Knockout mutations of the LuxR regulator PFL_3627 and the sRNA *phrS* causes pleiotropic effects on the biocontrol bacterium *Pseudomonas protegens* Pf-5

Huayu Qin
B. Sc, Nanjing Agriculture University
M. Sc (Biotechnology), University of Western Sydney

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Department of Chemistry and Biomolecular Sciences

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Abstract

The *Pseudomonas protegens* Pf-5 (previously known as *P.fluorescens* Pf-5) has shown potential as a biological control agent against plant pathogenic bacteria, fungi and nematodes. The production of anti-pathogen compounds and rhizosphere colonisation are two of the key traits that determine its biocontrol competency. Genetic and biochemical studies of secondary metabolite biosynthesis combined with analysis of the genome sequence have clarified many aspects of the production of anti-pathogen compounds in Pf-5. However, there is only very limited data on the molecular factors important for rhizosphere colonisation. Successful rhizosphere colonisation is likely influenced by two features: motility and biofilm formation. In many bacteria, motility and biofilm are both regulated by quorum sensing (QS) and small RNAs (sRNA). This study focuses on a LuxR solo regulator PFL_3627 involved in QS, and a sRNA *phrS*. Gene knockouts of both of these genes reveal they have pleiotropic effects on both biofilm formation and motility. A microarray analysis of a PFL_3627 mutant revealed a large number of genes that are up- or down-regulated by this regulator. Generally, canonical QS regulation by LuxR is mediated by the exogenous or endogenous QS signalling molecules N-acyl-homoserine-lactone (AHL). The Pf-5 genome does not encode a LuxI homolog for AHL biosynthesis, and consistent with this no endogenous AHL production was detected using biosensor strains. An assay of exogenous addition of short or long chain AHLs suggested that the LuxR regulator PFL_3627 is not involved in sensing AHLs. Thus PFL_3627 is presumably involved in the sensing of an as yet unknown molecule, possibly of plant origin.

Statement of candidate

I certify that the work in this thesis entitled “Knockout mutations of the LuxR regulator PFL_3627 and the sRNA *phrS* causes pleiotropic effects on the biocontrol bacterium *Pseudomonas protegens* Pf-5” has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution other than Macquarie University.

I also certify that the thesis is an original piece of research and it has been written by me. Any help and assistance that I have received in my research work and the preparation of the thesis itself have been appropriately acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Huayu Qin (42785014)

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Chapter 1. Introduction

1.1 Biocontrol

1.1.1 The demand for biocontrol in agriculture

To intensify the agricultural productivity for the growing food demand under an increasing global population, we require effective control of the devastation imposed by plant pathogens on crops and other agricultural products. However, the traditional major pest management strategy involves the enormous use of pesticide chemicals, which gives rise to severe environmental pollution as well as human health issues. Besides, there are certain pathogens that are not effectively controlled by existing pesticides(Gerhardson 2002). Therefore, upon the requirement of developing pollution-free strategies for pathogen management with minimized negative impacts, biocontrol has been proposed as one alternative or supplemental way to reduce agrochemical usage (Gerhardson 2002, Welbaum et al. 2004).

Biocontrol is the exploitation of living organisms as agents to suppress the survival of plant pathogens (Flint et al. 1998). A number of microbes have been utilized for plant disease management and pathogen control. One of the most widely used microbial species, the gram-positive bacteria *Bacillus thuringiensis* which can dwell on leaf surfaces, marine aquatic environments, animal feces, insect rich environments, flour mills and grain storage facilities has insecticidal properties and is used as an environmentally friendly method for controlling selectively the harmful insects including mosquito and nematodes (Fillinger et al. 2003, Crickmore 2005). Apart from that, the *Pseudomonas* bacteria include several strains with intriguingly effective suppression on some notorious fungal and oomycete plant pathogens such as *Gaeumannomyces, Thielaviopsis, Rhizoctonia, Fusariumoxysporum,* and *Pythium* sp. (Cook et al. 1995, Haas and Defago 2005, Mercado-Blanco and Bakker 2007).

As the interest in biocontrol is growing, more than 40 strains with biocontrol properties from various bacterial genera including *Agrobacterium, Aspergillus, Bacillus, Coniothyrium, Gliocladium, and Pseudomonas* have been commercialized and applied to manage plant diseases in agriculture (Fravel 2005). These strains use various mechanisms to achieve their anti-pathogen activity.

