Signal blind mutant (triangles, dashed line) and signal null mutant (circles, solid line) were mixed with wild type at various frequencies with 5×10^4 cfu μl^{-1} total spores in the inocula. Relative fitness of QS mutant is plotted against initial frequency of mutant in the inoculum. Relative fitness of QS mutant decreased significantly with increased initial frequency of mutant. QS mutants had higher relative fitness when they were rare at 5×10^4 cfu μl^{-1} initial spore density. Error bars represent one standard error about the mean.

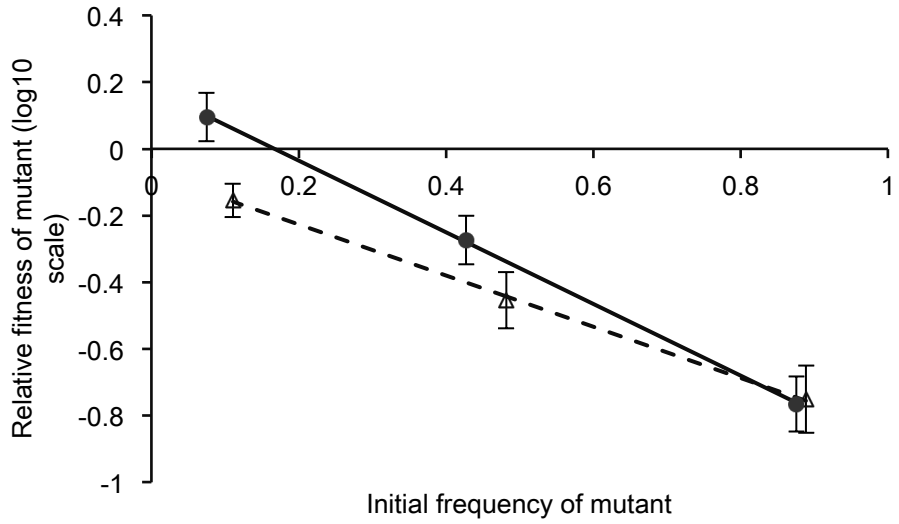


Fig 3.5 Rapid upsurge in relative fitness of mutant when the initial frequency of QS mutant dropped below 6.4%. The relative fitness of the signal null mutant (circles) and signal blind mutant (triangles) after 7 days growth in homogenized insect cadavers at 30℃ was plotted against initial frequency of the QS mutants. The relative fitness of QS mutants were correlated with a simple logarithmic function of the initial frequency of mutant (solid line). The relative fitness of QS mutant decreased significantly as the initial frequency of mutant increased ($F_{1,101}$ = 34.48, p < 0.001, n = 103).

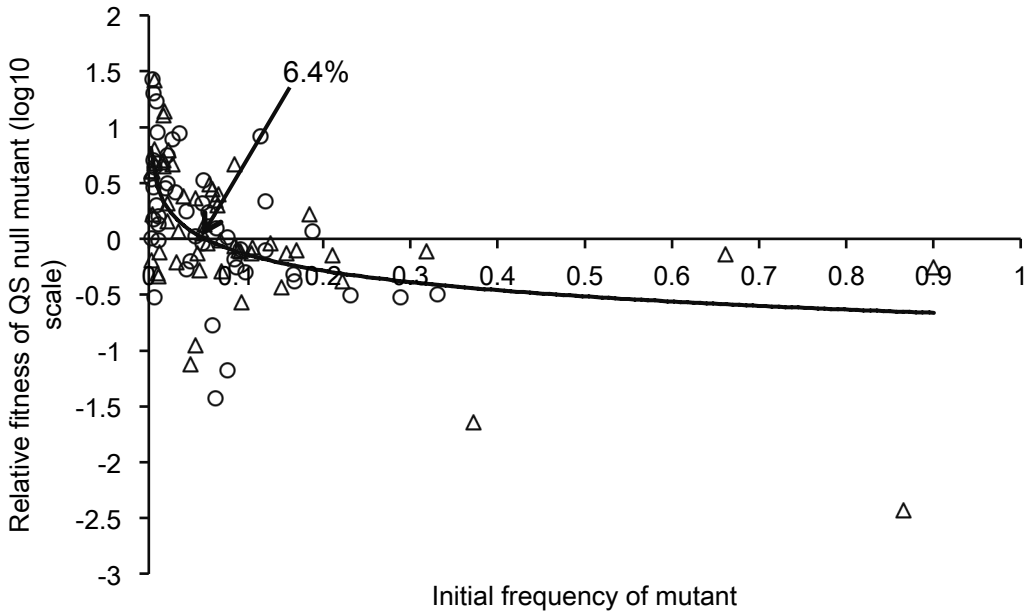


Fig 3.6 Spatial heterogeneity limited QS mutants from exploiting QS wild type. Insect homogenates were inoculated with either 10% signal null or 11% signal blind mutants and incubated at 37℃ for 24 hours under either shaken condition (grey boxes) at 200 rpm or unshaken condition (black boxes). Relative fitness of QS mutants and biofilm production (measured as absorbance at 595nm) were plotted under shake and unshaken conditions. (**A**) Both mutants had significantly higher relative fitness in shaken insect homogenates than that in unshaken insect homogenates ($F_{1,62}$ = 9.85, p < 0.003, n = 64). Mutants can only invade a wild type population in shaken insect homogenates. (**B**) Absorbance of unshaken insect homogenates was significantly higher than that in shaken insect homogenates ($F_{1,62}$ = 24.58, p < 0.001, n = 64). Error bars represent one standard error about the mean.

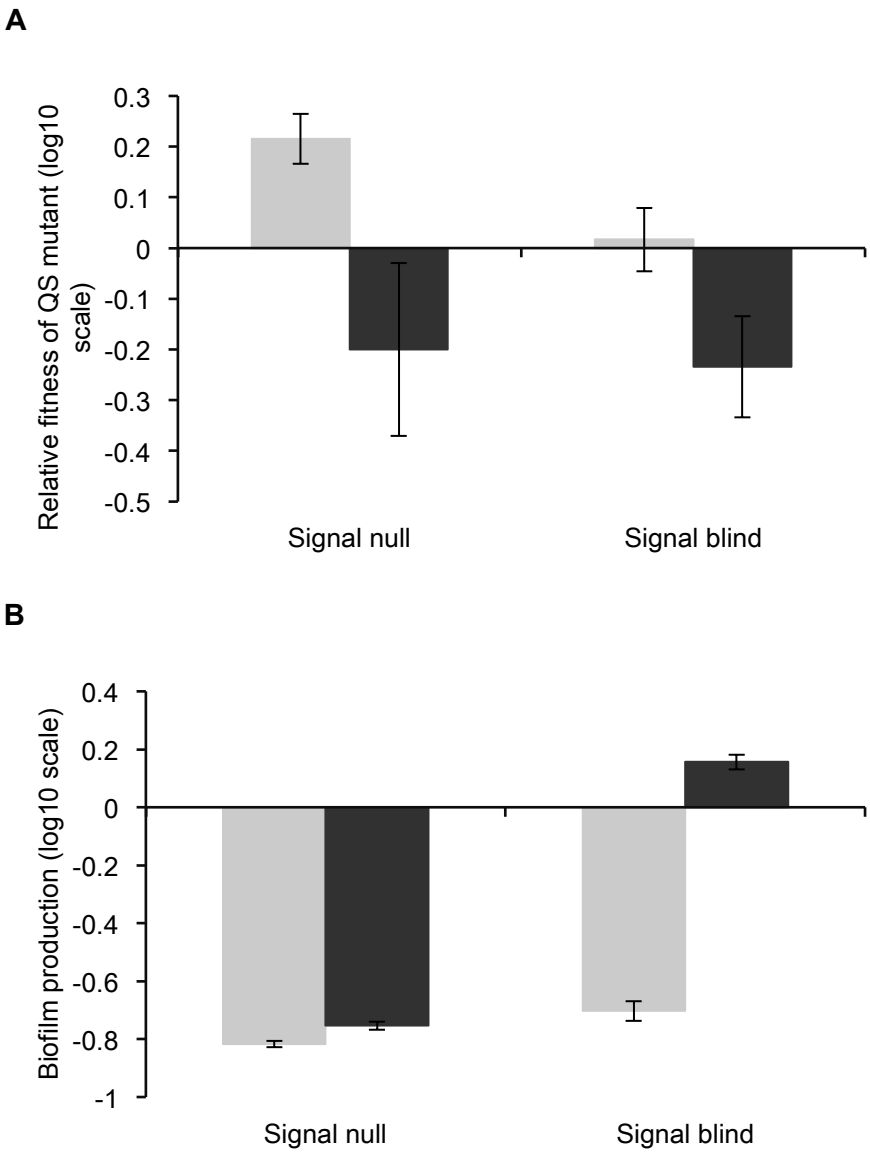
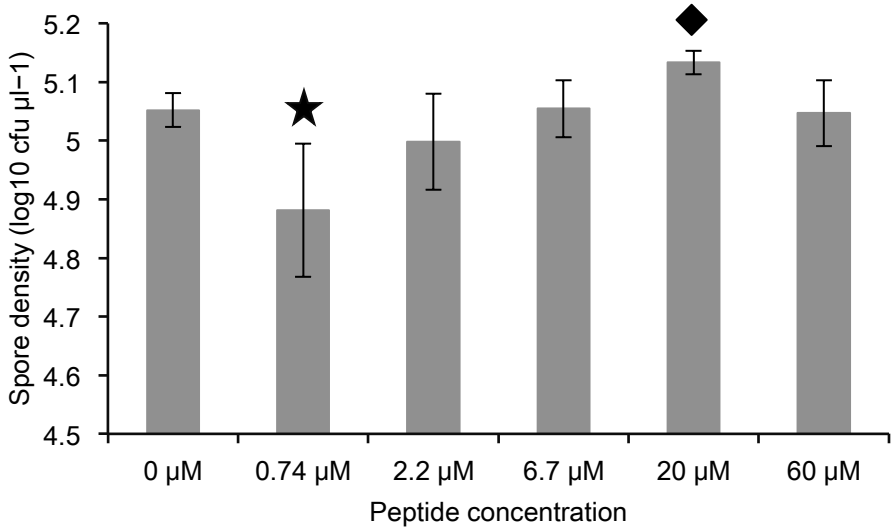


Fig 3.7 Productivity of signal null mutant was higher when supplemented with 20 μM signalling peptides. Spore density of signal null mutant after 4 days growth at 30℃ in insect homogenates was plotted under various peptide concentrations. Addition of signalling peptide did not have a major improvement in the productivity of signal null mutant in homogenized insect larvae. At 0.74 μM peptide concentration (indicated by star), the productivity of signal null mutant was significantly lower compared to the control treatment without peptides (t = –1.819, d.f. = 162, p = 0.0708). At 20 μM peptide concentration (indicated by diamond), the productivity of signal null mutant was significantly higher (t = 1.735, d.f. = 162, p = 0.0845). Error bars represent one standard error about the mean. This experiment was repeated twice.



3.4 Discussion

The PlcR regulon regulates the production of various extracellular proteins, many of which are involved in virulence (Gohar et al., 2008). Social evolution theory predicts that the extracellular proteins regulated by QS, while providing a benefit to the local group, can be exploited by selfish individuals who do not engage in public goods production (Diggle et al., 2007a, West et al., 2006). When there are more public goods for selfish individuals to exploit, the fitness of QS mutants should increase (Ross-Gillespie et al., 2009). On the other hand, the fitness of QS mutants should decrease with initial frequency of QS mutants in the population as public goods deplete faster with more QS mutants (Ross-Gillespie et al., 2007). I investigated the productivity and fitness of QS strains in diamondback moth larvae to investigate above predictions. Corroborating previous work with QS cheats of *P. aeruginosa* (Diggle et al., 2007b), single infections with QS wild type resulted in significantly higher productivity than those with QS mutants (Fig 3.1 A). QS wild type with functioning *plcR* and *papR* genes had higher group level fitness than its isogenic QS mutants. However, the distribution of productivity in cadavers for single strain infections was strongly bimodal, with data clustering near zero counts and also around 5×10^5 cfu per cadaver (Fig 3.1 B, C & D). At zero counts, the larvae probably have died as a result of gut paralysis caused by the Cry toxin. The bacteria have not colonized the cadavers successfully, confirming an important role of the public goods regulated by the PlcR in establishing successful infections (Salamitou et al., 2000). In support of this idea, analysis with the infection success showed a significantly negative correlation with the initial frequency of QS mutants (Fig 3.2 A & B) and a significantly positive relationship with the initial density of QS wild type (Fig 3.2 C). Studies with *Galleria mellonela* larvae demonstrated a drastic reduction in virulence of the QS mutants in orally infected larvae but not in larvae infected through injecting the bacteria into the hemocoel (Fedhila et al., 2003, Salamitou et al., 2000), indicating the role of the PlcR regulon in pathogenicity in the early stage of oral infection. Proteomics and transcriptomics analyses of the genes regulated by the PlcR regulon suggested the role of PlcR regulated public goods in the development of the bacteria in the larval gut lumen and in breaking down the gut

barrier (Gohar et al., 2008). Proteomic studies of the extracellular proteins secreted by *B. cereus* strains uncovered a reduction of flagellin proteins in signal blind mutant, even though the flagellin gene is not directly regulated by the PlcR regulon (Gohar et al., 2002). In *B. thuringiensis*, flagellin proteins allow the cells to attach to host (Zhang et al., 1995). Studies on the adhesion of *Bacillus* strains to epithelial cells suggested that adhesion could be the first step to virulence in oral infection, which allowed the interaction of PlcR regulated virulence factors with epithelial cell, causing cytotoxicity to epithelial cells (Ramarao and Lereclus, 2006). Although adhesion is not directed regulated by the PlcR regulon and not dependent on motility (Ramarao and Lereclus, 2006), the pleiotropic effect of PlcR regulon on multiple traits could potentially prevent QS mutants from exploiting the products of wild type strains. Pleiotropic effects of a single gene has been reported to partially prevent social cheating in other QS systems such as social amoeba *Dictyostelium discoideum* (Foster et al., 2004) and *V. fischeri* (Visick et al., 2000). Studies on rabbit eye infection of *B. thuringiensis* showed a proliferation of nonmotile QS mutants and reduced virulence, suggesting the pleiotropic effect of PlcR regulon on motility as a potential target for clinical intervention. However, in the studied QS system, mutants defective in adhesion was as motile as PlcR-PapR QS wild type (Ramarao and Lereclus, 2006), suggesting the pleiotropic effect of PlcR regulon is on adhesion/invasion rather than on motility.

In addition to productivity in cadavers and infection success, I measured the relative fitness of QS mutants in all the competition assays. The relative fitness of mutant is the estimated growth rate of mutant in relation to that of the wild type. Analysis of the relative fitness of QS mutants reported a significantly positive density dependent relationship between the initial density of QS wild type and signal null mutants (Fig 3.3 A). As hypothesized, the relative fitness of signal null mutant increased with the abundance of public goods. The relative fitness of QS mutants also decreased significantly increased initial frequency of mutants (Fig 3.4). In other words, QS mutants grew faster when they were rare and when there were more public goods

for them to exploit, corroborating previous work on QS cheats of *P. aeruginosa* (Diggle et al., 2007b). However, in the studied PlcR-PapR QS system, the QS mutants did not behave as cheats in host and could not out compete QS wild type *in vivo*, contrasting the work with *P. aeruginosa*, where cheats could invade a wild type population even when the cheats were at 73% frequency (Diggle et al., 2007b). Also previous work using a burned-mouse model in which infections were injected directly under the skin, QS mutants of *P. aeruginosa* were able to exploit the virulence factors produced by virulent producer (Rumbaugh et al., 1999). QS mutants of the PlcR-PapR QS system in diamondback moth larvae were ineffective cheats. In the reported *in vivo* competition assay, QS mutants could not out compete QS wild type. Signal null mutants could only out grow QS wild type when they were at 7.5% initial frequency. The results brought a puzzling question that why the QS mutants do not invade a wild type population in the host even when they are very rare?

One of the possibilities is that apart from the extracellular proteins, the intracellular proteins regulated by the PlcR regulon put a metabolic constraint on social cheating. Studies on the LasR-LasI QS system in *P. aeruginosa* demonstrated that QS-controlled expression of cellular enzymes suppressed social cheating even when the cellular enzymes were not required to break down resources for growth (Dandekar et al., 2012). However, in Chapter 2, *in vitro* competition assay of QS mutants and QS wild type showed that the signal blind mutants could out compete QS wild type in LB broth media, suggesting that the cellular proteins regulated by the PlcR regulon were not a metabolic constraint on social cheating *in vitro*.

Another possibility is that the PlcR regulon is suppressed in late infection (Dubois et al., 2012), preventing the invasion of QS mutants. Previous work on *P. aeruginosa* has shown that when the QteE-QscR QS system was not required for population growth, the QS-controlled expression of extracellular proteins was tuned down,

preventing the invasion of both the QS wild type and social cheats (Gupta and Schuster, 2013). The signalling peptide data have shown that the productivity of signal null mutant when supplemented with a small amount of peptides (0.74 μM) was significantly reduced compared to the productivity of those without the administration of peptides, suggesting that the peptides can suppress the bacterial growth at low dose. *In vitro* competition assay in chapter 2 revealed the inability of signal null mutant to invade a wild type population in LB broth media, suggesting the signalling peptide, PapR, could be also potentially involved in other regulation circuits of the PlcR-PapR QS system. Down regulation mechanisms for the LuxI/LuxR-type QS system has also been reported in *P. aeruginosa* as well as in *A. tumefaciens* (Pearson et al., 1999, Zhu and Winans, 1999, Zhu and Winans, 2001). Although the role of the PlcR regulon in pathogenicity has been reported to be in the early stage of oral infection (Fedhila et al., 2003, Salamitou et al., 2000) and the PlcR regulon is suppressed at late infection (Dubois et al., 2012), whether the PlcR regulon is down regulated after the bacteria have entered the host hemocoel is subject to further investigation. The anti-bacteria peptides regulated by the PlcR regulon (Gohar et al., 2008) could possibly hinder the commensal bacteria from entering the host hemocoel and inhibit the growth of commensal bacteria in the cadaver. It is more likely that the PlcR regulon is expressed at various stages of the *B. thuringiensis* life cycle.

Apart from the aforementioned hypotheses, the hypothesis that spatial heterogeneity limited the invasion of QS mutants was tested. Previous studies have also indicated that relative fitness of siderophore cheats of *P. aeruginosa* was greatly reduced in a host environment than it is *in vitro* (Griffin et al., 2004, Harrison et al., 2006), suggesting that a nature host environment is different from that *in vitro*. In homogenized cadavers, QS mutants can invade a wild type population when their frequency dropped below 6.4% at various initial spore densities (Fig 3.5), suggesting that cheat invasion in the hemocoel is achievable. The results indicated that if spatial structure was limiting the relative fitness of QS mutants, it is likely to be

in insect midgut rather than in the hemocoel. In homogenized larvae, the biofilm formation was significantly reduced in shaken condition with glass beads (Fig 3.6 B), suggesting that the experimental manipulation was efficient at disrupting the structure of biofilm. When biofilm structures were disrupted, the relative fitness of QS mutants increased significantly (Fig 3.6 A). Previous studies of biofilm and spatial heterogeneity using the computational models have suggested that cheats can invade a wild type population under conditions leading to high mixing while they are at a disadvantage when wild type and cheats are well segregated (Mitri et al., 2011, Nadell et al., 2010). Experiments on the growth of antibiotic-producing *Streptomyces* species and biofilm producing *P. fluorescens* have pointed the importance of spatial structure in maintaining cooperative behaviour and preventing the invasion of social cheats (Brockhurst et al., 2007, Wiener, 2000). Corroborating with these computational models and experimental studies, QS mutants can invade a wild type population in shaken insect homogenates but fail to do so in unshaken insect homogenates (Fig 3.5 A).

CHAPTER 4: SPATIAL HETEROGENEITY IN THE MIDGUT

4.1 Introduction

Spatial heterogeneity is common in nature (Dale, 1999). Organisms rarely distribute either uniformly nor randomly in nature, but instead form sort of spatial pattern (Dale, 1999). In microorganisms, cells readily form patches in space under a wide range of conditions (Nadell et al., 2010). Several models and experiments have reported the role of spatial structure in maintaining cooperative behaviour (Brockhurst et al., 2007, Doebeli and Knowlton, 1998, Foster and Wenseleers, 2006, Wiener, 2000, Yamamura et al., 2004). As suggested in chapter 3, spatial heterogeneity can one of the factors that limit the fitness of PlcR-PapR QS signal null and signal blind mutants *in vivo*. In order for spatial heterogeneity to impact on the relative fitness of signal null and signal blind mutant, it has to occur when the PlcR regulon is activated. Previous studies have reported the pathogenicity of the PlcR regulon in early stage of oral infection (Fedhila et al., 2003, Salamitou et al., 2000), possibly by allowing the cells to germinate in the gut lumen and to cross the gut barrier (Salamitou et al., 2000). The different ability between QS mutants and wild type to adhere to epithelial cells suggests a possibility of forming distinct patchy population by wild type and QS mutants at the attachment site (Ramarao and Lereclus, 2006). It would allow QS wild type to attach and interact with host epithelial cells more efficiently, possibly permitting them to cross the midgut first, while QS mutants are unable to utilize the public goods expressed by the wild type cells (Ramarao and Lereclus, 2006, Salamitou et al., 2000). There is a distinct possibility that the PlcR regulon is activated in the gut lumen while infection is spatially structured.

To investigate this idea, in this chapter, I will first construct plasmids with either green or red fluorescent genes and test the stability of these plasmids. In order to

differentiate cells expressing the green fluorescent gene from cells making the red fluorescent protein, I will also constructs the green or red plasmids to carry a erythromycin resistant gene or a tetracycline resistant gene respectively. I will use the plasmids I have constructed to infect diamondback moth larvae and visually examine the infection pattern in the insect midgut at early stage and later stage of infection. I hypothesize that at early stage of infection, which is in the insect midgut, bacteria populations are spatially separated, allowing the public goods to be shared between cooperative cells while limiting the fitness of QS mutants.