1.1.2 Mechanisms of biocontrol

Biocontrol microbes have several recognized modes to achieve effective suppression against plant pathogens: i) competition over the niche for colonisation, ii) induction of inherent plant resistance to reduce or eliminate the severity of diseases, and iii) antagonism against pathogens mediated by synthesized allelochemicals with various functions (Compant et al. 2005). The suppression of plant pathogens by induction of ISR or biosynthesis of biological control compounds has been depicted in Figure 1.1.

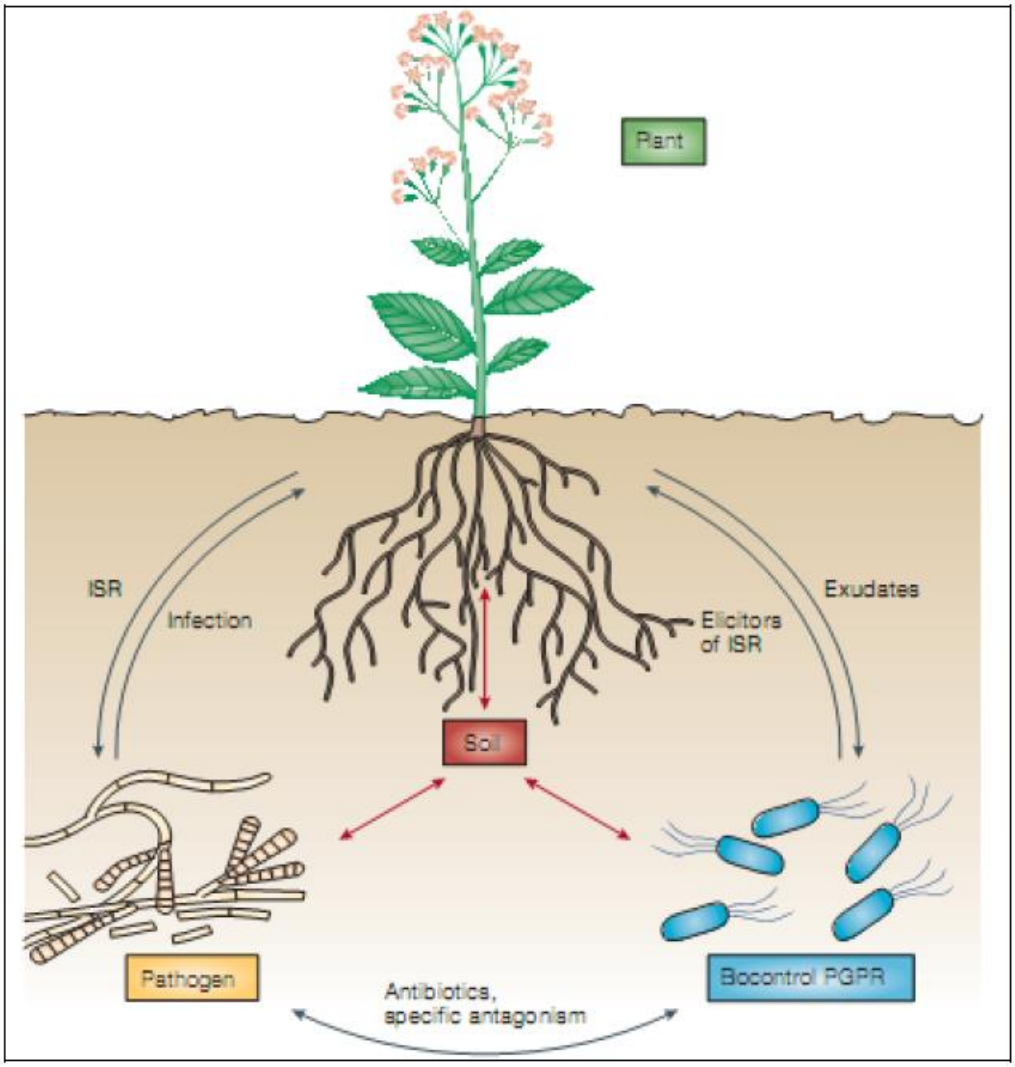

Figure 1.1: Suppression of plant pathogens by the induction of ISR or the production of anti-pathogen compounds. The biocontrol plant-growth promoting rhizobacteria (PGPR) can synthesize antibiotics which antagonise plant pathogens to eliminate their infection on plants, or induce systemic resistance which will defend plants from pathogens. Illustration is adapted (Haas and Defago 2005).