First of all, in order to visualize the infection process, I will take advantage of fluorescent microscopy and construct plasmid carrying either a green or red fluorescent gene. The PlcR-PapR QS strains, *B. thuringiensis* 407 Cry$^-$ (Lereclus et al., 1989), *B. thuringiensis* 407 Cry$^-$ A'Z Δ*papR* (Slamti and Lereclus, 2002) and *B. thuringiensis* 407 Cry$^-$ Δ*plcR* (Salamitou et al., 2000), are transformed with a plasmid that carries either a green fluorescent gene or a red fluorescent gene. The chosen plasmid is pHT315, which is a relatively small and stable plasmid for *B. thuringiensis* (Arantes and Lereclus, 1991). The plasmid pHT315 is 6.5 kilobase pairs long and has a copy number of 15 ± 5 per cell (Arantes and Lereclus, 1991). Having a moderate copy number reduces the burden of carrying the plasmid on the productivity of *B. thuringiensis* and ensures stability in cells. This is important because during the course of bacterial infection, cells in host undergo generations of replication without being selected by antibiotics. The plasmid, which demonstrate stable replication in *B. thuringiensis*, is therefore a desirable shuttle vector for the study (Arantes and Lereclus, 1991). Finally, the plasmid pHT315 originally provides the resistance to erythromycin to Gram positive bacteria and the resistance to ampicillin to *E. coli* (Arantes and Lereclus, 1991). The erythromycin resistant gene in pHT315 is substituted with tetracycline resistant gene from the pHT1618 plasmid (Lereclus and Arantes, 1992) for the red fluorescent gene.

For fluorescent microscopy, the selected fluorescent genes are a variant of the green jellyfish gene *gpf-mut1* and the red reef coral gene *dsred*, the products of which are the GFPmut1 and DsRed proteins respectively. The GFPmut1 protein has excitation and emission peak at 488 nm and 507 nm respectively (Cormack et al., 1996), while the DsRed protein has an excitation maximum at 558 nm and an emission maximum at 583 nm (Matz et al., 1999). The emission maximum of GFPmut1 is clearly separately from that of the DsRed, making the combination of GFPmut1 and DsRed suitable for double labelling studies with negligible cross talk. Either of the two fluorescent genes is inserted into the multiple cloning site of pHT315 with a transcriptional fusion of a constitutive promoter p*aphA3'* from the pGDC783 plasmid (Guerout-Fleury et al., 1995). Previous studies on *B. cereus* carrying the plasmid pHT315- p*aphA3'-gfp* have shown a constitutive expression of the *gfp* gene (Daou et al., 2009). The strain *B. thuringiensis* 407 harbouring the plasmid pHT315 that carries the transcriptional fusion between p*aphA3'* and either of the aforementioned fluorescent genes is likely to preserve the constitutive expression of *gpf-mut1* and *dsred* during infection.

After the above-stated QS strains have been transformed with the plasmid pHT315 containing either *gpf-mut1* or *dsred*, the stability of the genetically engineered plasmids in the QS strains are tested. With the addition of the constitutive promoter p*aphA3'* and the fluorescent genes, it is necessary to confirm that the genetically engineered plasmids maintain the stable property in the QS strains *B. thuringiensis* 407 of the study. To test the stability of the plasmid pHT315 with either *gpf-mut1* or *dsred*, the QS strains harbouring the genetically engineered plasmid are passaged 6 times successively in LB without any antibiotics. The presence of the plasmid in the QS strains in every passage is inspected via streaking colonies of the transformed QS strains onto both LB agar plates and LB agar plates with the appropriate antibiotics.

In addition to plasmid stability, the fitness cost of carrying the plasmids with different fluorescent genes on the productivity of *B. thuringiensis* 407 is evaluated. As for later experiments, *Plutella* larvae are inoculated with green and red *B. thuringiensis* 407 for multichannel visualization, the fitness difference incurred by the two fluorescent genes will confound the results with different growth rate between the green and red *B. thuringiensis*. Studies with *E. coli* expressing a GFP-variant EGFP or DsRed has shown that *E. coli* with DsRed has significantly smaller cell size and population size than those expressing EGFP, suggesting that the fitness cost of DsRed is higher than that of EGFP in *E. coli* (Jakobs et al., 2000). Whether *B. thuringiensis* 407 with *dsred* experience a higher fitness cost than *B. thuringiensis* 407 with *gfp-mut1* in *Plutella* larvae is investigated by competing bacteria with the two genes in homogenized insect larvae. A change of frequency is expected if the strain *B. thuringiensis* 407 bearing either of the fluorescent genes suffered different fitness costs.

Finally, to investigate spatial heterogeneity in the midgut of *Plutella* larvae, 3rd instar larvae are inoculated with green and red *B. thuringiensis* 407 using a droplet feeding method with green food dye. Each larva is fed on a precise volume of food droplet for a controlled period. Droplet feeding method allows precise control of the amount of bacteria to be introduced into the larvae. In addition, each food droplet contains certain amount of harmless and edible green food dye. Once the larvae have ingested the droplet, the food can be seen inside larval gut by naked eyes. Larvae that have eaten the food droplet are clearly different to those that have not eaten the food. Therefore every larvae sampled have been inoculated with desired amount of bacteria. Infected larvae are frozen in O.C.T compound and cut into thin sections at roughly 24 h ~ 36 h and 48 h ~ 50 h post infection for examination under the microscope. The time points are chosen because from previous observation, at about 24 h ~ 36 h post infection, *Plutella* larvae start producing melanin pigment, which is a distinctive feature of bacteria infection (Nappi and Vass, 1993), marking possibly the beginning of the infection process. It is likely that at 24 h ~ 36 h post

infection, *B. thuringiensis* 407 remain in the midgut. At about 48 h ~ 50 h post infection, *Plutella* larvae become completely melanised, suggesting that the bacteria have crossed the gut barriers and entered the hemocoel. The structural changes in bacterial population between the two time points are in the interests of this study. As proposed, spatial heterogeneity limited the invasion of PlcR-PapR QS signal null and signal blind mutants. In order for the effect of spatial heterogeneity to incur, segregation of PlcR-PapR QS wild type is likely to begin at 24 h ~ 36 h and end at 48 h ~ 50 h post infection, possibly allowing the QS wild type to cross the midgut barriers first (Salamitou et al., 2000) and restricting mutant access to the public goods produced by the QS wild type.

4.2 Materials and methods

4.2.1 Construction of the green fluorescent B. thuringiensis strains

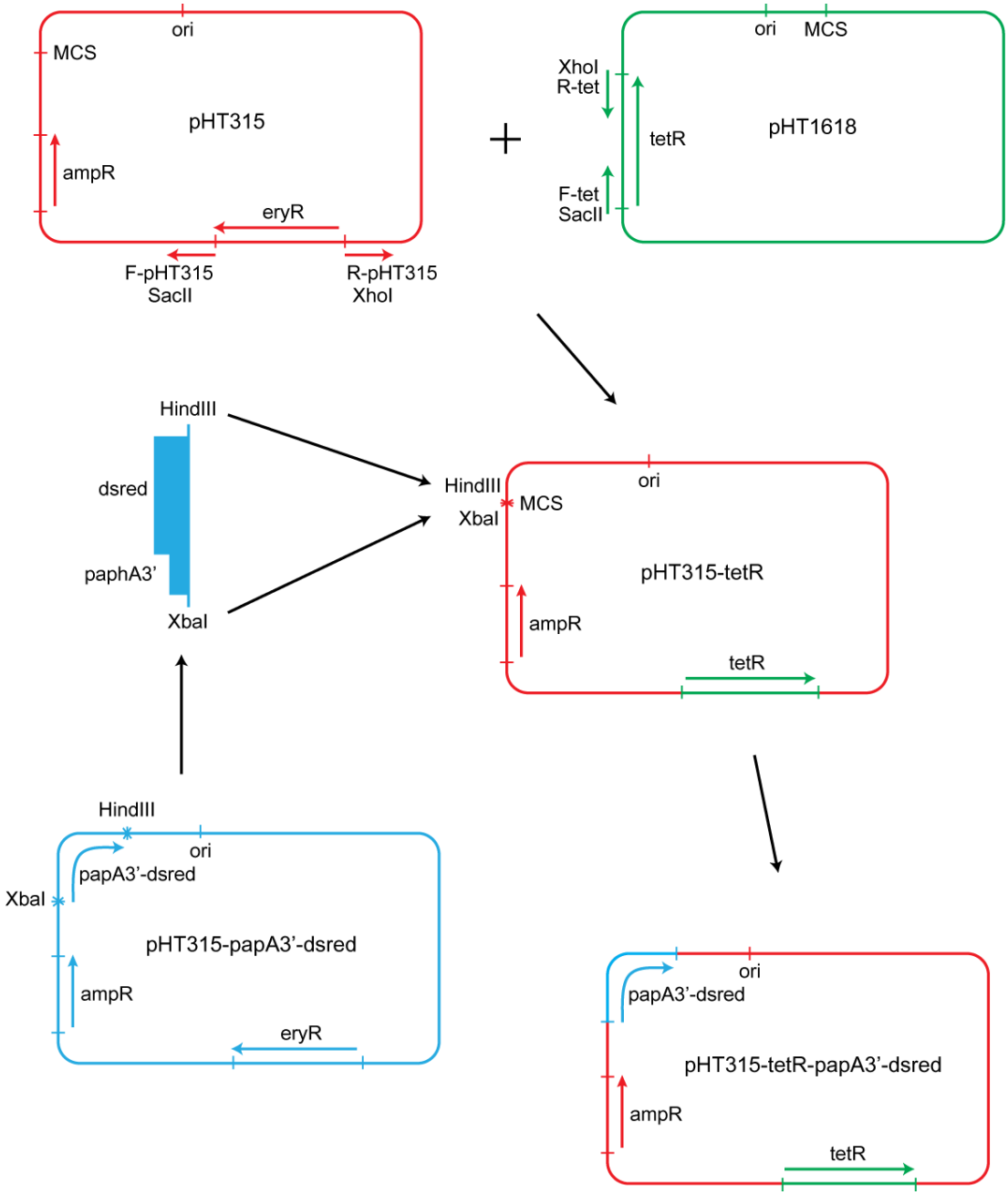
The strain *E. coli* DH5α carrying the plasmid pHT315-p*aphA3'-gfp* (Daou et al., 2009) was cultured in 10 ml 2% LB broth overnight, with vigorous shaking (200 rpm) at 37 ℃. The antibiotic used was ampicillin (100 μg ml^{-1} for *E. coli*). The plasmid pHT315-p*aphA3'-gfp* was then isolated with QIAprep Spin Miniprep Kit (Qiagen) and used to transform the strain *E. coli* ET12567 (Stratagene, La Jolla, CA, USA). *E. coli* DH5α methylates plasmid DNA while *E. coli* ET12567 provides nonmethylated plasmid DNA. Nonmethylated plasmid DNA is essential for efficient transformation of *B. thuringiensis* (Macaluso and Mettus, 1991). The gene p*aphA3'-gfp* was sequenced using primers M13PU (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') and M13PR (5'-AGCGGATAACAATTTCACACAGGA-3'). M13PU and M13PR were primers that bind to the regions outside the multiple cloning side of pHT315, where the p*aphA3'-gfp* gene was inserted. No nucleotide change was detected and the plasmid pHT315-p*aphA3'-gfp* from *E. coli* ET12567 was used to transform the strains *B. thuringiensis* 407 Cry^- (Lereclus et al., 1989), *B. thuringiensis* 407 Cry^- A'Z Δ*papR* (Slamti and Lereclus, 2002) and *B. thuringiensis* 407 Cry^- Δ*plcR* (Salamitou et al., 2000) via electroporation. The antibiotic used for selection was erythromycin (10 μg ml^{-1} for *B. thuringiensis*). Transformants were selected as erythromycin-resistant colonies and the colonies were examined through PCR using primers M13PU and M13PR for the presence of the gene p*aphA3'-gfp*. After verifying the existence of p*aphA3'-gfp*, erythromycin-resistant colonies were then streaked over 2% LB agar plates containing 10 μg ml^{-1} erythromycin for the isolation of single colonies. The strains *B. thuringiensis* 407 Cry^-, *B. thuringiensis* 407 Cry^- A'Z Δ*papR* and *B. thuringiensis* 407 Cry^- Δ*plcR* carrying the plasmid pHT315-p*aphA3'-gfp* was named as wild type GFP, signal null GFP and signal blind GFP respectively. A single colony of wild type GFP, signal null GFP and signal blind GFP was then transferred individually to 5 ml autoclaved 2% LB broth using 10 μl sterile disposable inoculating loops (Greiner Bio-One) and was allowed to grow

overnight at 30℃. After overnight growth, 250 µl of the overnight culture were mixed with 750 µl of 80% glycerol (Sigma) in 1.5 ml cryotubes (Thermo Scientific). The glycerol stocks of wild type GFP, signal null GFP and signal blind GFP were stored at – 80℃.

4.2.2 Construction of the red fluorescent B. thuringiensis strains

In order to differentiate mutant strains from the wild type strain, I reconstructed the plasmid pHT315 to carry the red fluorescent gene *dsred* from reef coral (Matz et al., 1999) and the tetracycline resistant gene *tetR* from the plasmid pHT1618 (Lereclus and Arantes, 1992). The plasmid pHT315 originally provides the resistance to ampicillin to *E. coli* and the resistance to erythromycin to *B. thuringiensis* (Arantes and Lereclus, 1991). I replaced the erythromycin-resistant gene *eryR* with the tetracycline-resistant gene *tetR*. The *tetR* gene was amplified from the plasmid pHT1618 using the primers F-tet (5'-CCGCTCGAGCCATATTGTTGTATAAGTGATG-3') and R-tet (5'-TCCCCGCGGCAATAACCCTGATAAATGCTTC-3') with Phusion® High-Fidelity DNA Polymerase (New England Biolabs). In order to remove the gene *eryR* from pHT315, we amplified pHT315 with Phusion® High-Fidelity DNA Polymerase using primers that extend outwards from the gene *eryR*. The primers used were F-pHT315 (5'-TCCCCGCGGCGTTACTAAAGGGAATGG-3') and R-pHT315 (5'-CCGCTCGAGCTTAATTACAAATTTTTAGC-3'). All the PCR products were purified with QIAquick PCR Purification Kit. The pHT315 PCR fragments and *tetR* PCR fragments were then digested with restriction enzymes *Sac*I (New England Biolabs) and *Xho*I (New England Biolabs). *TetR* were ligated with pHT315 at the site where *eryR* was deleted. The resulting plasmid pHT315-*tetR* was transformed into *E. coli* DH5α through heat-shock transformation (Inoue et al., 1990) and the antibiotics used for selection were ampicillin (100 µg ml^{-1}) and tetracycline (10 µg ml^{-1}). Transformants were selected as tetracycline-resistant colonies and examined via

Fig 4.1 Construction of the plasmid pHT315-*tetR*-p*aphA3'-dsred*. Details are given in the text. Origin of replication and multiple cloning site of each plasmid are abbreviated as ori and MCS in the diagram.



*Sac*II and *Xho*I. The gene *tetR* was sequenced using primers F-tet and R-tet. The sequence of *tetR* was aligned with the original sequences of pHT1618. No nucleotide change was found.

After confirmation of the presence of plasmid pHT315-*tetR* in *E. coli* DH5α, the strain *E. coli* DH5α bearing the plasmid pHT315-p*aphA3'-dsred* (kindly provided by Andrei Papkou, University of Kiel, Germany) and the strain *E. coli* DH5α with the plasmid pHT315-*tetR* were grown in 5 ml 2% LB broth overnight, with vigorous shaking (200 rpm) at 37 ℃. The antibiotic used was ampicillin (100 μg ml^{-1} for *E. coli*). The plasmids pHT315-p*aphA3'-dsred* and pHT315-*tetR* were then isolated with QIAprep Spin Miniprep Kit (Qiagen) and digested with *Xba*I (New England Biolabs) and *Hin*dIII (New England Biolabs). The gene fragments p*aphA3'-dsred* and pHT315-*tetR* were identified by size and purified from gel electrophoresis. The gene fragment p*aphA3'-dsred* was inserted into the multiple cloning site of pHT315-*tetR*. The resulting plasmid pHT315-*tetR*-p*aphA3'-dsred* was transformed into a nonmethylating strain *E. coli* GM2163 by electroporation and the antibiotics used for selection were ampicillin (100 μg ml^{-1}) and tetracycline (10 μg ml^{-1}). Upon successful cloning, the plasmid pHT315-*tetR*-p*aphA3'-dsred* was then isolated from *E. coli* GM2163 and was used to transform the strains *B. thuringiensis* 407 Cry^{-}, *B. thuringiensis* 407 Cry^{-} A'Z Δ*papR* and *B. thuringiensis* 407 Cry^{-} Δ*plcR* via electroporation as previously described (Peng et al., 2009). The antibiotic used for selection was tetracycline (10 μg ml^{-1} for *B. thuringiensis*). Transformants were selected as tetracycline-resistant colonies and the colonies were checked with PCR using primers M13PU and M13PR for the presence of the gene p*aphA3'-dsred*. Upon verification of p*aphA3'-dsred*, tetracycline-resistant colonies were then streaked over 2% LB agar plates containing 10 μg ml^{-1} tetracycline for the isolation of single colonies. The strains *B. thuringiensis* 407 Cry^{-}, *B. thuringiensis* 407 Cry^{-} A'Z Δ*papR* and *B. thuringiensis* 407 Cry^{-} Δ*plcR* carrying the plasmid pHT315-*tetR*-p*aphA3'-dsred* was named as wild type RFP, signal null RFP and signal blind RFP respectively. A single colony of wild type RFP, signal null RFP and signal blind RFP

was picked up individually and was allowed to grow in 5 ml sterile 2% LB broth overnight at 30℃. After overnight growth, 250 µl of the overnight culture were mixed with 750 µl of 80% glycerol (Sigma) in 1.5 ml cryotubes (Thermo Scientific). The glycerol stocks of wild type RFP, signal null RFP and signal blind RFP were stored at – 80℃.

4.2.3 Plasmid stability

The plasmid pHT315-p*aphA3'-gfp* and the plasmid pHT315-*tetR*-p*aphA3'-dsred* was checked for their stability in *B. thuringiensis* 407 Cry⁻ (Lereclus et al., 1989), *B. thuringiensis* 407 Cry⁻ A'Z ΔpapR and *B. thuringiensis* 407 Cry⁻ ΔplcR (Salamitou et al., 2000, Slamti and Lereclus, 2002). Wild type GFP, signal null GFP, signal blind GFP, wild type RFP, signal null RFP and signal blind RFP were streaked from the previously prepared glycerol stocks to antibiotic selective 2% LB agar plates, with 10 µg ml^{-1} erythromycin for the GFP strains and 10 µg ml^{-1} tetracycline for the RFP strains. A single colony of the GFP and RFP strains were transferred individually to 5 ml sterilized 2% LB broth without antibiotics using 10 µl sterile disposable inoculating loops (Greiner Bio-One) and were allowed to grow for 24 h at 30℃. After 24 h growth, 5 µl of the 1st passage culture cells were pipetted into fresh 5 ml sterile 2% LB broth without antibiotics and allowed to grow for 24 h at 30℃. The process was replicated 6 times, giving 6 serial passages for each GFP and RFP strains. All the passages were serial diluted and 100 µl of each serial dilution were plated on 2% LB agar plates, leaving one 2% LB agar plate for each 100 µl serial dilution. The plates were incubated overnight (16 h) at 26℃. For plates containing 30 to 200 colonies, the number of colonies on the plate was counted. For each GFP and RFP strains, 100 colonies from the counted plates were streaked using sterile toothpicks onto both 2% LB agar plates and 2% LB agar plates containing 10 µg ml^{-1} erythromycin and tetracycline for the GFP and RFP strains respectively. The streaked plates were incubated overnight at 26℃. The number of streaks that appeared on the 2% LB agar plates and the antibiotic 2% LB agar plates were noted.

4.2.4 Spore preparation for the GFP and RFP strains

For succeeding experiments, spores of the GFP and RFP strains were acquired by culturing cells separately on the *B. cereus* selective agar plates (previously described in chapter 2) for 5 to 7 days at 37℃. The *B. cereus* selective agar plates were prepared with the addition of 10 μg ml^{-1} erythromycin and tetracycline for the GFP and RFP strains respectively. As described in chapter 2, after 5 to 7 days, the cells were harvested with a flame-sterilized spatula and were suspended in 1.5 ml micro-centrifuge tubes with 0.75 ml 0.85% NaCl (Fisher Scientific). The cell suspensions were then centrifuged at 6000 g min^{-1} for 3 minutes and the supernatants were discarded. Following 2 serial resuspensions and centrifugations, the cells were resuspended in 1 ml 0.85% NaCl solution. The cell resuspensions were aliquoted and heat-treated at 65℃ for 20 minutes. All the aliquots were stored at – 20℃. The spore concentrations of the aliquots were measured through serial dilution and spreading 100 μl of serial dilutions on 2% LB agar plates. Within the countable dilution (30 – 200 colonies), 6 plates were counted to estimate the spore concentration in each aliquot. The aliquots that were defrosted for subsequent experiments were placed at 4℃ and were discarded after one month. Fresh aliquot for each GFP and RFP strains was taken out from – 20℃ and same method was used to estimate the spore concentrations in the fresh aliquots.

4.2.5 Competition assay in homogenized insect larvae

To establish whether either the GFP strains or the RFP strains had a growth benefit in 2% LB broth, wild type GFP were mixed with wild type RFP and the mixed cultures were allowed to grow for 24 h at 30℃. To prepare the mixed cultures, an aliquot of wild type GFP and wild type RFP were defrosted. The spore concentrations in the aliquots were counted via serial dilution and plating as stated above. According to the spore concentrations in the aliquots, wild type GFP was mixed with wild type RFP at 53% wild type GFP. The initial frequencies of wild type GFP in the mixed inocula were confirmed by serial dilution and plating on 2% LB agar plates. As the colonies of wild type GFP and wild type RFP appeared green

and red to the naked eye, the frequencies of wild type GFP can be counted on 2% LB agar plates. For the competition assay, two later 3rd instar larvae were homogenized in 1 ml sterile distilled water using TisseLyser II (Qiagen) at 22.5 Hz for 4 minutes. A total of 72 later 3rd instar larvae were homogenized, giving 36 independent pools of insect homogenates. For each treatment 12 insect homogenates broth were infected with 10 µl of the respective mixed inoculum containing 7×10^4 cfu μl^{-1}. Eighteen inoculated insect homogenates, 6 for each treatment, were incubated in one 24-well plate and the rest of the wells of the 24-well plate were filled with 0.85% NaCl. A total of two 24-well plates were prepared and incubated for 24h at 37℃.

4.2.6 In vivo competition, histology and microscopy

In order to follow the infection process, we mixed the wild type strain *B. thuringiensis 407 Cry⁻* harbouring the plasmid pHT315-p*aphA3'-gfp* (WT gfp) and the mutant strain *B. thuringiensis* 407 Cry⁻ Δ*plcR* carrying the plasmid pHT315-*tetR*-p*aphA3'-dsred* (MT rfp) at a 1:1 ratio. We also prepared an inoculum with the wild type strain *B. thuringiensis 407 Cry⁻* containing the plasmid pHT315-*tetR*-p*aphA3'-dsred* (WT gfp) and the mutant strain *B. thuringiensis* 407 Cry⁻ Δ*plcR* with the plasmid pHT315-p*aphA3'-gfp* (MT rfp) at a 1:1 ratio. Both inocula were prepared at a spore concentration of 1 x 10^5 cfu μl^{-1} and a Cry1Ac toxin concentration of 0.0262 µg μl^{-1} using a green mixture as a diluent. The green mixture contained 15.5% v/v green food colour (Dr Oekter, United Kingdom), 82.5% v/v cabbage extract and 20 mM sucrose. The green inocula were then mixed with pre-warmed (50℃) 0.8% w/v agar (Agar Bacteriological, Oxoid) at a 1:1 ratio, after which 1.5 µl of the mixture was quickly pipetted into each well of a 24-well plate. After air-dried at room temperature, only one 3rd instar *P. xylostella* larva was allowed to feed on a green droplet for 12 hours and the larva became distinctly green in the gut area upon ingestion of the droplet. A total of 48 larvae were infected for each inoculum. After 12 h, the larvae were removed and placed onto antibiotic free semi-synthetic diet as previously described.

After feeding on the diet for 24 ~ 36 hours, 24 larvae from each treatment were embedded in O.C.T. compound (VWR, United Kingdom) and frozen using dry ice. The O.C.T mounted larvae were sliced longitudinally (head to posterior end) into 7 µm thick sections using a Leica 2800E cryostat and mounted on SuperFrost Plus slides (Menzel, Germany). The sections were then fixed in 100% cold methanol (4℃) for 30 seconds, followed by three washes with PBS (5 min each). After that, the sections were mounted with VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories, United Kingdom). The sections were examined using a Nikon upright fluorescence microscope (ECLIPSE TE300), equipped with a cooled charge-coupled device (CCD) Hamamatsu Orca camera controlled through SimplePCI software (Hamamatsu, USA). The images were analyzed and processed using HCImageLive image analysis software (Hamamatsu, USA). After feeding on the diet for 48 ~ 50 hours, the remaining 24 larvae from each treatment were dead and processed in the same method.

4.2.7 Data analysis

Data analysis was carried out in R v 2.15.2 (http://www.r-project.org/). For plasmid stability data, the proportion of stable plasmids that were present in the bacteria in every passage were computed as the number of streaks on the antibiotic LB agar plates divided by the number of streaks on the LB agar plates. The data were analyzed with a generalized linear model with binomial errors. For the competition assay in homogenized insect larvae, the relative fitness of wild type GFP is calculated by $v = x_2(1 - x_1)/x_1(1 - x_2)$, where x_1 is the initial frequency of wild type GFP in the population and x_2 is the final frequency of wild type GFP (Ross-Gillespie et al., 2007). The relative fitness of wild type GFP is the estimated growth rate of wild type GFP in relation to that of wild type RFP. The relative fitness of wild type GFP was transformed with the logarithm to base 10 and analysed with a general linearized model. All model assumptions were checked with graphical analyses of error distribution and normality.

4.3 Results

4.3.1 Construction of the green fluorescent B. thuringiensis strains

To make the green fluorescent *B. thuringiensis* strains, the plasmid pHT315-p*aphA3'-gfp* was first extracted from the methylating *E. coli* DH5α and used to transform the nonmethylating *E. coli* ET12567. Fig 4.2 A shows the resulting nonmethylated plasmid pHT315-p*aphA3'-gfp* isolated from *E. coli* ET12567 (lane 2 & 3, 1 µl plasmid DNA in each lane), giving three different forms of the plasmid: relax circular form (top band), linearized form (middle band) and supercoiled form (bottom band). The nonmethylated plasmid pHT315-p*aphA3'-gfp* from *E. coli* ET12567 was then used to transformed *B. thuringiensis* 407 Cry⁻ QS strains. Transformants were selected as erythromycin-resistant colonies and screened via PCR. Fig 4.2 B showed the PCR products (10 µl in each lane) of the gene p*aphA3'-gfp* from transformants of *B. thuringiensis* 407 Cry⁻ A'Z (lane 2 & 3), *B. thuringiensis* 407 Cry⁻ (lane 4 & 5), *B. thuringiensis* 407 Cry⁻ A'Z Δ*papR* (lane 6 & 7) and *B. thuringiensis* 407 Cry⁻ Δ*plcR* (lane 8 & 9) respectively. The gene p*aphA3'-gfp* was 1.2 kilobase pairs (kb) long and agreed with the band sizes observed in Fig 4.2 B, suggesting that the screened erythromycin-resistant transformants carried the plasmid pHT315-p*aphA3'-gfp*.

4.3.2 Construction of the red fluorescent B. thuringiensis strains

To make the red fluorescent *B. thuringiensis* strains with tetracycline-resistant gene, firstly by means of PCR, the erythromycin-resistant gene *eryR* was removed from pHT315 and the tetracycline-resistant gene *tetR* was amplified from pHT1618. Fig 4.3 showed the PCR products of *tetR* (Fig 4.3 A, lane 2, 1671 bp) and pHT315 (Fig 4.3 A, lane 5 & 6, 5564 bp). The PCR products of *tetR* and pHT315 were then purified, digested and ligated. The resulted plasmid pHT315-*tetR* was used to transform *E. coli* DH5α. Transformants were selected as tetracycline-resistant colonies and confirmed through plasmid digestion. Fig 4.3 B showed the digested

DNA fragments of plasmids isolated from tetracycline-resistant *E. coli* DH5α transformants. Lane 10 and 11 showed band sizes of 1662 bp and 5555 bp, suggesting that in these 2 lanes, the tetracycline-resistant *E. coli* DH5α transformants carried the plasmid pHT315-*tetR* (Fig 4.3 B).

After confirmation of the presence of pHT315-*tetR* in *E. coli* DH5α, the plasmid was isolated and digested at the multiple cloning site. The DNA fragments of the gene p*aphA3'-dsred* digested from the plasmid pHT315-p*aphA3'-dsred* and the digested plasmid pHT315-*tetR* were purified from gel. Fig 4.4 A and B showed the gel pictures of purified DNA fragments of pHT315-*tetR* (Fig 4.4 A, lane 2, 7.2 kb) and p*aphA3'-dsred* (Fig 4.4 B, lane 1 to 4, 1.2 kb). The DNA fragments of pHT315-*tetR* and p*aphA3'-dsred* were then ligated and the resulted plasmid pHT315-*tetR*-p*aphA3'-dsred* was transformed into *E. coli* GM2163. Transformants were selected as tetracycline-resistant colonies and screened for the presence of p*aphA3'-dsred* via PCR. Fig 4.4 C showed the PCR results of the gene p*aphA3'-dsred* from tetracycline-resistant *E. coli* GM2163 transformants. Lane 1, 4, 6 and 7 showed band sizes of 1.2 kb, suggesting that the screened tetracycline-resistant *E. coli* GM2163 transformants carried the plasmid pHT315-*tetR*-p*aphA3'-dsred*. The nonmethylated plasmid pHT315-*tetR*-p*aphA3'-dsred* from *E. coli* GM2163 was then transformed into *B. thuringiensis* 407 Cry⁻ QS strains. Transformants were selected as tetracycline-resistant colonies and screened via PCR. Fig 4.5 showed the PCR products (10 µl in each lane) of the gene p*aphA3'-dsred* from transformants of *B. thuringiensis* 407 Cry⁻ (lane 4, 7 & 14), *B. thuringiensis* 407 Cry⁻ A'Z Δ*papR* (lane 16, 19 & 23) and *B. thuringiensis* 407 Cry⁻ Δ*plcR* (lane 29 & 38) respectively. The gene p*aphA3'-dsred* was 1.2 kb long and agreed with the band sizes observed in Fig 4.5, suggesting that the screened erythromycin-resistant transformants carried the plasmid pHT315-p*aphA3'-dsred*.

4.3.3 Plasmid stability

To test the stability of the plasmid pHT315-p*aphA3'-gfp* and the plasmid pHT315-*tetR*-p*aphA3'-dsred* in the studied QS strains, the studied QS strains carrying the plasmids were passaged 6 times. The proportion of stable plasmids that were present in the cells in every passage was plotted against the passage number. As shown in Fig 4.6, the plasmid pHT315-p*aphA3'-gfp* and the plasmid pHT315-*tetR*-p*aphA3'-dsred* were very stable in *B. thuringiensis* 407 Cry⁻, *B. thuringiensis* 407 Cry⁻ A'Z ΔpapR and *B. thuringiensis* 407 Cry⁻ ΔplcR. Passaging did not have an effect on the stability of the tested plasmids in *B. thuringiensis* ($F_{5,30}$ = 0.019, p = 0.008, n = 36). After 6 passages, the plasmid pHT315-p*aphA3'-gfp* and the plasmid pHT315-*tetR*-p*aphA3'-dsred* remained 100% present in the studied QS strains (Fig 4.6).

4.3.4 Competition assay in homogenized insect larvae

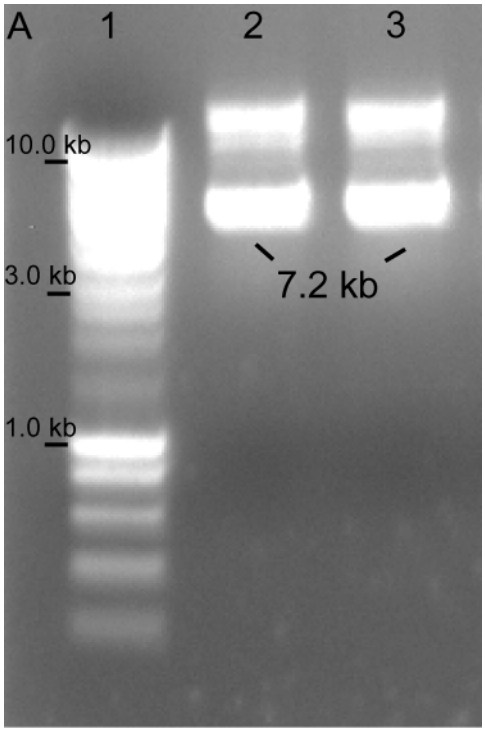
To examine whether wild type GFP grew better compared to wild type RFP, mixed inocula of wild type GFP and wild type RFP were used to inoculate homogenized insect larvae and the relative fitness of wild type GFP was measured. The initial frequency of wild type GFP was 53%. After 24 incubation in insect homogenates, based on 12 independent pools of homogenates, the frequency of wild type GFP increased to 73%. After logarithm transformation, the related fitness of wild type GFP was calculated to 0.39 ± 0.053. The relative fitness of wild type GPF measure the growth rate of wild type GFP in relationship to that of wild type RFP. After the 24 h incubation at 37℃, a positive fitness of wild type GFP suggests that wild type GFP grew faster than wild type RFP at 53% frequency in insect homogenates.

4.3.5 In vivo competition, histology and microscopy

To examine the distribution of QS wild type and signal blind mutant within host during the early stages of infection, 3rd instar larvae were inoculated with green QS

wild type and red signal blind mutant or vice versa using a droplet feeding method. As shown in Fig 4.7, the larva became distinctly green in the gut area upon ingestion of the droplet. Histological sections of infected larvae after 24 h and 48 h ingestion allowed visualization of bacterial population structure in insects. I examined 6 larvae and 60 sections, 10 sections per larva. I found a total of 25 ~ 30 wild type cells and 2 mutant cells. As seen in Fig 4.8, spatial heterogeneity was evident in the larval midgut after 24 h ingestion. Bacterial populations were founded on isolated bacteria patches comprised of 1 – 3 individual cells, mostly but not exclusively QS wild type (Fig 4.8, pointed by arrows). Signal blind mutant was rare but can be found (Fig 4.9, pointed by arrows). At 24 h post infection, signal blind mutant cells were also much smaller than QS wild type (Fig 4.8 and Fig 4.9). A mixture of QS wild type and signal blind mutant was observed 48 h post-ingestion (Fig 4.10). Compared to nearly intact host tissue and few isolated cells after 24 h ingestion (Fig 4.8 and Fig 4.9), 48 h post ingestion most host tissue have been broken down and a large number of vegetative cells was observed (Fig 4.10). QS wild type and signal blind mutant formed a well-mixed population in the larvae after 48 h ingestion. The result was independent of the markers used to identify QS wild type and signal blind mutant.

Fig 4.2 Gel pictures of the plasmid pHT315-p*aphA3'-gfp* from *E. coli* ET12567 and PCR products of the gene p*aphA3'-gfp* from erythromycin-resistant *B. thuringiensis* 407 Cry⁻ transformants. (**A**) Lane 1: 2-Log DNA Ladder (New England Biolabs, 5 µl) and lane 2 & 3: pHT315-p*aphA3'-gfp* from *E. coli* ET12567 (1 µl plasmid DNA in each lane). (**B**) Lane 1: 2-Log DNA Ladder (5 µl) and lane 2 – 9: PCR products (1.2 kb, 10 µl in each lane) of the gene p*aphA3'-gfp* from *B. thuringiensis* 407 Cry⁻ A'Z (lane 2 & 3), *B. thuringiensis* 407 Cry⁻ (lane 4 & 5), *B. thuringiensis* 407 Cry⁻ A'Z Δ*papR* (lane 6 & 7) and *B. thuringiensis* 407 Cry⁻ Δ*plcR* (lane 8 & 9).



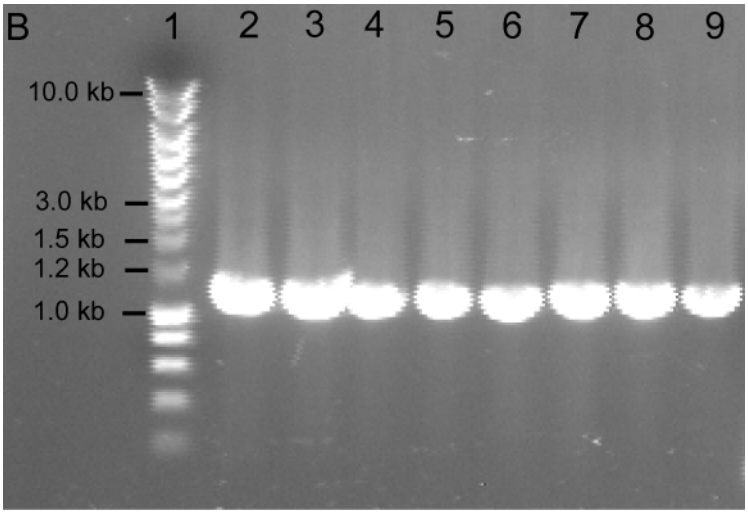
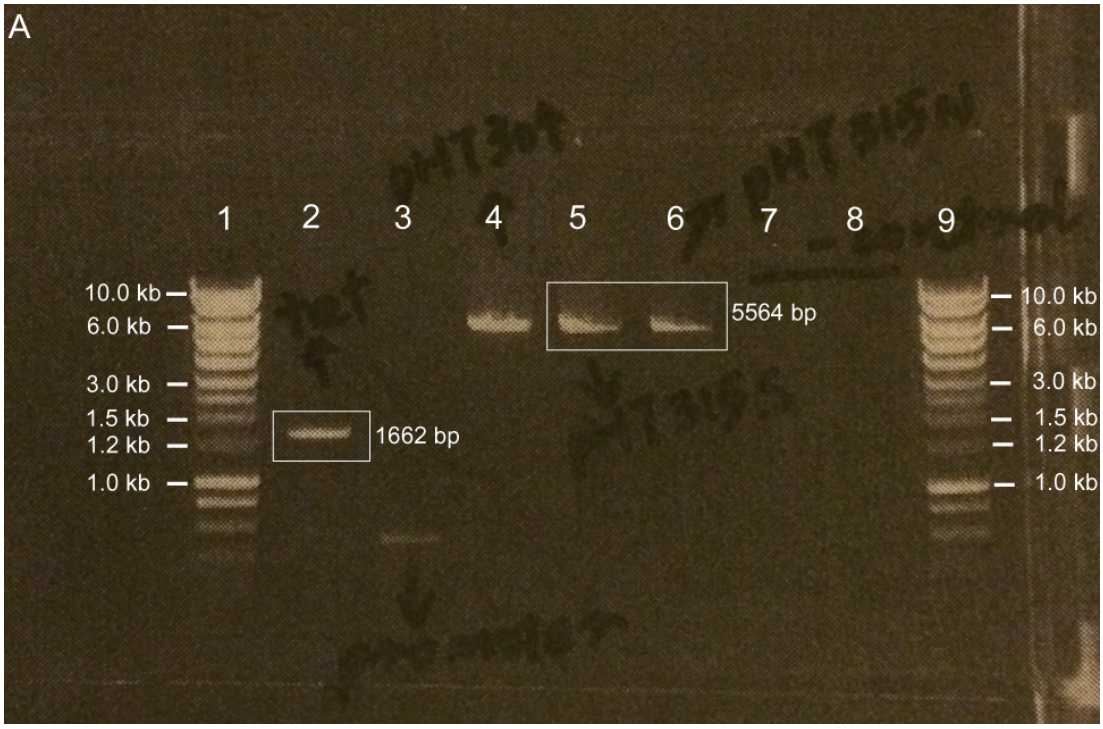


Fig 4.3 Gel pictures of PCR products of *tetR* and pHT315 and gel picture of digested plasmid DNA fragments of tetracycline-resistant *E. coli* DH5α transformants. (**A**) Lane 1 & lane 9: DNA Ladder (5 µl), lane 2: PCR products of *tetR* (1671 bp, 1 µl), lane 5 & 6: PCR products of pHT315 (5564 bp, 1 µl) and lane 7 & 8: PCR products of negative control (ddH_2O, 1 µl). (**B**) Lane 1: DNA Ladder (5 µl), lane 2 – 11: DNA fragments of digested plasmids isolated from tetracycline-resistant *E. coli* DH5α transformants (10 µl in each lane). Lane 10 and 11 shows bands sizes of 1662 bp (*tetR*) and 5555 bp (pHT315).



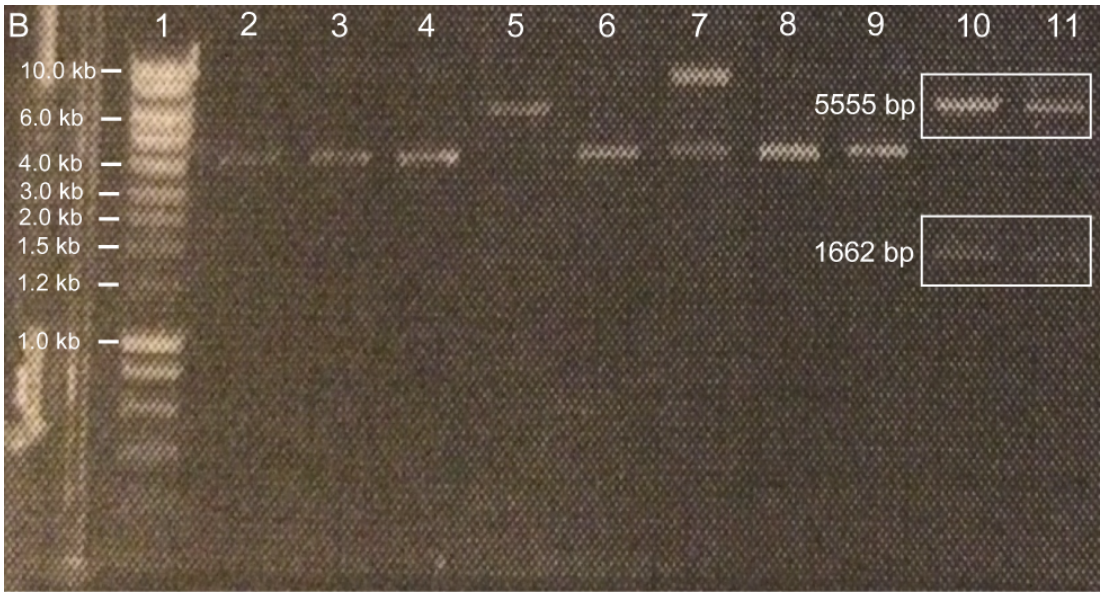


Fig 4.4 Gel pictures of digested plasmid fragments of pHT315-tetR and p*aphA3'-dsred* and gel picture of PCR products of p*aphA3'-dsred*. (**A**) Lane 1: DNA Ladder (5 µl) and lane 2: purified pHT315-*tetR* (7.2 kb, 1 µl) after digestion. (**B**) Lane 1 – 4: purified plasmid fragments of p*aphA3'-dsred* (1.2 kb, 1 µl) and lane 9: DNA Ladder (5 µl). (**C**) Lane 1 – 7: PCR products of p*aphA3'-dsred* from tetracycline-resistant *E. coli* GM2163 transformants (10 µl in each lane), lane 8 & 9: PCR products of negative control (ddH_2O, 10 µl) and Lane 10: DNA Ladder (5 µl). Lane 1, 4, 6 and 7 shows bands sizes of 1.2 kb, suggesting that the tested transformants carried that the plasmid pHT315-*tetR*-p*aphA3'-dsred*.