One mechanism to achieve pathogen suppression is the competition for niche occupation and essential nutrients. The plant root surface and surrounding rhizosphere are rich in carbon nutrients (Rovira 1965), therefore these are suitable niches with nutrients to attract microorganisms (Compant et al. 2005). Biocontrol bacteria can successfully compete against

pathogens for plant surface colonisation and nutrient utilisation and therefore suppress phytopathogenesis (Duffy 2001). One typical example is the biological control bacterium *Enterobacter cloacae* which competes for plant-derived unsaturated long-chain fatty acids as a nutrient with the causal agent oomycete *Pythium ultimum* for seed-rotting and thereby suppresses its infection on plants (van Dijk and Nelson 2000). Another example of suppression of pathogens by competition is the competition for iron-acquisition mediated by siderophores produced by plant commensal biological control bacteria. Siderophores are secondary metabolite compounds that chelate iron for its acquisition. The siderophore-iron complex can be recaptured by producing bacteria via their specific outer-membrane receptor proteins. Under conditions of iron scarcity, the siderophore-producing bacteria can competitively acquire ferric ion and thus control the growth of pathogens. For example, *Pseudomonas fluorescens* B10 produces the siderophore pseudobactin and is able to suppress diseases caused by *F.oxysporum f. sp.lini* and *G. graminis var. tritici* in soils through competition for iron (Kloepper et al. 1981).

The biocontrol bacteria can supress pathogenesis by producing a range of bacterial allelochemicals including antibiotics, lytic enzymes, and bacteriocins, which are able to antagonize the survival, endurance, and growth of phytopathogens. The biocontrol pseudomonads have been reported so far to generate amphisin, 2, 4-di-acetylphloroglucinol (DAPG), pyoluteorin, pyrrolnitrin, tensin, tropolone and cyclic lipopeptides (Raaijmakers et al. 2002, Nielsen and Sorensen 2003). Apart from them, the lytic enzymes released by a range of biocontrol microbes are another class of compounds attacking phytopathogens. The lytic enzyme chitinase produced by *S.plymuthica* C48 inhibits spore germination and germ-tube elongation in *Botrytis cinerea* (Frankowski et al. 2001). Apart from them, the bacteriocin is another class of anti-pathogen agents produced by microbes with described biocontrol properties. The protein toxin colicin-like bacteriocins, for instance, are produced by a few Gram-negative bacteria and can suppress pathogens that share a close phylogenetic relationship to their producers (Chavan and Riley 2007).

Some biocontrol agents including a few plant commensal bacteria have been shown to induce or stimulate the inherent capability of plants to defend against pathogens, the mechanism of which is defined as “Induced Systemic Resistance” (ISR) (van Loon et al. 1998). Upon the

induction of ISR, the host plant will enhance its defensive physiological features, including strengthening the cell wall, altering physiological and metabolic activities, or releasing anti-pathogen compounds (Ramamoorthy *et al*. 2001; Nowak and Shulae, 2003). One example is the *P. fluorescens* strain WCS417 protects carnation plants systemically against *F. oxysporum* f. sp. *Dianthi* (van Peer et al. 1991). This induction is suggested to activate upon the interaction between plants and their specific rhizobacterias, as the bacteria will release a range of signalling molecules for communication and stimulation (van Loon et al., 2007). A variety of these immunity-inducing molecules have been described, including lipopolysaccharides and siderophores (van Loon et al. 1998), flagella, biosurfactants, N-acyl-homoserine lactones (AHL), N-alkylated benzylamines, antibiotics and exopolysaccharides (EPS) (Vleesschauwer and Hofte, 2009). Such communication between plants and microbes bridged by signalling molecules now arises as a new emerging field of research called interkingdom signalling, which will be introduced in the following sections.