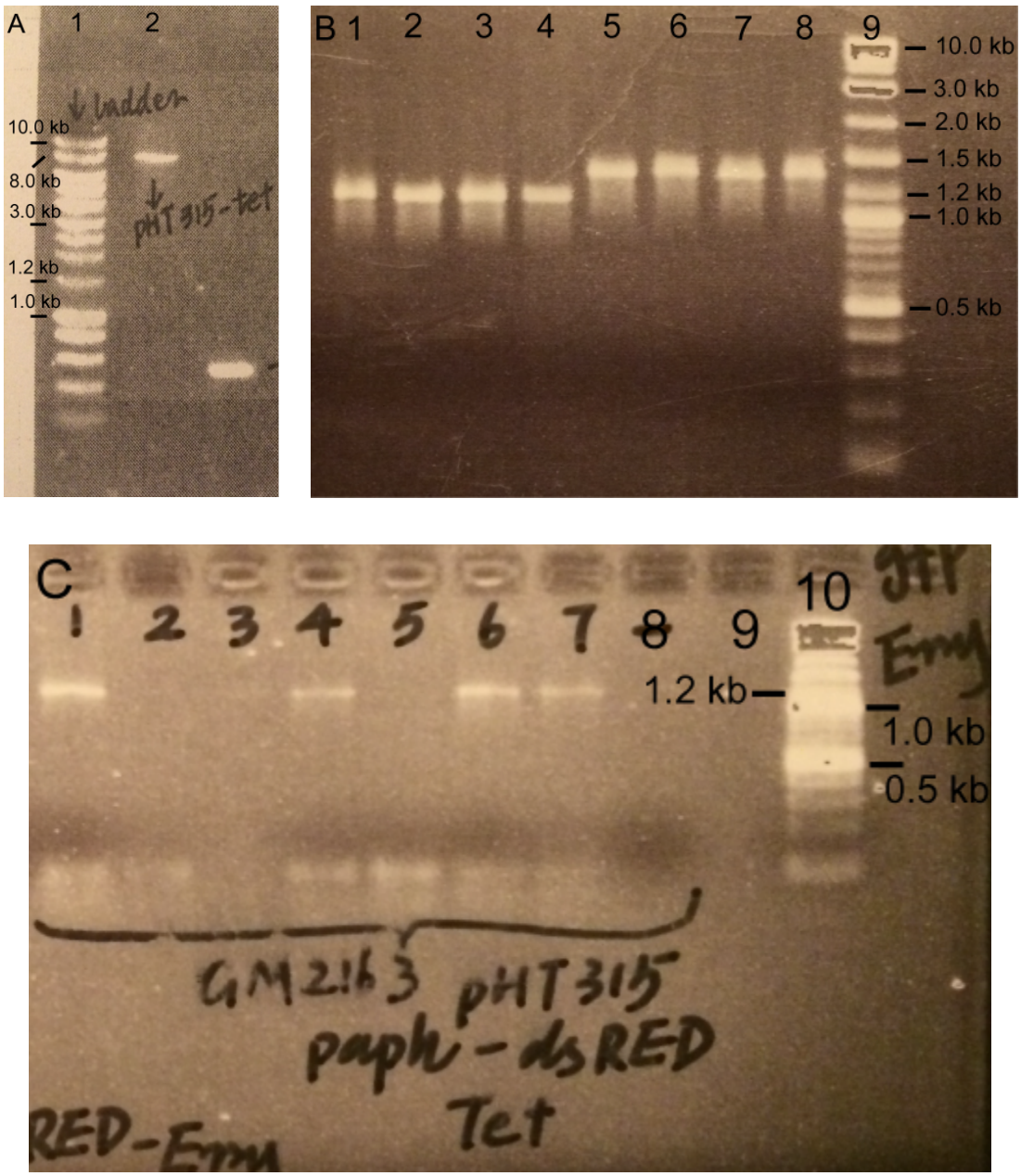


Fig 4.5 Gel pictures of PCR products of the gene p*aphA3'-dsred* from tetracycline-resistant *B. thuringiensis* 407 Cry⁻ transformants. Lane 4 – 43: PCR products (1.2 kb, 10 µl in each lane) of the gene p*aphA3'-dsred* from *B. thuringiensis* 407 Cry⁻ (lane 4 – 15), *B. thuringiensis* 407 Cry⁻ A'Z Δ*papR* (lane 16 – 26) and *B. thuringiensis* 407 Cry⁻ Δ*plcR* (lane 27 & 43). Solid arrow showed the p*aphA3'-dsred* digested from the plasmid pHT315-p*aphA3'-dsred* (1 µl). Lane 4, 7, 14, 16, 19, 23, 29 and 38 showed band sizes of 1.2 kb, suggesting that the tested transformants carried the plasmid pHT315-*tetR*-p*aphA3'-dsred*.

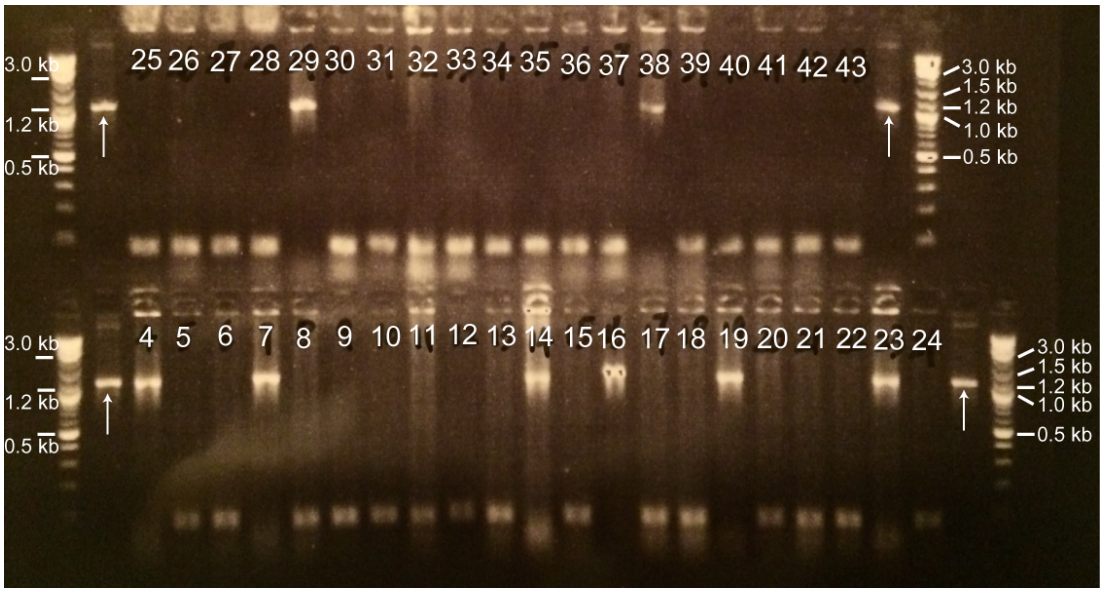


Fig 4.6 The green and red pHT315 plasmids were extremely stable in *B. thuringiensis*. The proportion of stable plasmids was plotted against the passage number. The plasmid pHT315-p*aphA3'-gfp* and the plasmid pHT315-*tetR*-p*aphA3'-dsred* were very stable in QS wild type, QS signal null and QS signal blind mutant. After 6 passages, 100% of the bacteria carried the green and red pHT315 plasmids.

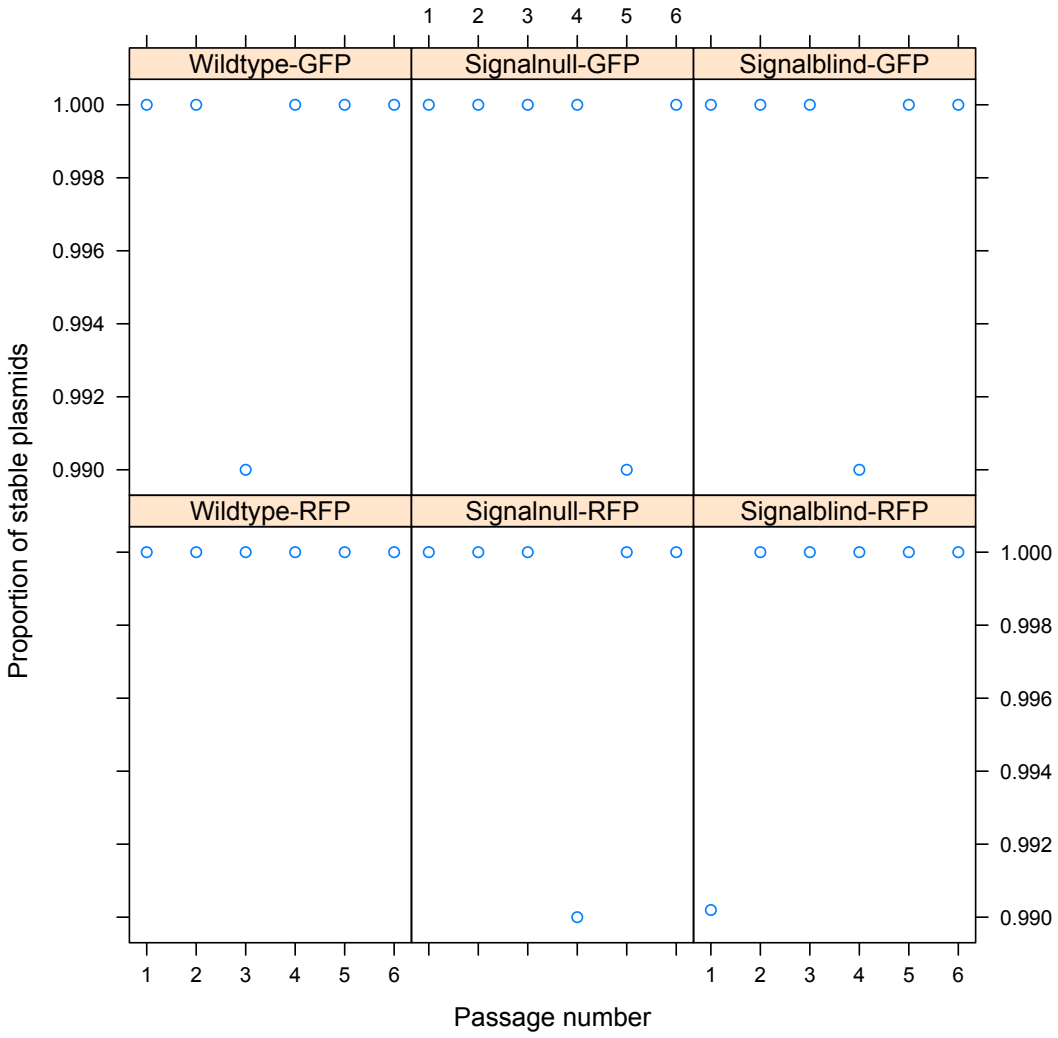


Fig 4.7 Image of infection 3rd instar larvae using the droplet feeding method. After the larvae have eaten 1 µl of the green food droplet. Images of inoculated larvae were taken in comparison to larvae that have not eaten the green food droplet. Larva that has not eaten the green food droplet is on top left, while larva that has ingested the food is on bottom right.

Fig 4.8 QS wild type was observed after 24h infection. Third instar diamondback moth larvae were inoculated with green QS wild type (WT GFP) and red isogenic signal blind mutant (MT RFP) mixed at a 1:1 ratio. Third instar insect larvae were also infected with red QS wild type (WT RFP) and green isogenic signal blind mutant (MT GFP) at a 1:1 ratio. After 24 h infection, the larvae were frozen in O.C.T compound and cut laterally through the gut into 7 μm sections. Wild type cells are pointed by arrows. Scale bar: 15 μm.

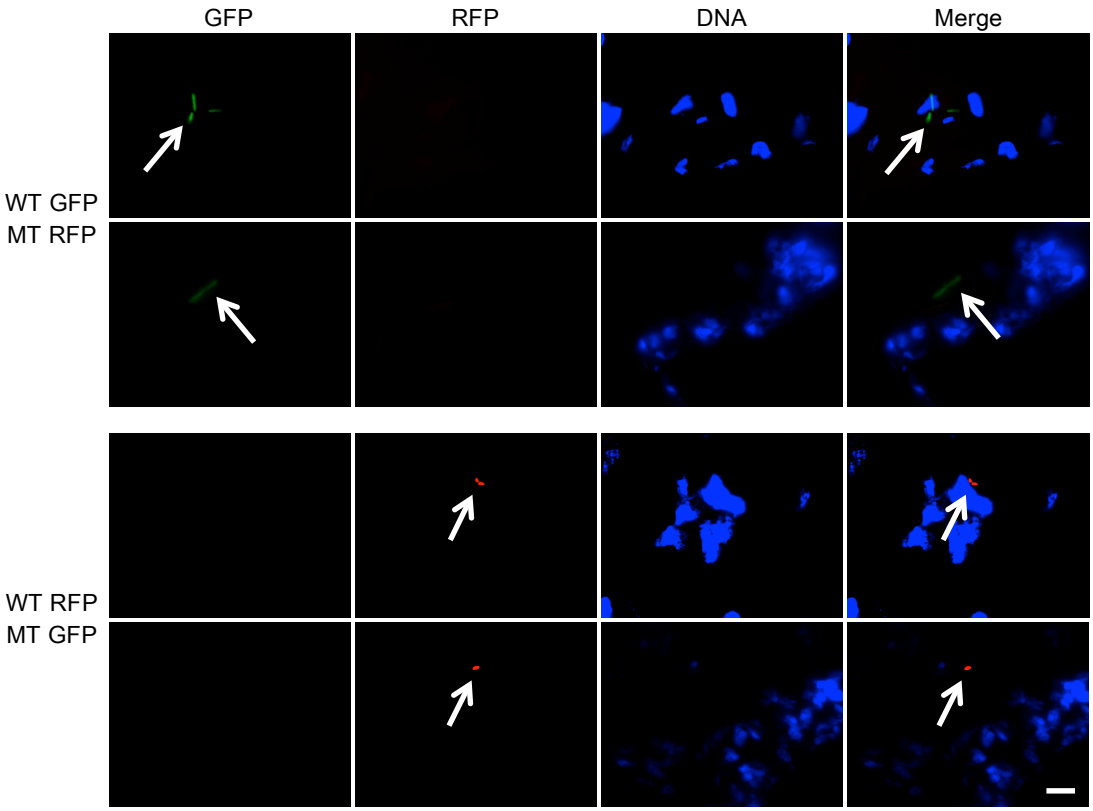


Fig 4.9 Signal blind mutant was rare but can be found at 24h post infection. Third instar diamondback moth larvae were inoculated with green QS wild type (WT GFP) and red isogenic signal blind mutant (MT RFP) mixed at a 1:1 ratio. After 24 h infection, the larvae were frozen in O.C.T compound and cut laterally through the gut into 7 µm sections. Mutants cell are pointed by arrows. Scale bar: 15 µm.

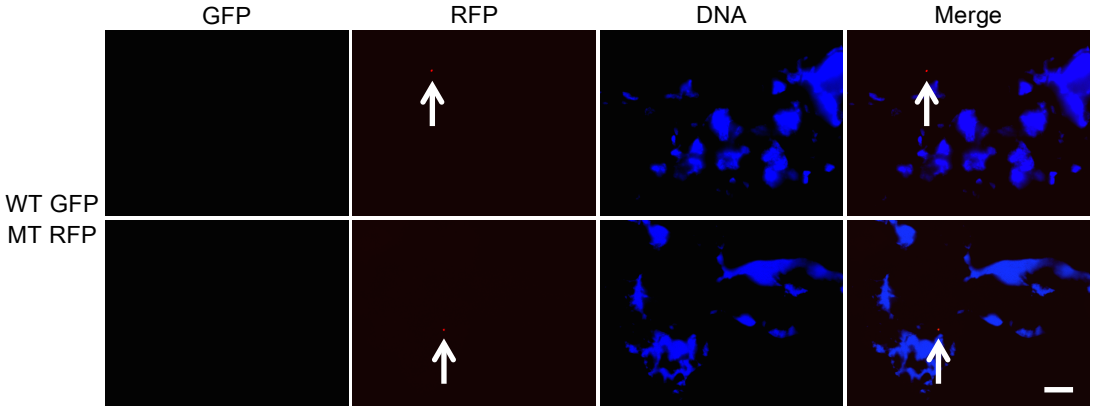
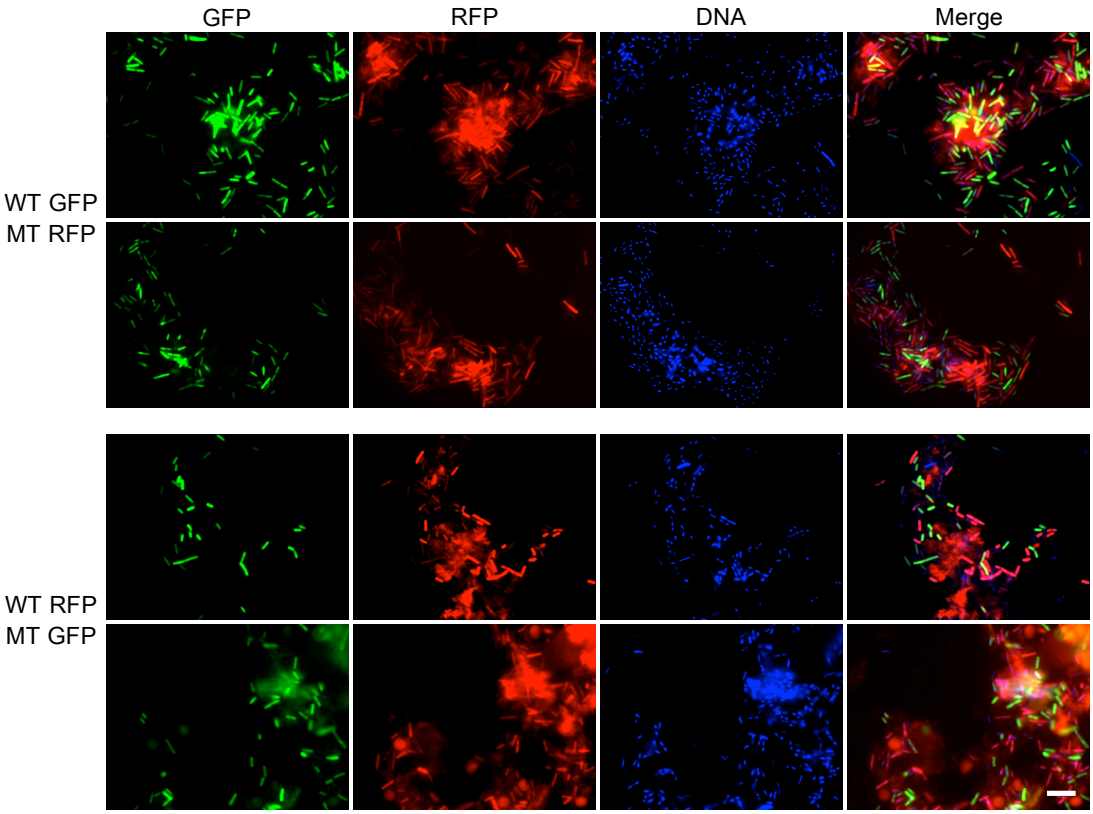


Fig 4.10 A mixture of QS wild type and mutant was observed upon 48h infection. Third instar diamondback moth larvae were inoculated with green QS wild type (WT GFP) and red isogenic signal blind mutant (MT RFP) mixed at a 1:1 ratio. Third instar insect larvae were also infected with red QS wild type (WT RFP) and green isogenic signal blind mutant (MT GFP) at a 1:1 ratio. After 48 h infection, the larvae were embedded in O.C.T compound and sliced laterally into 7 µm sections. Nuclear DNA (visualised using DAPI) is shown in blue. Scale bar: 15 µm.



4.4 Discussion

Before the investigation of spatial heterogeneity in bacterial population in host, plasmid stability in *B. thuringiensis* 407 of pHT315 carrying the transcriptional fusion between the constitutive promoter p*aphA3'* and either of the fluorescent genes *gfp-mut1* or *dsred* is first confirmed. Corroborating with previous studies, the plasmid pHT315 is exceptionally stable in *B. thuringiensis* 407 (Arantes and Lereclus, 1991). After 6 passages without any antibiotic selective pressure, the plasmid pHT315 remained 100% present in *B. thuringiensis* 407 (Fig 4.5). Apart from plasmid stability, the fitness costs of the two fluorescent genes *gfp-mut1* or *dsred* were also tested by competing QS wild type carrying GFP with QS wild type RFP at 53% of wild type GFP. Overall, QS wild type expressing GFPmut1 grew faster compared to wild type RFP in homogenized insect larvae with 5×10^4 cfu μl^{-1} initial spore density. Observational study under the microscope also showed that the cells of signal blind RFP were much smaller than those of wild type GFP, in support of the fitness results and also corroborated with previous studies in *E. coli* (Jakobs et al., 2000). For subsequent experiments under the microscope, *Plutella* larvae were inoculated with wild type GFP and signal blind mutant RFP or vice versa. The observed fitness difference between *gfp-mut1* and *dsred* is therefore unlikely to affect the image results.

To examine the distribution of QS wild type and signal blind mutant within hosts during the early stages of infection, 3rd instar *Plutella* larvae were inoculated with 7.5×10^4 viable spores in a single droplet with 1:1 ratio of QS wild type and signal blind mutant. Isolated patches of 1 – 3 individual cells were found in the midgut 24 h post infection (Fig 4.7 and Fig 4.8). The patches were comprised of mainly but not exclusively QS wild type (Fig 4.7 and Fig 4.8). Signal blind mutant was less frequently observed (Fig 4.8). At this stage of infection, signal blind mutant was clearly spatially separately from QS wild type, in support of the hypothesis that spatial heterogeneity limits the relative fitness of signal blind mutants. Although not examined in the study, signal null mutants are likely to behave differently from signal

blind mutants. The large secreted virulence factors regulated by the PlcR regulon are likely to be less diffusive than the small signalling peptides. Signal null mutants might be spatially separately from QS wild type, but signal null mutants are able to express the PlcR-regulated virulence factors with signalling peptides produced by the QS wild type. The relative fitness data from chapter 3 support the theory. The relative fitness of signal null mutants was higher than that of signal blind mutant. At 48 h post infection, most host tissue have been broken down and a large number of vegetative cells was pronounced, forming a well mixed population of QS wild type and signal blind mutant (Fig 4.9). This result was independent of the markers used to identify QS wild type and signal blind mutant. These data support the hypothesis that spatial heterogeneity occurs in the midgut of hosts, where the PlcR-PapR QS system is activated and PlcR-regulated virulence factors are required to cross the midgut barriers (Salamitou et al., 2000). In addition to spatial heterogeneity, the population bottleneck imposed by the midgut barriers is also likely to impact on the relative fitness of PlcR-PapR QS signal null and signal blind mutants. The larvae were inoculated with 7.5×10^4 viable spores, but the bacteria population within host were established from only countable patches of cells, indicating a sharp reduction in size of a population due to the barriers to infection imposed by hosts. Such sharp reduction in population size have been reported to encourage cooperation in *P. fluorescens*, with smaller population bottleneck size leading to higher level of cooperation (Brockhurst, 2007). As bacteria have clonal reproduction, the relatedness is likely to increase with fewer cells surviving the host infection barriers, thus favouring the evolution of QS in bacteria.

The ecology of the PlcR-PapR QS system is very different from the other established QS systems in *P. aeruginosa*, based on *in vitro* study in well-mixed liquid media and *in vivo* study in mice with burn wounds (Diggle et al., 2007, Rumbaugh et al., 2009). In the *in vitro* study of *P. aeruginosa*, QS mutants were allowed to interact freely with the QS wild type in shaken liquid media and QS mutants of *P. aeruginosa* outcompeted wild type bacteria over a wide range of

frequencies (Diggle et al., 2007, Rumbaugh et al., 2009). Spatial heterogeneity is unlikely to limit the invasion of *P. aeruginosa* QS mutants in the *in vitro* system. In the burned wound mouse system, *P. aeruginosa* was injected directly under the skin of mice, which overcame the normal infection barriers impose by hosts (Rumbaugh et al., 1999). The ecology of the mouse system is unusual in nature. Hosts usually display a range of resistant mechanisms to bacteria infection, leading to a narrow population bottleneck. Overall, the PlcR-PapR QS system in oral infected *Plutella* has several advantages over other established QS systems. While PlcR-regulated virulence factors are required for successful infections, spatial heterogeneity in host and population bottleneck could affect the fitness of QS mutants.

CHAPTER 5 POLYMORPHISM OF THE PLCR-PAPR QUORUM SENSING SYSTEM IN *B. THURINGIENSIS*

5.1 Introduction

Polymorphic signalling systems are widespread in both Gram-positive and Gram-negative bacteria (Kleerebezem et al., 1997, Manefield and Turner, 2002). Specificity and polymorphism have been previously described in the competence QS system in *B. subtilis* and in *Streptococcus* species (Pozzi et al., 1996, Tortosa et al., 2001, Whatmore et al., 1999) as well as in the virulence QS system in *Staphylococcus* species (Dufour et al., 2002, Ji et al., 1997). Genetic studies have suggested the polymorphism observed in the various QS systems evolved via accumulation of point mutations or recombinational changes in an element of the QS systems followed by positive selection of compensatory mutations in the second element of the same QS systems (Ansaldi and Dubnau, 2003, Dufour et al., 2002, Havarstein et al., 1997, Ichihara et al., 2006, Slamti and Lereclus, 2005, Tortosa et al., 2001). A polymorphism comprising four distinct communication groups (pherotypes) was also found in the *B. cereus* group (Slamti and Lereclus, 2005). Strains from the same pherotype exchange information efficiently while strains from different pherotypes communicate far less effectively (Bouillaut et al., 2008). While a wealth of studies in the genetics of the polymorphic QS systems, empirical evidence of the evolution and maintenance of polymorphism in quorum sensing is lacking. This chapter presents experimental studies of the polymorphic PlcR-PapR QS system in *B. thuringiensis* by allowing *in vivo* competition of different pherotypes in the diamondback moth *P. xylostella* and monitoring the change in frequency of the competing pherotypes. I hypothesize that the observed polymorphism in the PlcR-PapR QS system was maintained by facultative cheating or environmental dependent fitness.

Mathematical modelling of QS suggested that the polymorphism was positively selected by facultative cheating which allows cycles of alternative cheating and invasion of rare pherotypes (Eldar, 2011). Facultative cheating model have suggested that a rare pherotype is able to out-compete a common pherotype in a cooperative population (Eldar, 2011). In mixed infections, cells from the same pherotype will be able to communicate with each other effectively, stimulating the production of the extracellular proteins, the "public goods", regulated by the PlcR regulon. Public goods are costly to produce but benefit the local group surrounding the producers (Frank, 1998, West et al., 2007). Thus a rare pherotype in mixed infection should benefit from the public goods produced by the common pherotype. The fitness of the rare pherotype should be greater when they are rarer, as there would be more public goods for them to exploit (Ross-Gillespie et al., 2007). I hypothesize that the relative fitness of a pherotype should decrease with increasing initial frequency of the same pherotype but also not suffer any loss of fitness in single strain/clonal infections.

After investigating facultative cheating *in vivo*, the importance of spatial heterogeneity in limiting the fitness of rare pherotype is also evaluated. Studies of biofilm using the computational models have suggested that cheats are unable invade a wild type population when wild type and cheats are well segregated (Mitri et al., 2011, Nadell et al., 2010). Using this logic, a rare pherotype in a population would not be able to take advantage of the public goods produced by a common pherotype if the rare pherotype and the common pherotype are spatially separated. Thus a pairwise competition assay between group III and group IV pherotypes is conducted in insect homogenates under shaking conditions, as in chapter 3. In shaken insect homogenates, the two pherotypes are able to interact with each other freely and the rare pherotype should be able to out compete the common pherotype by utilizing the public goods produced by the common pherotype. A negative frequency dependent relationship between the initial frequency of a pherotype and the relative fitness of the same pherotype is expected

Apart from facultative cheating, I hypothesized that the polymorphism observed in the PlcR-PapR QS system is maintained by environment dependent fitness, such that particular pherotypes will have enhanced fitness in particular environmental conditions. Previous studies of an acetate-crossfeeding polymorphism in *E. coli* has shown that the acetyl CoA synthetase overproducing phenotype had higher fitness in glucose-limited media while the normal producer had higher fitness in glucose media (Treves et al., 1998, Turner et al., 1996). Signalling peptides are external signals so the environment and presence of competitors may affect signal efficacy and persistence. Studies have suggested the AHL lactonase, AiiA, from *Bacillus* species can inhibit QS between Gram-negative bacteria and attenuate virulence of *E. carotovora* (Dong et al., 2000, Dong et al., 2001, Dong et al., 2004). It raises a question whether signals from competitors in the other way around can possibly render QS between *Bacillus* species ineffective? To test idea that the presence of a competitor will affect the fitness of different pherotypes differently, I will conduct a pairwise *in vivo* competition assay of QS wild type, group III and group IV pherotypes with or without a gut bacteria strain *Enterobacter cloacae* and measure the change of fitness of different pherotypes when challenge with *E. cloacae*. *E. cloacae* is a common gut bacteria of the gastrointestinal tract of 40 to 80% of people and is widely distributed in the environment (Dudley et al., 1980, Gaston, 1988). The strain used in this experiment was isolated from the midgut of *P. xylostella*; it forms a persistent association with the larval midgut and competes with *B. thuringiensis* strains in the cadaver during septicaemic proliferation (Raymond et al., 2009). The hypothesis is that the addition of *E. cloacae* would affect the fitness of different pherotypes.

In order to conduct the competition assay *in vivo*, I first complemented the strain *B. thuringiensis* 407 Cry⁻ A'Z Δ*plcR-papR* with either a plasmid carrying group III *plcR-papR* gene or a plasmid carrying group IV *plcR-papR* gene. *B. thuringiensis* 407 Cry⁻ A'Z Δ*plcR-papR* has a disruption in both the *plcR* and *papR* genes, thus *B. thuringiensis* 407 Cry⁻ A'Z Δ*plcR-papR* is unable express neither the *plcR* gene nor

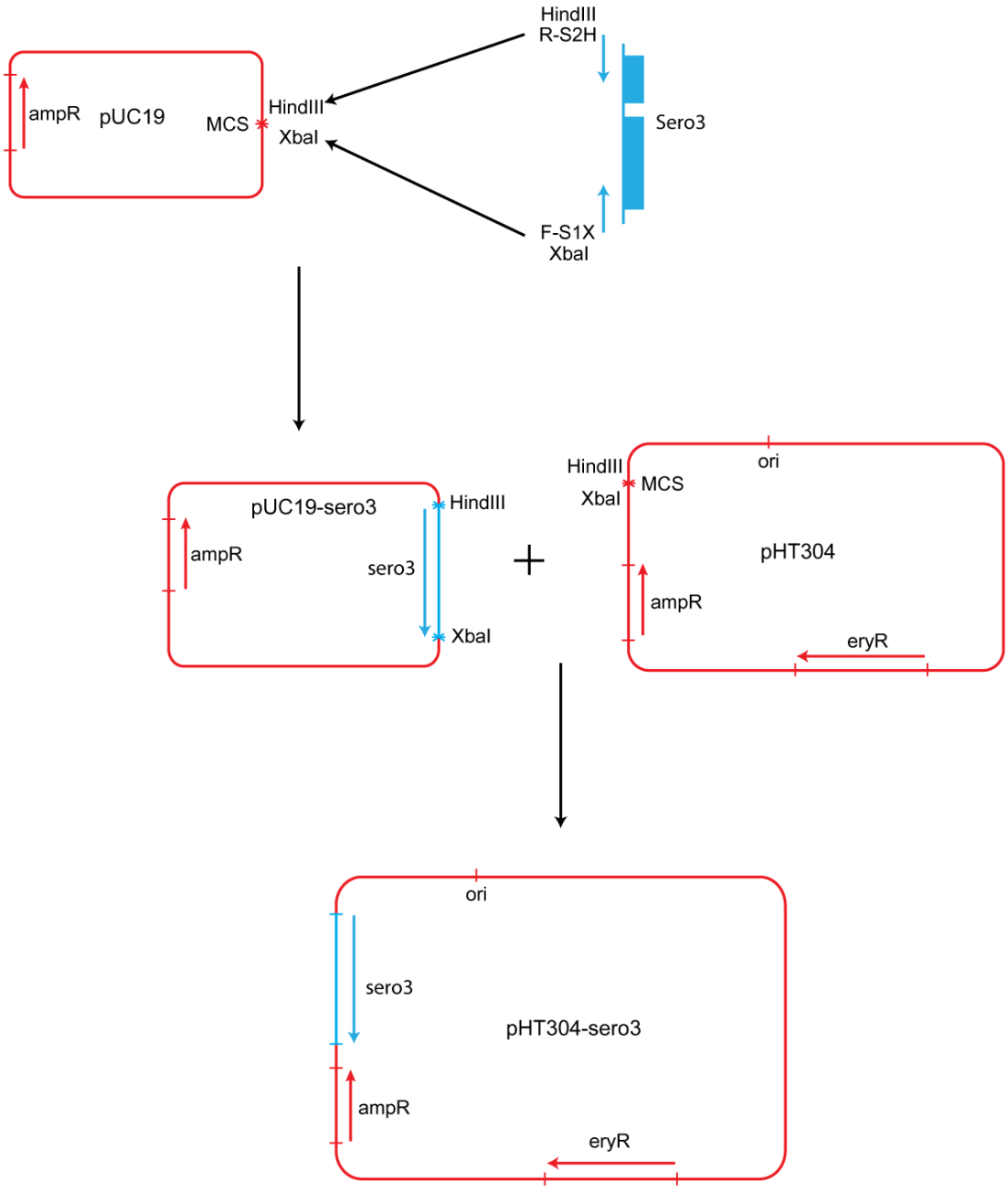
the *papR* genes (Bouillaut et al., 2008). The group III *plcR-papR* genes are from *B. thuringiensis* subsp. *kurstaki* HD-73 (Bouillaut et al., 2008) and the group IV *plcR-papR* genes are from *B. thuringiensis* serovar roskoldiensis (Slamti and Lereclus, 2005). The two groups are chosen because the cross talk between the two groups was minimal (Bouillaut et al., 2008). The signalling peptide from group IV, PapRIV, was only able to activate its own regulon, PlcRIV. While the signalling peptide from group III, PapRIII, was able to switch on its own regulon, PlcRIII, as well as regulons from group I and group II albeit less effectively, the cross talk between PapRIII and PlcRIV was negligible (Bouillaut et al., 2008). In addition, QS wild type studied in previous chapters belongs to group I pherotype. Similarly, PapRI is able to turn on PlcRI but significantly less potent for regulons from the other three pherotypes. As *B. thuringiensis* 407 Cry⁻ A'Z Δ*plcR-papR* is an isogenic strain of QS wild type, competition assay between QS wild type and *B. thuringiensis* 407 Cry⁻ A'Z Δ*plcR-papR* complemented with PapRIII or PapRIV draw a comparison of growth rate as a results of the different *plcR-papR* genes. Apart from the selection of pherotypes, the plasmid used to carry different *plcR-papR* genes is pHT304. Similar to pHT315, the plasmid pHT304 is a small and stable plasmid for *B. thuringiensis*, which is 6.5 kilobase pairs long and has a copy number of 4 ± 1 per cell (Arantes and Lereclus, 1991). As discussed in chapter 4, a low copy number and stable plasmid is desirable as a shuttle vector for the study. Although previous studies have suggested pHT304 to be very stable in *B. thuringiensis*, the stability of pHT304 will be confirmed by passaging the cells complemented with the plasmid 6 times successively in LB without antibiotic selection. Finally, the plasmid pHT304 also carries erythromycin resistant gene to Gram positive bacteria and ampicillin resistant gene to *E. coli* (Arantes and Lereclus, 1991). In order to differentiate cells expressing group III pherotype genes from cells of group IV pherotype, the erythromycin resistant gene in pHT304 is replaced with tetracycline resistant gene from the pHT1618 plasmid (Lereclus and Arantes, 1992) for group IV pherotype.

5.2 Materials and methods

5.2.1 Construction of PlcR-PapR group III B. thuringiensis strain

The strain *B. thuringiensis* 407 Cry⁻ A'Z Δ*plcR-papR* (Bouillaut et al., 2008) was complemented with the plasmid pHT304 containing group III *plcR* and *papR* genes and was constructed using the following steps. The group III *plcR-papR* genes, designated as *sero3*, were amplified from *B. thuringiensis* subsp. *kurstaki* HD-73 (Bouillaut et al., 2008) using the primers F-S1X (5'-GCTCTAGACTATTATTATATGTGAGATGAATTGTATG-3') and R-S2H (5'-CCCAAGCTTGTAAAGACGTTTGGATGTTACTCC-3') with Phusion® High-Fidelity DNA Polymerase (New England Biolabs). The *sero3* PCR products were purified using QIAquick PCR Purification Kit (Qiagen). The *sero3* PCR fragments and the plasmid cloning vector pUC19 (New England Biolabs) were then digested with restriction enzymes *Xba*I (New England Biolabs) and *Hin*dIII (New England Biolabs). The *sero3* PCR fragments were then ligated with the digested pUC19. The resulting plasmid pUC19-*sero3* was transformed into *E. coli* DH5α through heat-shock transformation using 100 μg ml^{-1} ampicillin for selection. Transformants were selected as ampicillin-resistant colonies and examined via PCR with primers M13PU and M13PR (chapter 4) for the presence of *sero3*. After confirmation of the presence of *sero3*, the strain *E. coli* DH5α carrying the plasmid pUC19-*sero3* and the strain *E. coli* DH5α with the plasmid pHT304 (Arantes and Lereclus, 1991) were cultured in 10 ml 2% LB broth containing 100 μg ml^{-1} ampicillin, with vigorous shaking (200 rpm) overnight at 37 ℃. The plasmid pHT304, similar to pHT315, provides the resistance to ampicillin to *E. coli* and the resistance to erythromycin to *B. thuringiensis* (Arantes and Lereclus, 1991). The plasmids pUC19-*sero3* and pHT304 were then isolated with QIAprep Spin Miniprep Kit (Qiagen) and were digested with *Xba*I (New England Biolabs) and *Hin*dIII (New England Biolabs). The gene fragments *sero3* and pHT304 were identified by size via gel electrophoresis and purified from gel. *Sero3* was then inserted into the multiple cloning site of

Fig 5.1 Construction of the plasmid pHT304-*sero3*. Details are given in the text. Origin of replication and multiple cloning site of each plasmid are abbreviated as ori and MCS in the diagram.

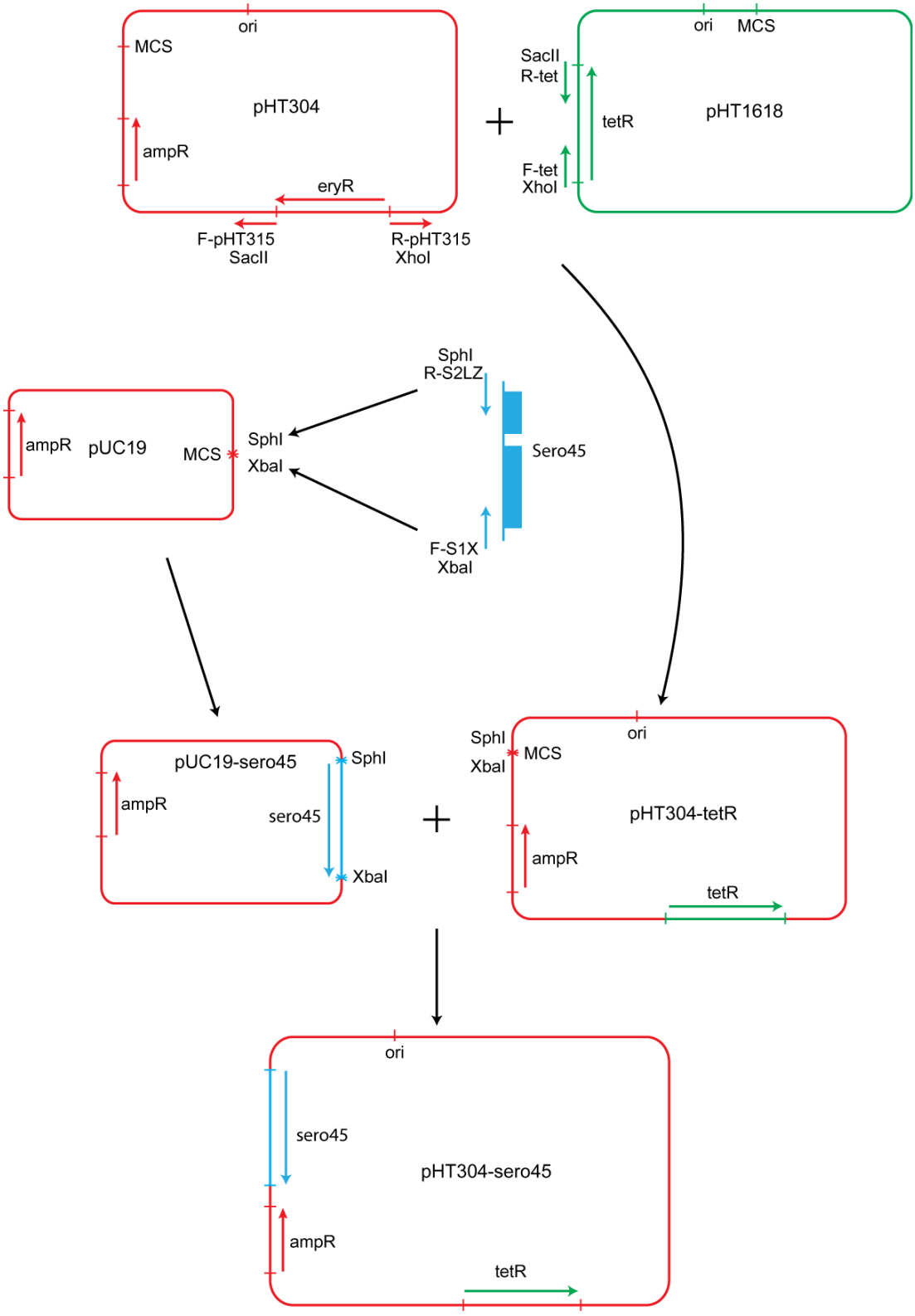


pHT304. The resulting plasmid pHT304-*sero3* was then used to transform *E. coli* DH5α through heat-shock transformation. Transformants were selected as colonies resistant to 100 μg ml^{-1} ampicillin and examined via PCR using primers M13PU and M13PR for the presence of *sero3*. Following validation of the existence of *sero3*, the strain *E. coli* DH5α carrying the plasmid pHT304-*sero3* were cultured in 10 ml 2% LB broth containing 100 μg ml^{-1} ampicillin overnight at 37 ℃ and shaking at 200 rpm. The plasmid pHT304-*sero3* was then isolated with QIAprep Spin Miniprep Kit (Qiagen) and used to transform the nonmethylating strain *E. coli* ET12567 (Stratagene, La Jolla, CA, USA). The gene *sero3* in the plasmid pHT304-*sero3* isolated from *E. coli* DH5α was also sequenced using primers M13PU and M13PR. No nucleotide change was detected and the plasmid pHT304-*sero3* from *E. coli* ET12567 was used to transform the strain *B. thuringiensis* 407 Cry^- A'Z Δ*plcR-papR* (Bouillaut et al., 2008) via electroporation. Transformants were selected as erythromycin-resistant (10 μg ml^{-1}) colonies and examined through PCR using primers M13PU and M13PR for the presence of the gene *sero3*. Following PCR, erythromycin-resistant colonies were then streaked over 2% LB agar plates containing 10 μg ml^{-1} erythromycin for the isolation of single colonies. The strain *B. thuringiensis* 407 Cry^- A'Z Δ*plcR-papR* complemented with the plasmid pHT304-*sero3* was named as Sero3. A single colony of Sero3 was transferred to 5 ml autoclaved 2% LB broth using 10 μl sterile disposable inoculating loops (Greiner Bio-One) and was allowed to grow overnight at 30℃ with vigorous shaking at 200 rpm. After overnight growth, 250 μl of the overnight culture of Sero3 were mixed with 750 μl of 80% glycerol (Sigma) in 1.5 ml cryotubes (Thermo Scientific). The glycerol stock of Sero3 was stored at – 80℃.

5.2.2 Construction of PlcR-PapR group IV B. thuringiensis strain

In order to distinguish the PlcR-PapR group IV *B. thuringiensis* strain from the PlcR-PapR group III *B. thuringiensis* strain, the plasmid pHT304 was reconstructed to carry the tetracycline resistant gene *tetR* from the plasmid pHT1618 (Lereclus and Arantes, 1992) and the group IV *plcR-papR* genes from *B. thuringiensis* serovar

Fig 5.2 Construction of the plasmid pHT304-*sero45*. Details are given in the text. Origin of replication and multiple cloning site of each plasmid are abbreviated as ori and MCS in the diagram.



roskoldiensis (Slamti and Lereclus, 2005). First of all, the erythromycin-resistant gene *eryR* in pHT304 was replaced with *tetR* by amplifying the plasmid pHT304 outwards from *eryR* and the tetracycline-resistant gene *tetR* from pHT1618 with Phusion® High-Fidelity DNA Polymerase (New England Biolabs). The primers used for pHT304 amplification were F-pHT315 and R-pHT315 (chapter 4) and for *tetR* amplification were F-tet and R-tet (chapter 4). The PCR products of pHT304 and *tetR* were purified with QIAquick PCR Purification Kit (Qiagen) and digested with restriction enzymes *Sac*I (New England Biolabs) and *Xho*I (New England Biolabs). *TetR* were ligated with pHT304 and the resulting plasmid pHT304-*tetR* was transformed into *E. coli* DH5α through heat-shock transformation. The antibiotics used for selection were ampicillin (100 μg ml^{-1}) and tetracycline (10 μg ml^{-1}). Transformants were examined via digesting the plasmids isolated from the tetracycline-resistant colonies with *Sac*I and *Xho*I. The gene *tetR* in pHT304 was also sequenced using primers F-tet and R-tet. No nucleotide change was found.