1.2 Biocontrol pseudomonads

Pseudomonas is a large genus of Gram-negative aerobic Gammaproteobacteria, consisting of diverse species including *P. aeruginosa*, *P. syringae*, and *P. fluorescens* (Euzeby 1997). Pseudomonads survive ubiquitously in various niches ranging from terrestrial soil, the marine aquatic environment to the human body (Poirier et al. 2008). Based on their production of the yellow–green, brightly fluorescent pigment pyoverdine, pseudomonads are divided into fluorescent and non-fluorescent subgroups (Elliott 1958). The soil inhabitant pseudomonads includes the plant pathogenic species *P. syringae,* which gives rise to a variety of phyto-diseases, such as galls, blasts, cankers and lesions on the infected plants (Gross and Loper 2009). Besides the plant pathogenic species, other pseudomonads includes species with biocontrol properties for plant growth promotion, covering a range of strains defined as plant growth-promoting rhizobacteria (PGPR). This category includes some *P. aeruginosa* strains (Buysens et al. 1996, Bano and Musarrat 2003), *Pseudomonas brassicacearum* (Ortet et al. 2011), and predominantly *P. fluorescens* (Loper et al. 2007). One instance is the *P. protegens* strain CHA0 which is capable of protecting crop roots from the invasion by fungal pathogen *Pythium ultimum* (Figure 1.2). The biocontrol pseudomonads are intricately enmeshed in the plants and soil with their diverse anti-pathogen features. In the following section I examine representative biocontrol pseudomonad strains.

Figure 1.2: The biocontrol strain *P. protegens* CHA0 protects wheat roots from infection by *Pythium ultimum* (Haas and Defago 2005). From left to right: control plant without added microorganisms; diseased plant in the presence of *P. ultimum*; plant protected by the wild type CHA0 from *P. ultimum.*

1.2.1 Examples of biocontrol pseudomonads

The *P. fluorescens* group includes a subset of strains list in table 1.1 which are sequenced and featured for their biocontrol property including *P. fluorescens* A506, SS101, F113, *P. chlororaphis* 30-84 and *P. chlororaphis* O6 (Loper et al. 2012, Redondo-Nieto et al. 2012).

Table 1.1. Examples of Biocontrol Strains from *Pseudomonas Fluorescens* Group

Biological control species & strains	Biocontrol mechanism	Diseases suppressed	Reference source(s)
P. fluorescens A506	Competitive exclusion of the pathogen from sites of colonization	Fire blight of the Rosaceae caused by *Erwinia amylovora*	Lindow and Suslow 2003
P. fluorescens SS101	Synthesis of the biocontrol compound cyclic lipopeptide antibiotic (CLP) massetolide A	Hyacinth root rot caused by *Pythium intermedium*	De Souza et al. 2003
P. chlororaphis 30-84	Production of biological compounds phenazine-1-carboxylic acid	Take-all disease of wheat caused by the fungal pathogen	Pierson and Thomashow 1992

	(PCA), 2-hydroxy-phenazine-1-carboxylic acid (2OHPCA), and a small amount of 2-hydroxy-phenazine (2OHPZ)	*Gaeumannomyces graminis* var. *tritici* (*Ggt*)	
P. chlororaphis O6	Induction of systemic resistance and secretion of phenazines, pyrrolnitrin, and hydrogen cyanide	Soft-rot caused by *Erwinia carotovora subsp. Carotovora*	Kang et al. 2007, Han et al. 2006, Lee et al. 2011, Park et al. 2011
P. fluorescens F113	Niche competition with pathogens and release of anti-pathogen compounds 2, 4-Diacetylphloroglucinol and siderophores	Inhibition on growth of root pathogens including root pathogens *oxysporum f. sp. Radicis-lycopersici/Lycopersicum esculentum* in tomato and *Phytophthora cactorum/Fragaria vesca* in strawberry	Shanahan et al. 1992, Barahona et al. 2011
P. protegens Pf-5	Production of a range of anti-pathogen compounds	Seedling emergence	Howell & Stipanovic 1979

The well-studied biocontrol strain *P. fluorescens* A506 was initially isolated from asymptomatic leaves of a commercial pear tree in a Californian orchard (Wilson and Lindow, 1993). It is a plant epiphyte and is now commercially available (BlightBan A506) by Frost Technology Corporation, USA and Plant Health Technologies, USA (Fravel 2005) to manage the devastation from fire blight, a disease of the Rosaceae caused by *Erwinia amylovora* (Lindow and Suslow 2003). A506 protects the plant host from pathogens by niche competition on the plant surface, so if it is applied on surface of flowers, it can effectively exclude the pathogen from infection sites (Wilson and Lindow, 1993). Apart from that, it is also able to suppress several other pathogens, including infection on watermelon blossoms and seeds by *Acidovorax avenae* subsp. Citrulli (Fessehaie and Walcott, 2005), mummy berry disease caused by *Monilinia vaccinii-corymbosi* (Scherm *et al*., 2004), and speck on tomato caused by *P. syringae* pv. *tomato* (Wilson *et al*., 2002).