After the construction of pHT304-*tetR*, the group IV *plcR-papR* genes, designated as *sero45*, were amplified from *B. thuringiensis* serovar roskoldiensis (Slamti and Lereclus, 2005) using the primers F-S1X and R-S2LZ (5'-ACATGCATGCCTAGTAAAGACGTTTGGATGTTACTCC-3') with Phusion® High-Fidelity DNA Polymerase (New England Biolabs). The purified *sero45* PCR fragments and the plasmid pUC19 (New England Biolabs) were digested with *Xba*I (New England Biolabs) and *Sph*I (New England Biolabs). *Sero45* was then inserted into pUC19 and the ligated plasmid pUC19-*sero45* was transformed into *E. coli* DH5α through heat-shock transformation. Transformants were selected as colonies resistant to 100 μg ml^{-1} ampicillin and examined via PCR using primers M13PU and M13PR for the presence of *sero45*. After confirmation of the presence of *sero45* in pUC19, plasmids pUC19-*sero45* and pHT304-*tetR* isolated from *E. coli* DH5α were digested with *Xba*I (New England Biolabs) and *Sph*I (New England Biolabs). The gene fragments *sero45* and pHT304-*tetR* were identified by size and purified from gel electrophoresis. *Sero45* was then ligated with pHT304-*tetR*. The resulted

plasmid pHT304-*tetR-sero45* was then used to transform *E. coli* DH5α through heat-shock transformation. Transformants were selected as ampicillin-resistant colonies on 2% LB agar plates containing 100 μg ml^{-1} ampicillin and examined by PCR using primers M13PU and M13PR. After confirming the existence of *sero45* in pHT304-*tetR*, the plasmids pHT304-*tetR-sero45* from *E. coli* DH5α were used to transform the nonmethylating strain *E. coli* ET12567 (Stratagene, La Jolla, CA, USA). The gene *sero45* was also sequenced using primers M13PU and M13PR and no nucleotide changes were found. The plasmid pHT304-*tetR-sero45* from *E. coli* ET12567 was then used to transform the strain *B. thuringiensis* 407 Cry^- A'Z Δ*plcR-papR* (Bouillaut et al., 2008) via electroporation. Transformants were selected as colonies resistant to 10 μg ml^{-1} tetracycline and examined through PCR using primers M13PU and M13PR for *sero45*. After verifying the existence of *sero45*, tetracycline-resistant transformants were streaked over 2% LB agar plates containing 10 μg ml^{-1} tetracycline for the isolation of single colonies. The strain *B. thuringiensis* 407 Cry^- A'Z Δ*plcR-papR* complemented with the plasmid pHT304-*tetR-sero45* was named as Sero45. A single colony of Sero45 was transferred to 5 ml autoclaved 2% LB broth using 10 μl sterile disposable inoculating loops (Greiner Bio-One) and was allowed to grow overnight at 30℃ with vigorous shaking at 200 rpm. The glycerol stock of Sero45 was prepared by mixing 250 μl of the Sero45 overnight culture with 750 μl of 80% glycerol (Sigma) in 1.5 ml cryotubes (Thermo Scientific). The glycerol stock of Sero45 was stored at – 80℃.

5.2.3 Plasmid stability

The plasmid pHT304-*sero3* and the plasmid pHT304-*tetR-sero45* were checked for their stability in *B. thuringiensis* 407 Cry^- A'Z Δ*plcR-papR*. As previously described in chapter 4, after selected on 2% LB agar plates containing either 10 μg ml^{-1} erythromycin for Sero3 or 10 μg ml^{-1} tetracycline for Sero45, Sero3 and Sero45 were allowed to grow in 5 ml sterile 2% LB broth without antibiotics for 24 h at 30℃ with vigorous shaking at 200 rpm. After 24 h growth, the culture cells were passaged 6 times successively by pipetting 5 μl of the culture cells (passage 1) into

fresh 5 ml sterile 2% LB broth without antibiotics (passage 2) for 24 h periods of time. All passages were serial diluted and 100 µl of each serial dilution were plated onto individual 2% LB agar plates. The plates were incubated overnight (16 h) at 26℃. For dilutions giving 30 to 200 colonies on plates, the number of colonies on the plate was counted. A hundred colonies from the counted plates were streaked using sterile toothpicks onto both 2% LB agar plates and 2% LB agar plates containing either 10 µg ml^{-1} erythromycin or 10 µg ml^{-1} tetracycline for Sero3 or Sero45 respectively. The streaked plates were incubated overnight at 26℃. The number of streaks that appeared on the 2% LB agar plates and the antibiotic 2% LB agar plates were noted.

5.2.4 Spore preparation for Sero3 and Sero45

For following experiments, spores of Sero3 and Sero45 were prepared by culturing cells separately on the *B. cereus* selective agar plates (chapter 2) with addition of either 10 µg ml^{-1} erythromycin or tetracycline respectively for 5 to 7 days at 37℃. After 5 to 7 days, the cells were harvested and suspended in 1.5 ml micro-centrifuge tubes with 0.75 ml 0.85% NaCl (Fisher Scientific). The cells were washed by centrifuging the cells at 6000 g min^{-1} for 3 minutes and resuspending the pellets with 0.85% NaCl solution after centrifugation. Following 2 serial washes, the cells were resuspended in 1 ml 0.85% NaCl solution and aliquoted. The aliquots were heat-treated at 65℃ for 20 minutes and stored at – 20℃ after heat-treatment. The spore concentrations of the aliquots were measured through serial dilution and plating 100 µl of serial dilutions on 2% LB agar plates. For dilution giving 30 – 200 colonies on plates, 6 plates were counted to estimate the spore concentration in each aliquot. One aliquot of each Sero3 and Sero45 was defrosted for subsequent experiments. The defrosted aliquots were stored at 4℃ and were discarded after one month. Fresh aliquot for Sero3 and Sero45 was taken out from – 20℃ and same method applied to estimate the spore concentrations in the freshly defrosted aliquots.

5.2.5 In vivo competition assay

To establish whether the polymorphism of the PlcR-PapR QS system in *Bacillus* were maintained by frequency-dependent selection, Sero3 was mixed with Sero45 at various densities and frequencies. Previously prepared spore aliquots of Sero3 and Sero45 were taken out from – 20℃ and defrosted. The spore concentrations of the defrosted aliquots were measured through serial dilution and plating as detailed above. Based on estimated spore concentrations, using 0.85% NaCl solution, Sero3 was mixed with 10%, 50% or 90% of Sero45 for final spore densities of 5×10^4 cfu μl^{-1}, 1×10^4 cfu μl^{-1}, and 2×10^3 cfu μl^{-1} respectively. As Sero3 and Sero45 is incapable of toxin production, the crystal toxin Cry1Ac purified from the previously described *E. coli* JM109 strain carrying the *Cry1Ac* gene (kindly provided by Neil Crickmore, University of Sussex, United Kingdom) was added to all the inocula at a final concentration of 1.25 µg ml^{-1}. All inocula were serial diluted and 100 µl of the serial dilutions were spread on 2% LB agar plates and 2% LB agar plates containing either 10 µg ml^{-1} erythromycin or tetracycline. The plates were incubated overnight (16h) at 26℃. At the suitable dilution (with 30 – 200 colonies per plate), 6 LB agar plates and 6 LB agar plates with the appropriate antibiotics were counted to estimate the spore density and initial frequency of Sero45 in the inocula.

For larvae infection, sloppy semi-synthetic diet was prepared using the protocol described in chapter 2 with addition of 0.4% w/v Vanderzant vitamin mixture for insects (Sigma) and 0.36% w/v L-Ascorbic acid (Sigma). Ninety-six well plates were prepared to have 60 µl of the sloppy diet and 4 µl of the inocula per well. Negative control for the experiment was 4 µl of sterile 0.85% NaCl solution with the same concentration of toxin instead of inocula. One 96-well plate was used for one inoculum. After that, 3rd instar larvae of *P. xylostella* were introduced to the sloppy diet plates, with one larva per well. The plates were then covered with dry tissue and sealed tightly to minimize the movement of larvae from one well to another, after which all the 96-well plates were incubated at 23°C for 48 h. After 48 h of infection, cadavers of *Plutella* larvae were incubated in collection microtubes

(Qiagen) containing 10 µl sterilized distilled water for 2 weeks at 30°C to make sure that the bacteria within the cadavers had fully sporulated. After 2 weeks incubation, with addition of 490 µl of 0.85% NaCl solution and a 4 mm sterile stainless steel ball in each microtubes, cadavers were homogenized by shaking the collection microtubes using TisseLyser II (Qiagen) at 22.5 Hz for 4 minutes. The homogenized cadavers were serial diluted and 10 µl of the serial dilutions were plated out on 2% LB agar plates containing as well as 2% LB agar plates containing either 10 µg ml^{-1} erythromycin or tetracycline for Sero3 or Sero45 respectively. All the LB agar plates were incubated overnight (16h) at 26℃ and counted after to estimate the spore density and final frequency of Sero45 in each cadavers. The experiment was replicated once. A total of 361 third instar *P. xylostella* larvae were inoculated with the above-stated inocula.

5.2.6 Competition assay in homogenized insect larvae

To establish whether spatial heterogeneity in insect larvae could be one of factors that limit the invasion of rare PlcR-PapR group, Sero3 was allowed to compete with 10%, 50% or 85% Sero45 respectively at a initial spore density of 1×10^3 cfu μl^{-1}. Mixed inocula of Sero3 and Sero45 were prepared via adding appropriate quantities of freshly defrosted spore aliquots of Sero3 and Sero45 to 0.85% NaCl solution. The initial frequencies of Sero45 in the mixed inocula were confirmed by serial dilution and plating on 2% LB agar plates and 2% LB agar plates containing 10 µg ml^{-1} tetracycline. For the competition assay, insect homogenates were prepared via mashing 2 late 3rd instar larvae in 1 ml sterile distilled water using TisseLyser II (Qiagen) at 22.5 Hz for 4 minutes. After that, insect homogenates were pipetted into 24-well plates and inoculated with 10 µl of the mixed inocula. For each inocula, 24 insect homogenates were infected, one 24-well plate per inocula. A total of 72 pools of insect homogenates were prepared. Inoculated insect homogenates were incubated for 4 days at 37℃.

5.2.7 In vivo competition assay with E. cloacae

To examine whether the polymorphism of the PlcR-PapR QS system in *Bacillus* were adapted to different ecological environments, I conducted an experiment in which different ecological environments were created through introducing the insect larvae with the gut bacteria strain *E. cloacae*. A factorial experiment was conducted in which an environmental treatment (with and without *E. cloacae*) was crossed with a pairwise competition treatment of Sero3, Sero45 and QS wild type. QS wild type has been described previously. Sero3, Sero45 and QS wild type belongs to PlcR-PapR group III, group IV and group I respectively (Slamti and Lereclus, 2005, Bouillaut et al., 2008). First of all, mixed inocula of Sero3, Sero45 and QS wild type were prepared using freshly defrosted spore aliquots of the three strains. The spore concentrations of the defrosted aliquots were measured through serial dilution and plating as detailed above. Based on estimated spore concentrations of each aliquots, using 0.85% NaCl solution, Sero3 was mixed with 60% Sero45 or 10 % QS wild type for final spore densities of 5×10^3 cfu μl^{-1} and 2×10^4 cfu μl^{-1} respectively. Sero45 was also mixed with 10% QS wild type for a final spore density of 3×10^4 cfu μl^{-1}. The crystal toxin Cry1Ac was added to all inocula at a final concentration of 1.25 μg ml^{-1}. The initial frequencies and densities of all inocula were confirmed via serial dilution and plating 100 μl of the serial dilutions were spread on 2% LB agar plates and 2% LB agar plates containing either 10 μg ml^{-1} erythromycin or tetracycline. For treatments with the gut bacteria strain *E.cloacae*, *E.cloacae* was allowed to grow in 2% LB broth with 100 mg ml^{-1} rifampicin overnight at 30℃ with vigorous shaking at 200 rpm. The cell density of the overnight culture of *E. cloacae* was measured through serial dilution and plating. Based on the cell density of the overnight culture of *E. cloacae*, the inoculum of *E. cloacae* was prepared using 0.85% NaCl solution for a final cell density of 3×10^5 cfu μl^{-1}.

For larvae infection, vitamin diet pots containing 10 ml of the vitamin semi-synthetic diet per 55×14 mm Petri dish were prepared using the protocol described in chapter 2 with addition of 0.4% w/v Vanderzant vitamin mixture for insects (Sigma) and

0.36% w/v L-Ascorbic acid (Sigma). The vitamin diet pots were cut into quarters using a flame-sterilized spatula, leaving a quarter of the diet per pot. The quarter diet pots were treated with 100 µl of the inocula, giving 8 quarter-diet pots per inocula of the competition treatment. For each inocula, 4 out of the 8 treated quarter diet pots were further treated with 100 µl of the inocula of *E. cloacae*. The other 4 diet pots were treated with 0.85% NaCl solution. All treated diet pots were left to dry at laminar flow cabinet for 20 min. After that, twelve 3rd instar *Plutella* larvae were introduced to each inoculated quarter diet pots, 96 insect larvae per competition treatment. The quarter diet pots were then incubated at 23°C for 48 h. After 48 h post inoculation, cadavers of *Plutella* larvae were picked up using sterile toothpicks and put into collection microtubes (Qiagen) containing 10 µl sterilized distilled water. The collection microtubes were then incubated for 2 weeks at 30°C to make sure that the bacteria within the cadavers had fully sporulated. After 2 weeks incubation, cadavers were homogenized in 490 µl of 0.85% NaCl solution with a 4 mm sterile stainless steel ball in each microtubes using TisseLyser II (Qiagen) at 22.5 Hz for 4 minutes. The homogenized cadavers were serial diluted and 10 µl of the serial dilutions were plated out on 2% LB agar plates as well as 2% LB agar plates containing either 10 µg ml^{-1} erythromycin or tetracycline for Sero3 and Sero45 respectively. All LB agar plates were incubated overnight (16 h) at 26℃ and counted after to estimate the final frequency of Sero45 and Sero3 and the spore density in each cadavers. A total of 288 third instar *P. xylostella* larvae were inoculated.

5.2.8 Data Analysis

Data analysis was carried out in R v 2.15.2 (http://www.r-project.org/). For plasmid stability data, the proportion of plasmids that were present in the bacteria in every passage were computed as the number of streaks on the antibiotic LB agar plates divided by the number of streaks on the LB agar plates. The data were analyzed with a generalized linear model with binomial errors. For *in vivo* competition assay and competitive assay in homogenized insect, the relative fitness of Sero45 is

calculated by

$$v = \frac{x2(1 - x1)}{x1(1 - x2)}$$

where x_1 is the initial frequency of Sero45 in the population and x_2 is the final frequency of Sero45 (Ross-Gillespie et al., 2007). The relative fitness of Sero45 is the estimated growth rate of Sero45 in relation to that of Sero3. The relative fitness was transformed with the logarithm to base 10 and analysed with a general linearized model. For *in vivo* competition assay with *E. cloacae*, the relative fitness of Sero45 was calculated as above mentioned in relation to Sero3 or QS wild type when Sero45 was present in the initial population. In mixed culture where Sero45 was not present, the relative fitness of Sero3 was calculated in relation to QS wild type. As the addition of *E. cloacae* reduced the productivity of Sero3 and Sero45 in insect gut, to account for the lower productivity, the lenski fitness – relative fitness per generation – is calculated by

$$v' = \frac{\frac{x1(1 - x2)}{x1(1 - x2)}}{\log_2 d}$$

where *d* is the final population size. Lenksi fitness was then transformed with the logarithm to base 10 and analysed with the general linearized model. All model assumptions were checked with graphical analyses of error distribution and normality.

5.3 Results

5.3.1 Construction of PlcR-PapR group III and group IV B. thuringiensis strains

To allow competition between the isogenic strains *B. thuringiensis* 407 Cry⁻ A'Z Δ*plcR-papR* carrying two different PlcR-PapR group genes, the strain *B. thuringiensis* 407 Cry⁻ A'Z Δ*plcR-papR* was complemented with either pHT304-*sero3* or pHT304-*tetR-sero45*. First of all, *sero*3 and *sero45* were amplified from *B. thuringiensis* subsp. *kurstaki* HD-73 and *B. thuringiensis* serovar roskoldiensis respectively. Fig 5.3 A showed the PCR products of *sero3* (lane 1, 1 µl PCR products) and *sero45* (lane 2, 1 µl PCR products). The group III and group IV *plcR* and *papR* genes are 1.3 kilobase pairs (kb) long and agreed with the band sizes observed in Fig 5.3 A, suggesting that the PCR products were *sero3* and *sero45* respectively. The plasmid vector pUC19 used for carrying *sero3* and *sero45* is 2.7 kb long. Fig 5.3 B showed the gel pictures of pUC19 that had been digested with restriction enzymes *Xba*I and *Hin*dIII (lane 2 & 3, 10 µl plasmid DNA in each lane) or *Xba*I and *Sph*I (lane 4 & 5, 10 µl plasmid DNA in each lane). Fig 5.3 B showed the correct band sizes for pUC19 (2.7 kb), suggesting that the plasmids had been linearized and hence digested successfully. The PCR products of *sero3* and *sero45* were then purified, digested and ligated with pUC19. The resulted plasmids pUC19-*sero3* and pUC19-*sero45* were used to transform *E. coli* DH5α. Transformants were selected as ampicillin-resistant colonies and confirmed through PCR. Fig 5.4 showed the PCR results of *sero3* and *sero45* from ampicillin-resistant *E. coli* DH5α transformants. Lane 2, 6, 7, 9, 10 & 11 shows band size of 1.3 kb, suggesting that the screened ampicillin-resistant *E. coli* DH5α transformants carried that the plasmid pUC19-*sero3*. The band sizes of lane 22 & 23 also suggested that transformants carried that plasmid pUC19-*sero45*.

To differentiate Sero3 from Sero45, the plasmid pHT304 was reconstructed to carry the tetracycline-resistant gene *tetR* instead of *eryR*. To start with, *tetR* was

amplified from pHT1618 and pHT304 was amplified outwards from *eryR*. Fig 5.5 A showed the PCR products of *tetR* (Fig 5.5 A, lane 2, 1.7 kb) and pHT304 (Fig 5.5 A, lane 3 & 4, 5.7 kb). Lane 4 in Fig 5.5 B showed the correct band size for pHT304. The PCR products of *tetR* and pHT304 were then purified, digested and ligated. The resulted plasmid pHT315-*tetR* was used to transform *E. coli* DH5α. Transformants were selected as tetracycline-resistant colonies and confirmed through plasmid digestion. Fig 5.5 B showed the digested DNA fragments of plasmids isolated from tetracycline-resistant *E. coli* DH5α transformants. Lane 2 shows band sizes of 1.7 kb (*tetR*) and 5.6 kb (pHT304), suggesting the tetracycline-resistant *E. coli* DH5α transformants in lane 2 carried the plasmid pHT304-*tetR*.

After confirmation of the presence of pHT304-*tetR* in *E. coli* DH5α, the plasmids pHT304 and pHT304-*tetR* was isolated and digested at the multiple cloning site. Digesting plasmids pUC19-*sero3* and pUC19-*sero45* gave DNA fragments of *sero3* and *sero45* and pUC19. Fig 5.6 A showed band sizes of 1.3 kb (*sero3* and *sero45*) and 2.7 kb (pUC19). The bands with 1.3 kb were cut out from gel and purified. DNA fragment of *sero3* was ligated with pHT304, while *sero45* was ligated with pHT304-*tetR*. The resulted plasmids were used to transform *E. coli* DH5α. Transformants were selected as erythromycin-resistant colonies for *sero3* and tetracycline-resistant colonies for *sero45*. Transformants were examined through PCR. Fig 5.6 B and Fig 5.6 C showed the PCR results of *sero3* and *sero45* from erythromycin-resistant and tetracycline-resistant *E. coli* DH5α transformants respectively. Lane 2 – 7 and lane 9 in Fig 5.6 B showed the correct band size of 1.3 kb for *sero3*, suggesting the transformants carried pHT304-*sero3*. For Fig 5.6 C, lane 1 – 4, 6 and 8 shows the right band size of 1.3 kb for *sero45*, suggesting the transformants carried pHT304-*tetR*-*sero45*.

Upon successful cloning, plasmids pHT304-*sero3* and pHT304-*tetR*-*sero45* was isolated from *E. coli* DH5α and used to transform the nonmethylating strain *E. coli*

ET12567. The nonmethylated plasmids pHT304-*sero3* and pHT304-*tetR-sero45* from *E. coli* ET12567 were then used to transform *B. thuringiensis* 407 Cry⁻ A'Z Δ*plcR-papR*. Transformants were selected as erythromycin-resistant colonies for Sero3 and tetracycline-resistant colonies for Sero45. Transformants were screened via PCR. Fig 5.7 showed the PCR products (10 µl in each lane) of the gene *sero3* (lane 1 – 4, Fig 5.7 A) and *sero45* (lane 3 & 4, Fig 5.7 B). Both *sero3* and *sero45* are 1.3 kb long and agreed with the band sizes observed in Fig 5.7, suggesting that the screened transformants carried the plasmid pHT304-*sero3* or pHT304-*tetR-sero45*.

5.3.2 Plasmid stability

Similar to pHT315, the plasmids pHT304-*sero3* and pHT304-*tetR-sero45* were extremely stable in *B. thuringiensis* 407 Cry⁻ A'Z Δ*plcR-papR*. All of the streaked colonies of *B. thuringiensis* 407 Cry⁻ A'Z Δ*plcR-papR* complemented with either pHT304-*sero3* or pHT304-*tetR-sero45* carried the plasmids after 6 passages. Passaging did not have an effect on the stability of the tested plasmids in *B. thuringiensis* ($F_{1,10}$ = 0.183, p = 0.608, n = 12) (Fig 5.8).

5.3.3 In vivo competition assay

To investigate whether the polymorphism observed in the PlcR-PapR QS system in *Bacillus* is maintained by frequency-dependent selection, Sero45 was allowed to compete with Sero3 at various initial densities and frequencies. As shown in Fig 5.9, the relative fitness of Sero45 increased significantly with increasing initial frequency of Sero45 ($F_{1,430}$ = 55.65, p < 0.0001, n = 432) regardless of the initial density in inoculum. Sero45 grew faster than Sero3 when Sero45 were more common in the inocula.

5.3.4 Competition assay in homogenized insect larvae

To examine whether spatial heterogeneity limited the relative fitness of Sero45 when this pherotype was rare, Sero45 was mixed with Sero3 at various frequencies at the initial spore density of 1×10^3 cfu μl^{-1}. The mixed cultures were allowed to grow in insect homogenates under shaking condition. As shown in Fig 5.10, in shaken insect homogenates, relative fitness of Sero45 in mashed insects decreased significantly with increased initial frequency of Sero45 (solid line, $F_{1,70} = 102.06$, $p < 0.0001$, n = 72). Constrasting *in vivo* competition assay of Sero3 and Sero45, Sero45 had higher relative fitness when they were rare at 1×10^3 cfu μl^{-1} initial spore density.