The *P. fluorescens* isolate SS101 from the wheat rhizosphere in the Netherlands was initially investigated for its significant protection against hyacinth root rot caused by *Pythium intermedium,* as the SS101 strain produces the cyclic lipopeptide antibiotic (CLP) massetolide A (De Souza et al. 2003). Subsequent investigation of this strain revealed its suppression on the late blight disease of tomato plants caused by *Phytophthora infestans*

(Tran et al. 2007). In this study, it was also evidenced that the compound massetolide A serves as a biological control agent by induction of systemic resistance in plant. In addition to that, this strain can induce the systemic resistance of plants by salicylic acid signalling (van de Mortel et al. 2012).

P. chlororaphis 30-84, or previously *P. aureofaciens* 30-84, was initially isolated as a soilborne strain from the rhizosphere of wheat take-all suppressive soil in Kansas, USA (Pierson and Thomashow 1992). It has been described to produce the antibiotic phenazine-1-carboxylic acid (PCA), which can effectively suppress take-all wheat disease caused by *G. graminis* var. tritici (Mazzola et al. 1995). The phenazine class of compounds accomplishes their biological control effects by transforming reactions of oxidation-reduction to cause the accumulation of toxic superoxide radicals in the pathogen cells (Kerr, 2000, Price-Whelan et al., 2006).

The strain *P. chlororaphis* O6 was originally isolated from wheat roots cultivated in Logan, Utah (Tucker *et al*., 1995). This strain has been shown to be a viable biocontrol agent as it has a broad spectrum of target pathogens. It has been shown to induce systemic disease resistance against a bacterial soft-rot pathogen, *Erwinia carotovora subsp. Carotovora* (Kang et al. 2007). Specifically its successful colonisation can induce the ISR in tobacco plants including *E. carotovora* which causes soft rot (Han et al. 2006). Its production of anti-pathogen compounds including phenazines, pyrrolnitrin, and hydrogen cyanide (HCN) are toxic against plant pathogenic fungi and nematode or can induce the inherent immunity in plant (Lee et al. 2011, Park et al. 2011). Apart from phenazines mentioned above, pyrrolnitrin is another antibiotic compound which inhibits the growth of pathogens by attacking their respiratory electron transport system as it inactivates a series of oxidases and reductases (Tripathi and Gottlieb 1969). The cyanide compound is a cellular toxin due to its formation of stable complexes with metabolic enzymes and other substrates so as to inhibit cell growth and metabolism (Cipollone et al. 2008).

The plant epiphyte *P. fluorescens* F113 was originally isolated from the rhizosphere of sugar beet and described for its inhibition of fungal pathogens by producing the antibiotic 2, 4-Diacetylphloroglucinol (DAPG) (Shanahan et al. 1992). It also demonstrates biocontrol activities against the fungal root pathogens *oxysporum f. sp. Radicis-lycopersici/Lycopersicum esculentum* in tomato and *Phytophthora cactorum/Fragaria vesca* in strawberry (Barahona et al. 2011). Its targeted range of pathogens also includes the potato

cyst nematode *Globodera rostochiensis* (Cronin et al. 1997). However, to our knowledge hitherto few information about molecular mechanism of DAPG to suppress pathogenesis has been disclosed. Apart from its biocontrol properties, this strain has become a mode to study rhizosphere colonisation (Barahona et al. 2010). Its genome has been fully sequenced recently and reveals the genes correlated to its biocontrol traits (Redondo-Nieto et al. 2012).

1.2.2 *P. protegens* Pf-5

P. protegens Pf-5, previously known as *P. fluorescens* Pf-5, was originally isolated from soil in Texas. It was characterised for its production of pyrolnitrin and suppression of the cotton disease caused by *Rhizoctonia solani* (Howell and Stipanovic, 1979). Further studies revealed a broader range of biocontrol targets. For example, seed rot and seedling death caused by *Pythium ultimum* can also be managed by Pf-5 (Howell and Stipanovic, 1980). When inoculated onto wheat straw residue, Pf-5 suppresses ascocarp formation by the tan spot pathogen of wheat, *Pyrenophora tritici-repentis* (Pfender *et al*. 1993). Additionally, Pf-5 can control the dollar spot caused by *Sclerotinia homoeocarpa* and leaf spot caused by *Drechslera poae*, which are deteriorating and widespread diseases for grasses (Rodriguez and Pfender 1997). This strain can also suppress the Fusarium crown and root rot of tomato, caused by *Fusarium oxysporum* f. sp. *13adices-lycopersici* (Sharifi-Tehrani *et al*., 1998). Pf-5 has been the first bioncontrol strain with fully sequenced genome and a series of features correlated to its biocontrol properties subsequently have been provided (Paulsen et al. 2005).