5.3.5 In vivo competition assay with E. cloacae

To investigate whether the polymorphism of the PlcR-PapR QS system in *B. thuringiensis* evolved as adaption to different environment niches, I examined fitness of different pherotypes in insect gut with or without the gut bacteria *E. cloacae*. I analyzed the fitness data with the general linearized model using the following formula

glm(formula = fitness ~ jjbc, subset = (treatment == "")

where jjbc represented the gut bacteria *E. cloacae*. Fitness was either the relative fitness or the lenski fitness. Treatment was either 45/3, 3/wt or 45/wt. When competing with QS wild type, the addition of *E. cloacae* significantly reduced the relative fitness of Sero45 ($F_{1,85} = 12.70$, $p < 0.0006$, n = 87) but significantly improved the relative fitness of Sero3 ($F_{1,90} = 6.96$, $p < 0.0098$, n = 92) (Fig 5.11 A). When competing with Sero3, the relative fitness of Sero45 was significantly increased after introducing *E. cloacae* ($F_{1,88} = 36.44$, $p < 0.0001$, n = 90) (Fig 5.11 A). However, the productivity of Sero3 and Sero45 in host was significantly reduced when challenged with *E. cloacae* ($F_{1,267} = 19.34$, $p < 0.0001$, n = 269) (Fig 5.11 B). After accounting for the effect of *E. cloacae* on reproduction of Bt in the host, when competing with QS wild type, the lenski fitness of both Sero45 ($F_{1,85} = 74.88$, $p <$

0.0001, n = 87) and Sero 3 ($F_{1,90}$ = 7.49, p < 0.0075, n = 92) were significantly increased (Fig 5.11 C). The lenski fitness of Sero45 was significantly increased ($F_{1,88}$ = 36.93, p < 0.0001, n = 90) when competing with Sero3, (Fig 5.11 C).

Fig 5.3 Gel picture of PCR products of *sero3* and *sero45* and gel picture of digested pUC19. (**A**) Lane 1: *sero3* from *B. thuringiensis* subsp. *kurstaki* HD-73 (1.3 kb, 1 µl DNA), lane 2: *sero45* from *B. thuringiensis* serovar roskoldiensis (1.3 kb, 1 µl DNA) and lane 3: 2-Log DNA Ladder (5 µl). (**B**) Lane 1: 2-Log DNA Ladder (5 µl), lane 2 & 3: pUC19 digested with *Xba*I and *Hin*dIII (2.7 kb, 10 µl DNA) and lane 4 & 5: pUC19 digested with *Xba*I and *Sph*I (2.7 kb, 10 µl DNA).

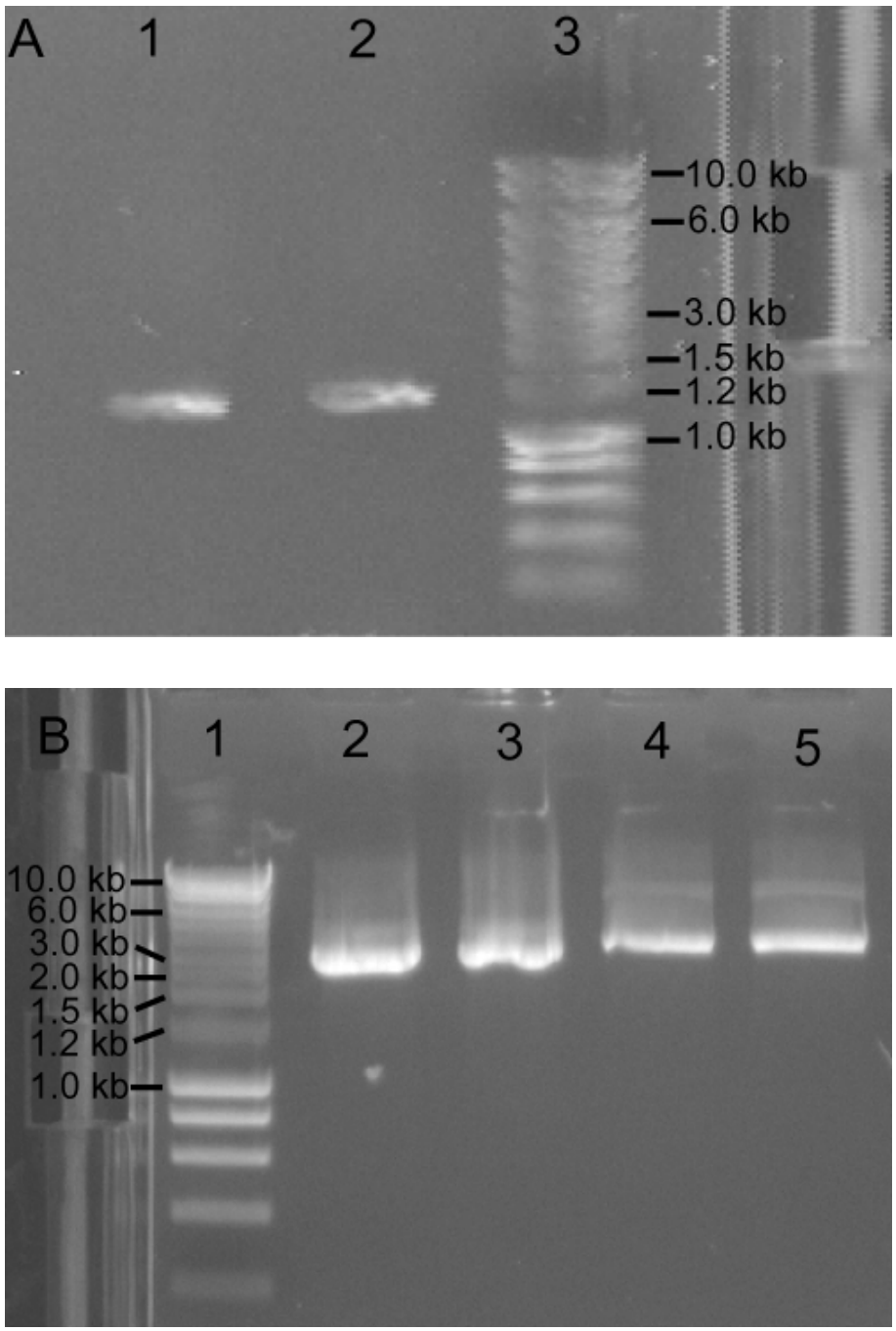


Fig 5.4 Gel picture of PCR products of *sero3* and *sero45* from ampicillin-resistant *E. coli* DH5α transformants. Lane 1, 20, 21 & 27: DNA Ladder (5 µl), lane 2 – 11: PCR products of *sero3* (1.3 kb, 1 µl) and lane 12 – 19 & 22 – 26: PCR products of *sero45* (1.3 kb, 1 µl). Lane 2, 6, 7, 9, 10 & 11 shows band sizes of 1.3 kb, suggesting transformants carried that the plasmid pUC19-*sero3*. For *sero45*, lane 22 & 23 shows correct band size of *sero45*, which is 1.3 kb.

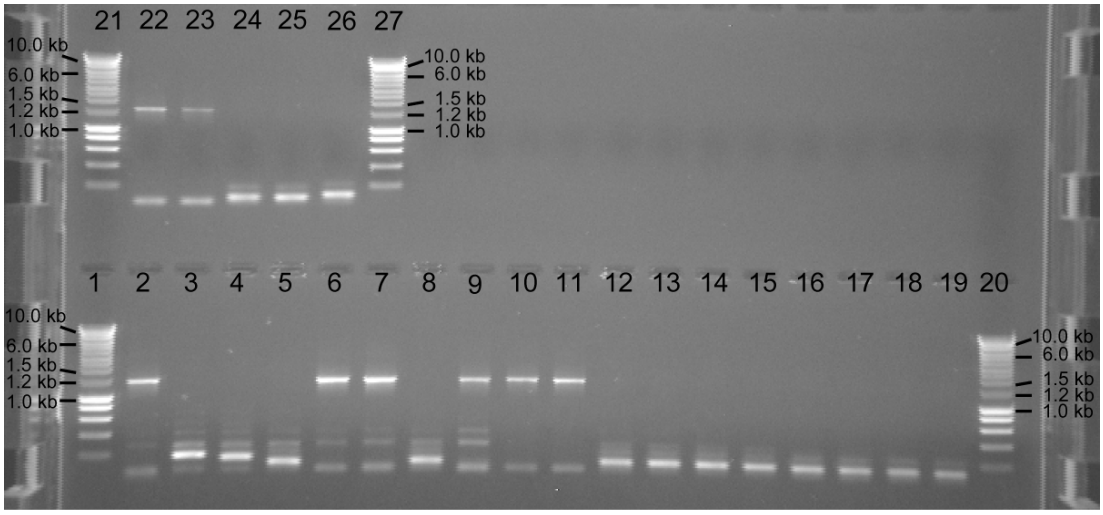


Fig 5.5 Gel pictures of PCR products of *tetR* and pHT304 and gel picture of digested plasmid DNA fragments of tetracycline-resistant *E. coli* DH5α transformants. (**A**) Lane 1: DNA Ladder (5 µl), lane 2: PCR products of *tetR* (1.7 kb, 1 µl) and lane 3 & 4: PCR products of pHT304 (5.6 kb, 1 µl). (**B**) Lane 1 – 8: digested DNA fragments of plasmids isolated from tetracycline-resistant *E. coli* DH5α transformants (10 µl in each lane) and lane 9: DNA Ladder (5 µl). Lane 2 shows band sizes of 1.7 kb (*tetR*) and 5.6 kb (pHT304), suggesting the tetracycline-resistant *E. coli* DH5α transformants corresponded to the result in lane 2 carried the plasmid pHT304-*tetR*.

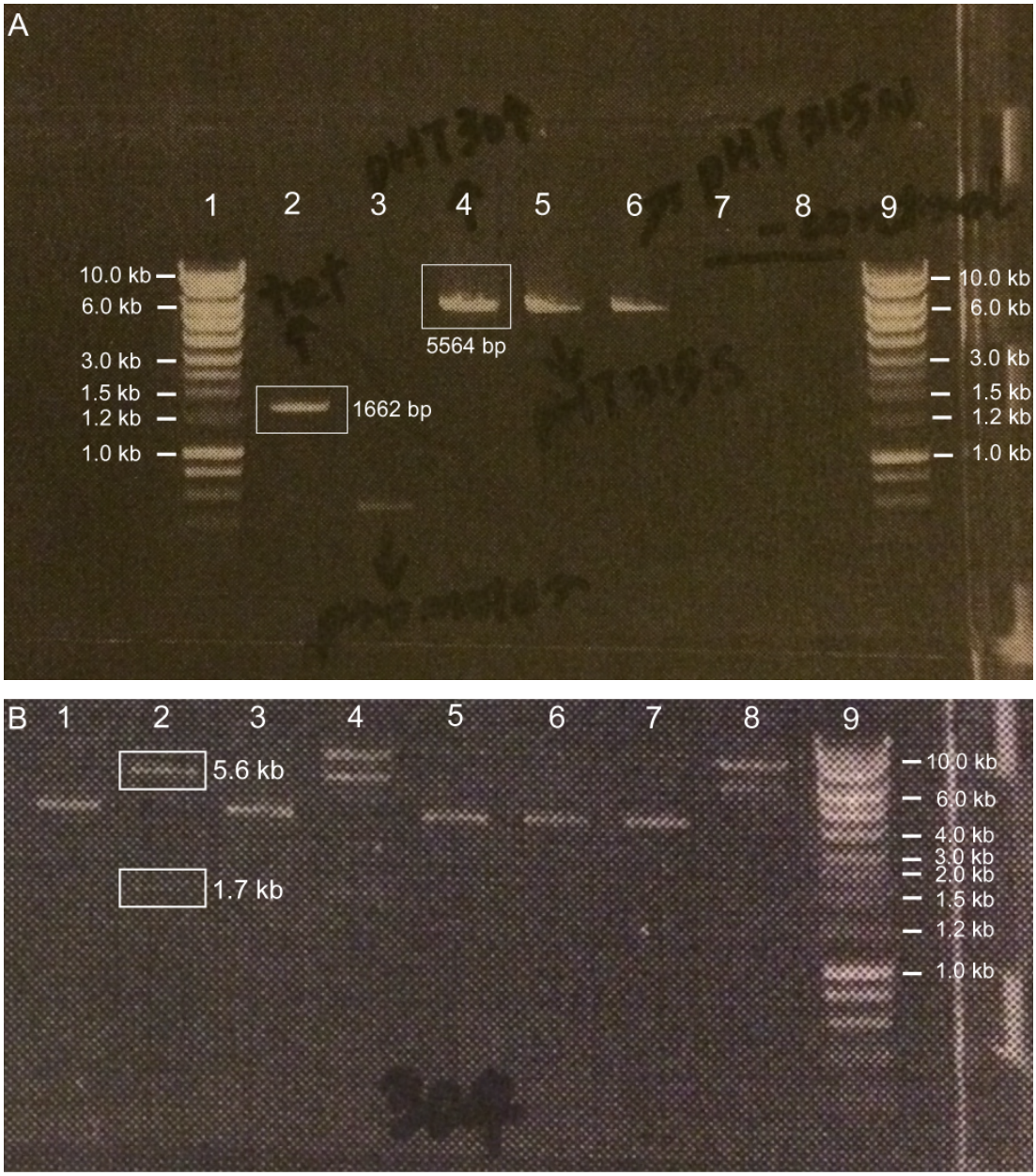
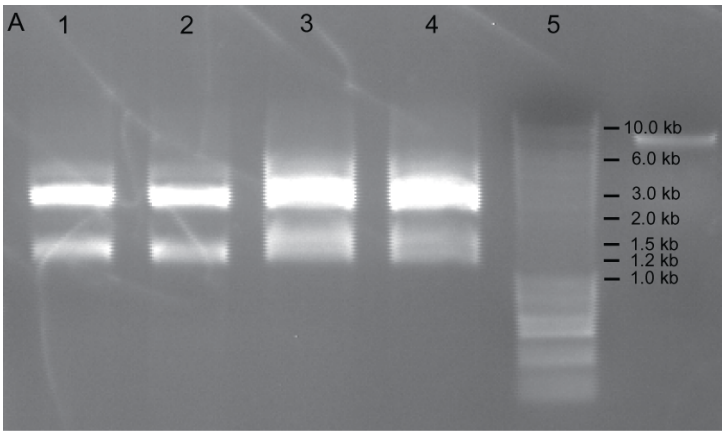
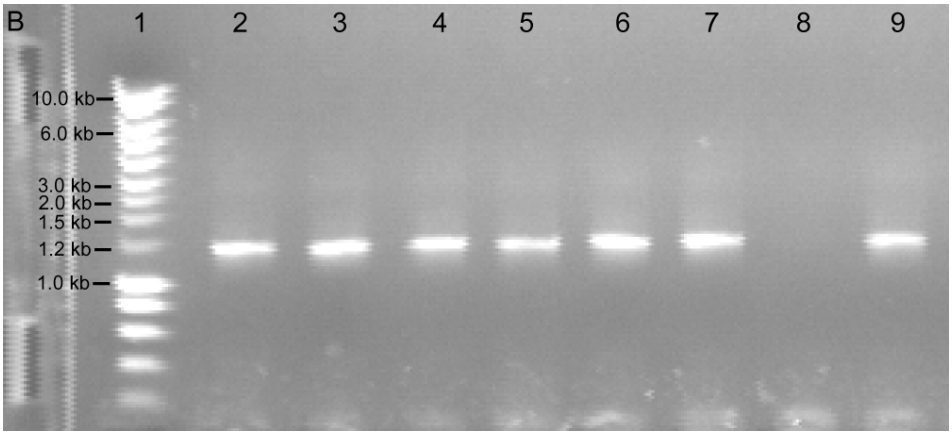


Fig 5.6 Gel picture of plasmid digest of pUC19-*sero3* and pUC19-*sero45*, gel pictures of PCR products of *sero3* and *sero45* *E. coli* DH5α transformants. (**A**) Lane 1 & 2: DNA fragments of digested pUC19-*sero3*, lane 3 & 4: DNA fragments of digested pUC19-*sero45* and lane 5: DNA Ladder (5 µl). (**B**) Lane 1: DNA Ladder (5 µl) and lane 2 – 9: PCR products of *sero3* (1.3 kb, 1 µl). Lane 2 – 7 and lane 9 shows the correct band size of 1.3 kb. (**C**) Lane 1: DNA Ladder (5 µl), lane 2 – 9: PCR products of *sero45* (1.3 kb, 1 µl). Lane 1 – 4, 6 and 8 shows the right band size of 1.3 kb.





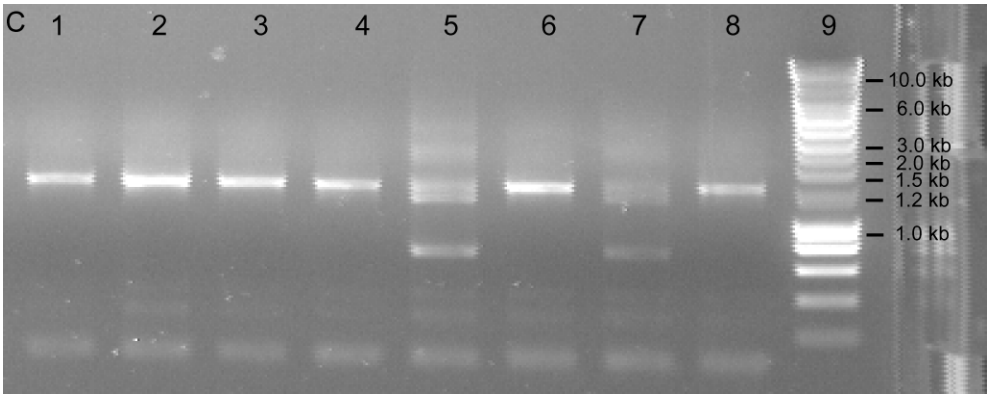
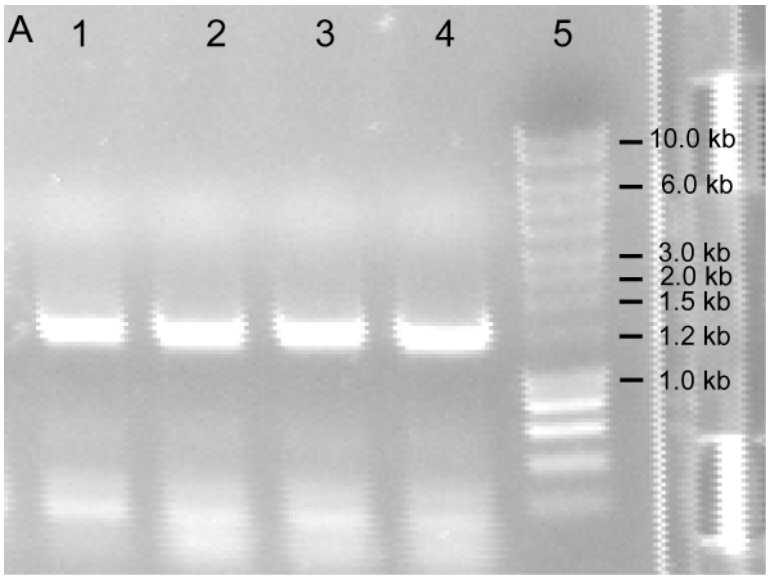


Fig 5.7 Gel pictures of PCR products of the gene *sero3* or *sero45* from transformants of *B. thuringiensis* 407 Cry⁻ A'Z Δ*plcR-papR*. (**A**) Lane 1 – 4: PCR products (1.3 kb, 10 µl in each lane) of *sero3*, and lane 5: 2-Log DNA Ladder (New England Biolabs, 5 µl). Lane 1 – 4 shows the band size of 1.3 kb, suggesting that the tested transformants carried the plasmid pHT304-*sero3*. (**B**) Lane 1: 2-Log DNA Ladder (5 µl) and lane 2 – 4: PCR products (1.3 kb, 10 µl in each lane) of the gene *sero45*. Lane 3 & 4 shows the band size of 1.3 kb, suggesting that the plasmid pHT304-*tetR-sero45* was present in the screened transformants.



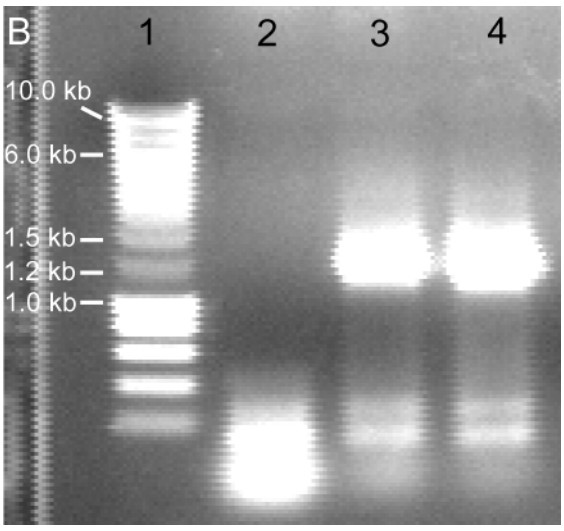


Fig 5.8 The plasmids pHT304-*sero3* and pHT304-*tetR-sero45* demonstrated very stable replication in *B. thuringiensis* 407. The proportion of stable plasmids in Sero3 and Sero45 was plotted against the passage number. The plasmid pHT304-*sero3* and the plasmid pHT304-*tetR*-sero45 were very stable in *B. thuringiensis* 407 Cry⁻ A'Z Δ*plcR-papR*. After 6 passages, 95% of the bacteria carried the pHT304 plasmid.