1.3 Biocontrol products produced by Pf-5

The published fully available genome sequence of Pf-5 provides clues of the genetic factors underlying its biocontrol properties (Figure 1.3). Approximately 6% of the Pf-5 genome are the gene clusters encoding biosynthesis of secondary metabolites and other anti-pathogen exoenzymes and bioactive compounds (Loper et al. 2007). These products play their dominant and indispensable role in suppressing target pathogens encompassing fungi, bacteria and insects. Overall, this strain produces the siderophores pyoverdine (Marschner et al. 1997) and enantio-pyochelin (Youard et al. 2007), and a number of antimicrobial secondary metabolites including 2, 4-diacetylphloroglucinol (Nowakthompson et al. 1994), pyoluteorin (Howell and Stipanovic, 1980), pyrrolnitrin (Howell and Stipanovic 1979), cyclic lipopeptide peptide orfamide A (Gross et al. 2007), and hydrogen cyanide (Kraus and Loper 1992).

1.3.1 siderophores

Siderophores are metal chelating compounds with a high affinity for iron which serve as iron acquisition systems (Loper and Gross 2007). Under low iron conditions, the siderophore will be synthesized and released out of cell to chelate iron, and the formed complex will be recaptured by the producing bacteria via specific outer-membrane receptor proteins (Poole et al. 1993). The production of siderophores by biocontrol pseudomonads restricts the availability of iron as an essential nutrient to pathogens and therefore suppresses their growth.