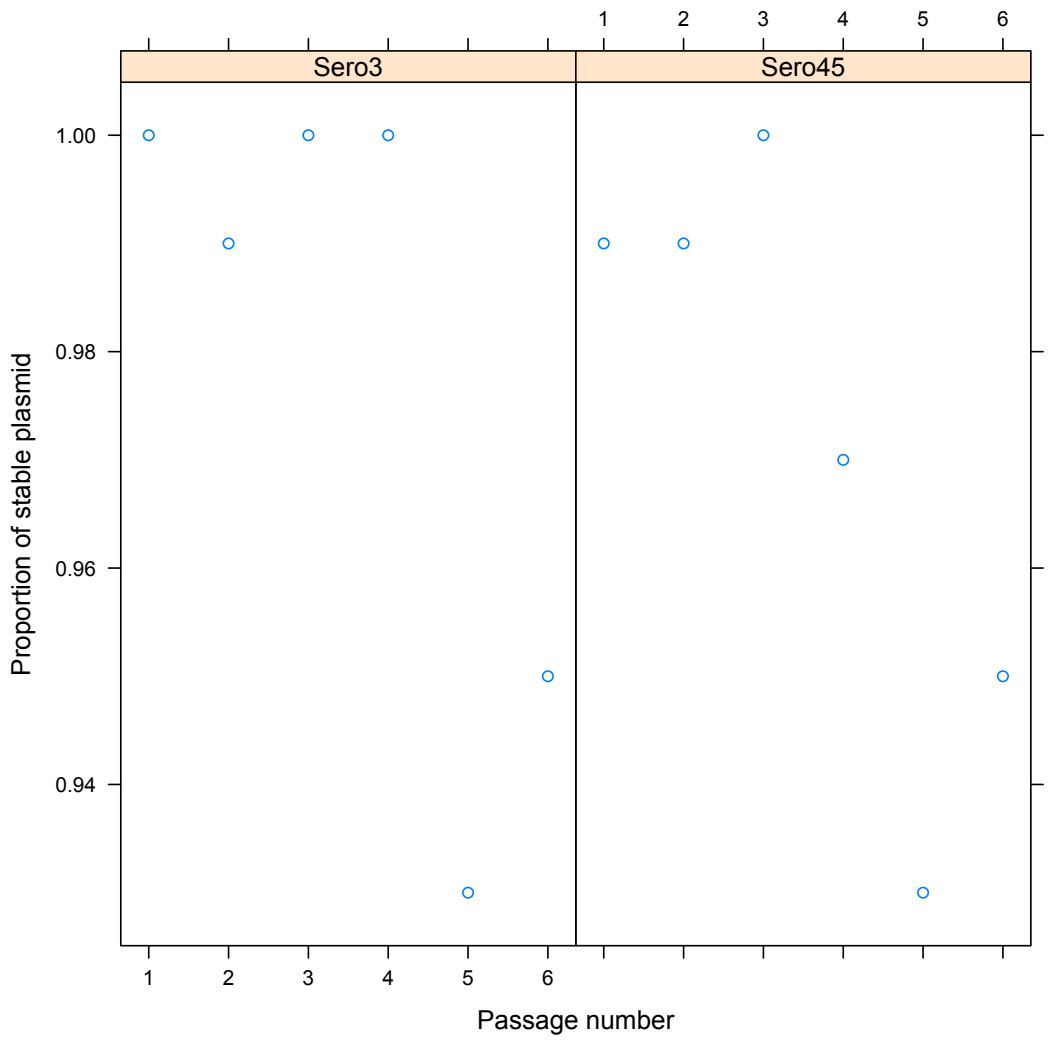


Fig 5.9 The relative fitness of Sero45 increased with the initial frequency of Sero45. Sero3 was mixed with Sero45 at frequencies. Relative fitness of Sero45 is plotted against its initial frequency in the inoculum for data in experimental block 1 (open circles) and block 2 (solid circles). A significantly positive relationship between the relative fitness of Sero45 and initial frequency of Sero45 was observed (solid lines, $F_{1,430} = 55.65$, $p < 0.0001$, n = 432). Sero45 had higher relative fitness when they were common regardless of the initial density in inoculum. Error bars represent one standard error about the mean.

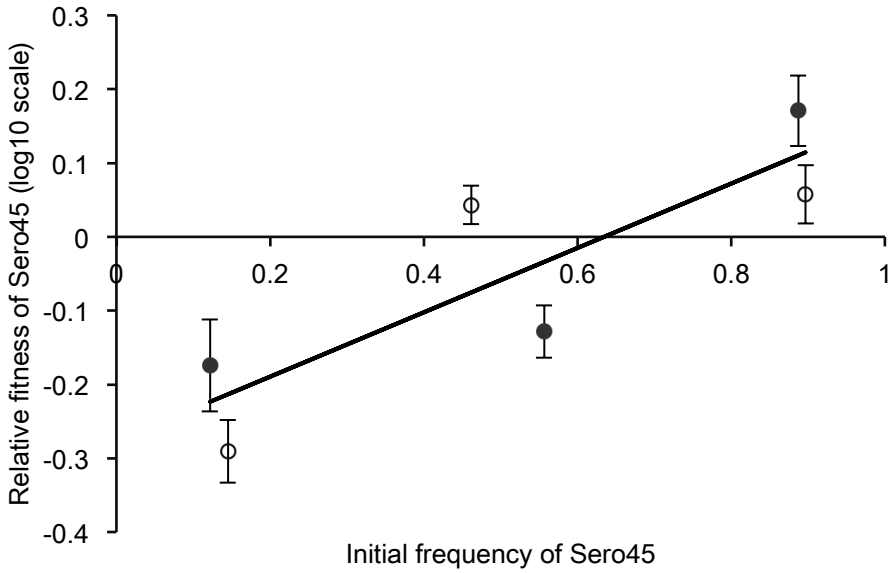


Fig 5.10 In homogenized insects the relative fitness of Sero45 decreased with the initial frequency of Sero45. Sero3 was mixed with Sero45 at various frequencies at the initial spore density of 1×10^3 cfu μl^{-1}. Relative fitness of Sero45 is plotted against its initial frequency in the inoculum. Relative fitness of Sero45 in mashed insects decreased significantly with increased initial frequency of Sero45 (solid line, $F_{1,70} = 102.06$, $p < 0.0001$, n = 72). Sero45 had higher relative fitness when they were rare at 1×10^3 cfu μl^{-1} initial spore density. Error bars represent one standard error about the mean.

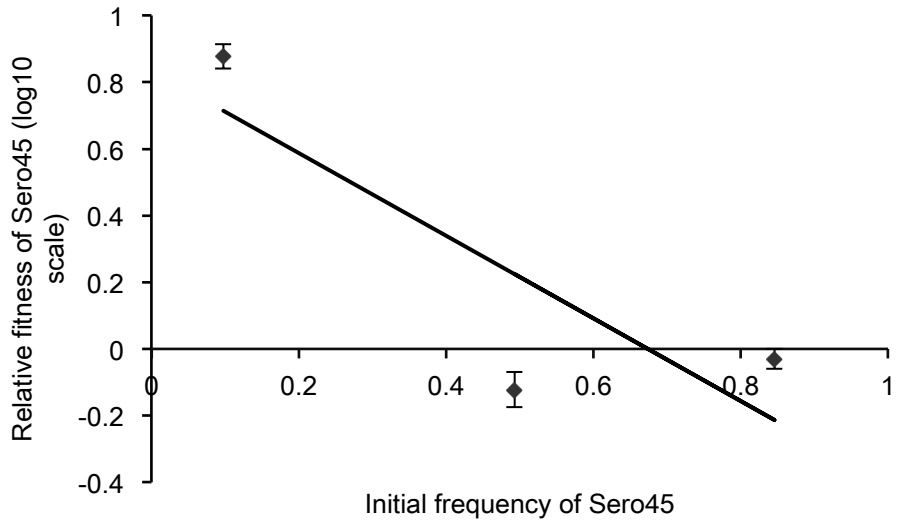
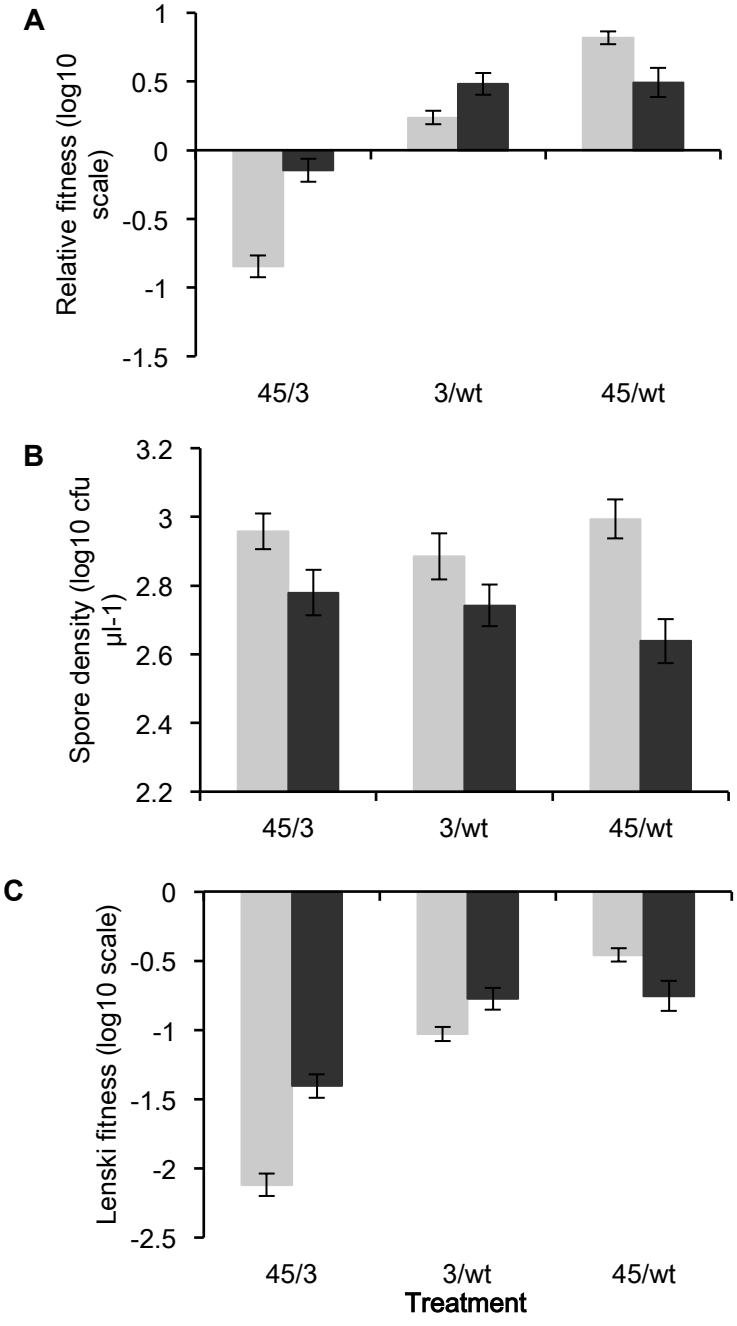


Fig 5.11 The addition of *E. cloacae* siginicatly affected the fitness of Sero3 and Sero45 in host. (**A**) The relative fitness and (**B**) the productivty of the mixed cultures in host are plotted with (black) or without (grey) *E. cloacae*. The relative fitness is plotted for the relative fitness of Sero45, Sero3 and Sero45 in mixed cultures of 45/3, 3/wt and 45/wt respectively. **C**) Fitness per generation is also plotted with (black) or without (grey) *E. cloacae*. Overall, introducing *E. cloacae* into insect gut greatly affected the fitness of different pherotypes. Error bars represent one standard error about the mean.



5.4 Discussion

The PlcR-PapR QS system in *B. cereus* group exhibits a polymorphism comprising four distinct pherotypes (Slamti and Lereclus, 2005). Strains from the same pherotype communicate efficiently while cross talk between different pherotypes is limited (Bouillaut et al., 2008). In order to study the evolution of the PlcR-PapR polymorphism, I constructed the strain *B. thuringiensis* 407 Cry⁻ A'Z Δ*plcR-papR* to carry either the plasmid pHT304-*sero3* or pHT304-*tetR-sero45*. Prior competition assay, the stability of the plasmids in *B. thuringiensis* 407 Cry⁻ A'Z Δ*plcR-papR* were first examined. Corroborating with previous study, the plasmid pHT304 was very stable in *B. thuringiensis* 407. After 6 successive passages without antibiotic selection, the plasmid remained present in 95% of the cells (Fig 5.8).

I tested the hypothesis that the PlcR-PapR polymorphism in *B. thuringiensis* was maintained by frequency dependent selection. According to the facultative cheating model, in mixed population, cells from the same pherotype communicate with each other efficiently, stimulating the production of public goods. A rare pherotype in mixed population should be able to exploit the public goods produced by the common pherotype, out-competing the common pherotype. The relative fitness of the rare pherotype should be greater when they are rarer as a result of more public goods in the population. I hypothesize that the relative fitness of a pherotype should decrease with increasing initial frequency of the pherotype – negative frequency dependent. Contradicting to the hypothesis, *in vivo* competition assay of group III and group IV pherotypes found a significant positive frequency dependent relationship between the relative fitness of group IV pherotype and the initial frequency of group IV pherotype in the inocula (Fig 5.9). However, competition assay of group III and group IV pherotypes in insect homogenates showed that the relative fitness of group IV pherotype was negatively dependent on the initial frequency of group IV pherotype in the inocula (Fig 5.10). I have only found support for the facultative cheating hypothesis in homogenized insects. In insect midgut where the PlcR regulon is likely to be activated (Fedhila et al., 2003, Gohar et al.,

2008, Salamitou et al., 2000), segregation of cells from the same pherotype, perhaps due to the adhesion of PlcR-PapR expressing QS cells to insect epithelial cells (Ramarao and Lereclus, 2006), could potentially favour the growth of group IV pherotype when they were common as the public goods were only shared between the same pherotype in the local group. Further studies of the expression of the PlcR regulon in insect gut is required to provide the basis for a comprehensive understanding of the evolution of the PlcR-PapR polymorphism.

Apart from facultative cheating hypothesis, the hypothesis that the polymorphism observed in the PlcR-PapR QS system was maintained by environment dependent fitness was also investigated. I hypothesized that the presence of the gut bacteria *E. cloacae* could render QS between *Bacillus* species ineffective and affect the fitness of a particular pherotype in a mixed population. The bacteria, *E. cloacae*, was an effective competitor: it significantly reduced the productivity of *B. thuringiensis* in cadavers (Fig 5.11 B), corroborating previous studies on *B. thuringiensis* infection of *P. xylostella* (Raymond et al., 2009). The addition of the gut bacteria *E. cloacae* significantly increased the fitness of group III pherotype but significantly decreased the fitness of group IV pherotype when competing with QS wild type. When competing with group III pherotype, the fitness of group IV pherotype was also significantly increased with the introduction of *E. cloacae*. Overall, the fitness of different pherotype was significantly affected by the presence of *E. cloacae*. However, there wasn't a single strain that was particularly favour by *E. cloacae*. The results were also confounded by the low frequencies of group III and group IV pherotypes in mixed culture of 3/wt and 45/wt respectively. The rare group III and group IV pherotypes in the mixed culture could be at a disadvantage when competing with QS wild type. Nonetheless, experimental conditions were otherwise identical in competition experiments that examined the effect of the presence or absence of *E. cloacae*. Studies of the Agr QS system in *Staphylococcus* species suggested that the polymorphism in the Agr QS system could be related to differences in disease patterns (Ji et al., 1997) perhaps due to different preferences

of colonization site of the virulence factors regulated by the Agr QS system (Shinefie.Hr et al., 1974). As the PlcR regulon also regulate various virulence factors as well as flagellin proteins potentially involved in the adhesion of *B. thuringiensis* to insect epithelial cells (Gohar et al., 2002, Gohar et al., 2008, Ramarao and Lereclus, 2006), it is likely the *E. cloacae* interferes differently with the colonization of different pherotypes in insect gut thereby potentially increasing the fitness of one pherotype while decreasing the fitness of the others. A healthy larval midgut is typically occupied by a diverse population of gut microbiota (Engel and Moran, 2013, Tang et al., 2012). The natural environment of insect gut is complex with each gut bacterium interacting differently with different pherotypes. Environment dependent fitness therefore has the potential be the force that maintains the polymorphism of the PlcR-PapR QS system in *B. thuringiensis*.

CHAPTER 6 GENERAL DISCUSSION

An increasing body of evidence suggests that bacteria can engage in multicellular behaviour via QS (Kleerebezem et al., 1997, Miller and Bassler, 2001, Whitehead et al., 2001). Many of the multicellular behaviours regulated by QS have a broad spectrum of impact of the daily lives of many people, such as the QS regulated virulence and antibiotic resistance of *S. aureus* and *P. aeruginosa*, both of which are opportunistic pathogens capable of causing serious infections in human (Pearson et al., 2000, Yarwood and Schlievert, 2003). Understanding these behaviours could provide alternative strategies to clinical intervention. In this thesis, I developed the diamondback moth larvae as an *in vivo* system for exploring the evolution and ecology of QS. I tested social evolution theory in this system by investigating the standard predictions of cheat invasion, density and frequency dependence. I also tested ideas of facultative cheating and environment dependent fitness in an attempt to explain the diversity and polymorphism of QS systems.

Social evolutionary studies on QS systems in the opportunistic pathogen *P. aeruginosa* have shown promising solutions to clinical intervention, with social cheats readily exploiting cooperative populations and reducing virulence effectively (Diggle et al., 2007, Rumbaugh et al., 1999). Similar findings have also been shown in *B. thuringiensis* infections in rabbit eyes (Callegan et al., 2005). However while effective cheating is prevalent in laboratory systems with well-mixed bacterial populations, such systems may be rare in nature. In addition, in the burned wound mouse and rabbit eye system, model hosts were infected by direct injection under the skin or eyes, bypassing the normal infection barriers imposed by the host. Hosts usually display a range of immune response to bacterial infection, leading to a narrow population bottleneck. Many of the studied QS systems indicated a high fitness of cheats. Incorrect notions of high cheater fitness in QS can also lead to theories that have limited application *in vivo*, such as the facultative cheating model

which has been suggested to maintain polymorphic QS systems (Eldar, 2011). Facultative cheating can not explain the evolution of polymorphic QS systems in natural host in the *B. cereus* group. In well-mixed populations, facultative cheating that increases the fitness of rare pherotypes may occur. However, the facultative cheating model has not considered the possible influence of host immune responses or competitors in the host, which can shape the evolution and ecology of competing pherotypes very differently. Mice treated with synthetic antagonistic peptides the pathogenic *S. aureus* showed resistance to *S. aureus* infection (Mayville et al., 1999), suggesting a novel strategy of quorum quenching in disease control. However, the mouse abscess model again bypasses host infection barriers. Overall, many of the studies in QS systems suggests effective cheating, which bring the puzzling question that why are QS systems so widespread?

The evolution and ecology of QS in natural *in vivo* system may not be intuitively obvious, as it varies with infection type, ecological conditions in the host and even time. I found that spatial structure and host defences prevent the invasion of cheats. Spatial segregation of QS wild type and QS mutants can prevent QS mutants from exploiting the public goods produced by the QS wild type. Host defences can shape the evolution and ecology of QS, potentially creating severe population bottleneck leading to higher relatedness and higher level of cooperation. Spatial structure and population bottlenecks have one shared consequence – high relatedness in the local group. According to Hamilton's rule, high relatedness favour the evolution of cooperative behaviours (Hamilton, 1964). Spatial structure and host defences could explain why QS is so widespread in bacteria when spatial structure and host defences are common in nature. Facultative cheating does not select for polymorphic QS system as spatial structure and competitors in host prevent the invasion of rare pherotypes. Sequence data of various polymorphic QS system suggested that the polymorphism evolved via point mutants or recombinational changes with a positive selection of compensatory mutations in other elements of QS systems (Ansaldi and Dubnau, 2003, Dufour et al., 2002, Havarstein et al., 1997,

Ichihara et al., 2006, Slamti and Lereclus, 2005, Tortosa et al., 2001). I suggest that environmental dependent fitness maintain the polymorphism in various QS systems. Different competitors in host can interact differently with different pherotypes, leading to a different fitness benefit of different pherotypes surviving various host conditions.

Understanding the social behaviour in bacteria could help to tackle infectious disease and provide novel medical interventions. One obvious strategy for clinical intervention is to reduce the expression of QS regulated traits by diminishing QS signalling effort. If wild type quorum sensing cells can be outcompeted by cheaters then it has been argued that this competitive interaction could be exploited so as to manipulate bacterial populations within hosts. This idea has been termed a Trojan horse strategy, as it may be a means of replacing virulent and potentially antibiotic resistant strains with antibiotic susceptible cheaters (Brown et al., 2009, Foster, 2005). A Trojan horse cheat is less virulent than its wild type counterpart, but is vulnerable to host immune response and antibiotic treatments (Brown et al., 2009). A Trojan horse cheats can out compete cooperative cells resulting in a less virulent infection. Once the Trojan horse cheats have successfully invaded a cooperative population, cheats can be eradicated by host immune response and antibiotic treatment. Evidence from experiments on QS regulated virulence and biofilm production of *P. aeruginosa* have shown that cheating in QS population can result in a reduced virulence in mouse (Rumbaugh et al., 2009) and a reduced biofilm formation which in turn increased antibiotic susceptibility of the opportunistic pathogen (Popat et al., 2012). However, the role of QS in natural infection may not be as immediately obvious as previous models have suggested. For instance, in infections of *S. aureus* in murine abscesses and wounds using a mouse model, mixed-strain infection with virulent producers and QS mutants led to a higher productivity in host as a result of mutant proliferation (Schwan et al., 2003). Invasion of QS mutants shifted virulence factor production towards biofilm formation, potentially prompting a chronic infection instead of an acute one (Papakyriacou et

al., 2000, Vuong et al., 2003). With effective cheating in well-mixed populations, QS mutants can readily exploit public goods producers and the Trojan horse approach maybe workable. However, spatial structure and host defences can, counter-intuitively, select for cooperative traits, leading to high virulence.

Another possible tactic to interfere with QS signalling has been described as quorum quenching. As QS signals are external, the environment and presence of competitors could affect QS efficiency and persistence. The ability of signalling molecules from one pherotype in inhibiting the growth of other pherotype from the same species has been noted in *S. aureus* (Lyon et al., 2002). Many *Bacillus* species are capable of cleaving the lactone rings from the AHL molecules using an N-acyl homoserine lactonase, AiiA, rendering QS between Gram-negative bacteria ineffective (Dong et al., 2000). Overexpression of AiiA in plant or co-culture of *B. thuringiensis* attenuated the QS regulated virulence of *E. carotovora* (Dong et al., 2001, Dong et al., 2004). In addition to interspecific and intraspecific quorum quenching in bacteria, human epithelial cells also showed quorum quenching ability towards *P. aeruginosa* LasI/LasR QS system (Chun et al., 2004). However, in a clinical assessment of cystic fibrosis patients, quorum quenching can select for high virulence and cooperation in *P. aeruginosa* as the use of QS-inhibitor prevented the invasion of less virulent cheats (Kohler et al., 2009). I found that infection with two pherotypes does not result in inhibition of growth of each other. The fitness of different pherotypes is dependent on the environment. The presence of competitors in host can reduce the productivity of QS strains in host and affect the frequency of different pherotypes, aiding host resistance to infection. Overall, quorum quenching as a novel medical therapy is promising if cheating is not prevalent and effective. If the virulence factors regulated by QS are crucial to a successful infection, a quorum quenching approach will be effective for disease control and will not select for high virulence.

In conclusion, bacterial quorum sensing systems are complex and often multiple QS circuits exist in a single cell. Bacteria live in a fluctuating environment filled with chemicals, some of which are signals and some of which are hostile molecules from host and competitors. The infection process of bacteria is a constant battle against the host immunity and other competitors. Conducting experiments in difficult 'non-model' organisms can provide much insight into how QS have evolved and are maintained in the face of selfish cheating strategies.

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