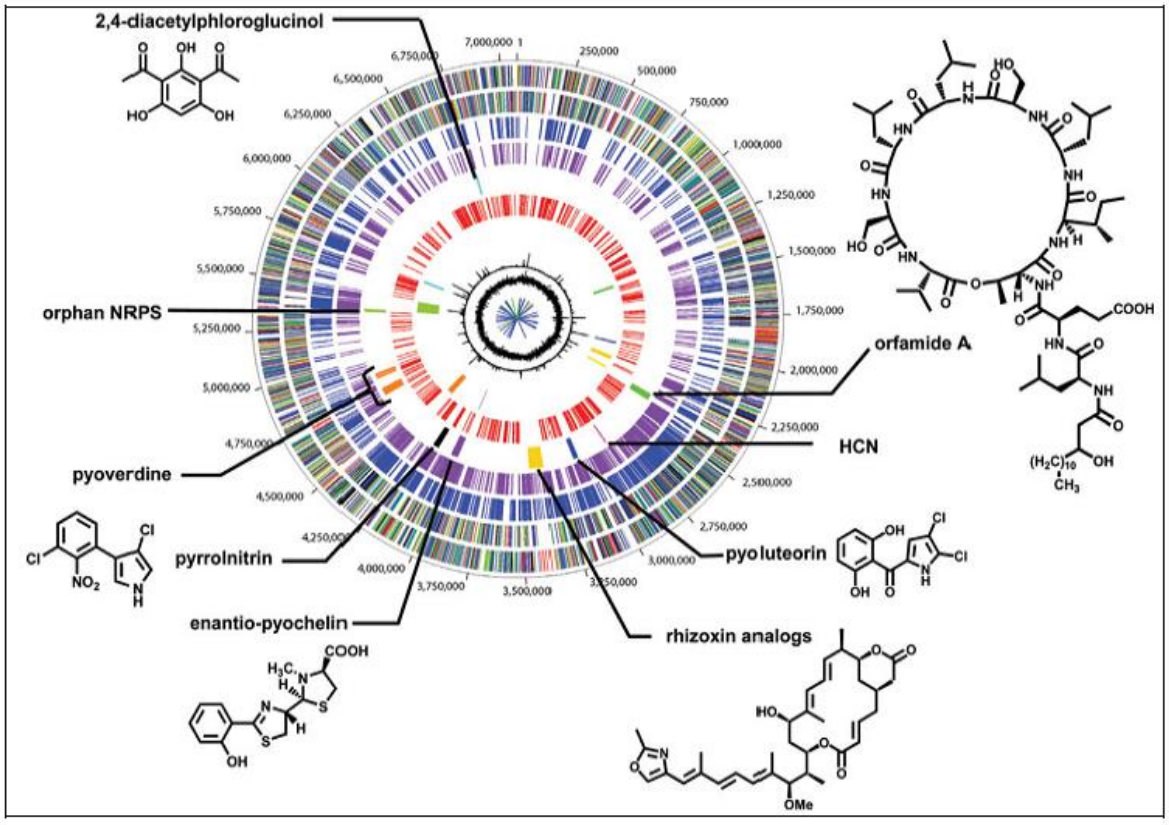

Figure 1.3: the genome of *P. protegens* Pf-5 depicts its biosynthesis of secondary metabolites including the ones acting as antimicrobials. The gene clusters encode products include 2,4-diacetylphloroglucinol, pyoverdine, pyrrolnitrin, enantio-pyochelin, pyoluteorin, orfamide A, HCN and rhizoxin analogs. Taken from (Gross and Loper 2009).

One of the siderophores produced by Pf-5 is pyoverdine. The genes for pyoverdine biosynthesis are included in three gene clusters in the Pf-5 genome (PFL_4079-98, PFL_4169-79 and PFL_4189-93), encoding both regulatory and transporter genes (Paulsen *et al*., 2005). In addition, the other siderophore produced by Pf-5 is enantio-pyochelin (E-pch), encoded by the gene cluster PFL_3473-504. E-pch is optical antipode of pyochelin produced

by *P. aeruginosa* (Youard *et al*. 2007). Pyochelin has been demonstrated to suppress damping-off disease of tomato plant caused by *P. splendens* (Buysens et al. 1996).

1.3.2 Antibiotics

Pf-5 produces DAPG (Nowak-Thompson *et al*., 1994) which is encoded by the gene cluster PFL_5951-58. DAPG is one of the most important secondary metabolites for biocontrol as it is active against a broad spectrum of pathogens. It has been shown to effectively control the diseases caused by oomycetes (Fenton et al. 1992), fungi (Keel *et al*. 1992), bacteria (Keel *et al*. 1992), as well as nematodes (Cronin et al. 1997). Apart from its antagonistic activities, it can induce the plant ISR in *Arabidopsis thaliana* against *Peronospora parasitica* (Iavicoli, et al. 2003).

Apart from DAPG, pyoluteorin is another secondary metabolite encoded by PFL_2784-801 gene cluster in Pf-5. Pyoluteorin is effective in management of the damping-off disease caused by the oomycete *P. ultimum* (Howell and Stipanovic, 1980). Hitherto our knowledge of the mechanism of pathogen control by pyoluteorin remains unrevealed.

Pyrrolnitrin is the first secondary metabolite discovered to be produced by Pf-5, characterised by its anti-fungal property as it was found to be capable of disrupting the fungal respiratory chains (Tripathi and Gottlieb 1969). In Pf-5 genome the PFL_3599-609 gene cluster is the loci for pyrrolnitrin biosynthesis.

The orfamides are a class of cyclic lipopeptides encoded by the gene cluster PFL_2142-51 in Pf-5 genome (Paulsen *et al*. 2005) and subsequently isolated from the culture (Gross et al. 2007). The orfamide family of compounds produced by Pf-5 includes the orfamide A which was found to lyse the zoospores of *Phytophthora ramorum*, the motile propagules of the plant pathogenic Oomycetes, and may therefore control the correlated phyto-diseases (Gross et al. 2007).

1.3.3 Exoenzymes/ proteinaceous bioactive compounds

Pf-5 produces the exoenzyme chitinase by gene PFL_2091, a homolog to chitinase-encoding gene *chiC*. Chitinase can degrade chitin which is the component of cell wall of many insects and fungi (Gooday, 1990). Such chitinolytic activity has been shown to suppress several

fungal pathogens including *Botrytis cinerea* (Frankowski et al. 2001), *Sclerotium rolfsii* (Ordentlich *et al*. 1988), and *Fusarium solani* (Lim et al. 1991).

Another exoenzyme produced by Pf-5 was identified as extracellular alkaline protease AprA, encoded by two homologous genes PFL_2483 and PFL_3210 (Loper *et al*. 2007). In the strain *P. protegens* CHAO which is closely related to Pf-5, AprA was shown as a suppressor for root knot nematode *Meloidogyne incognita* (Siddiqui et al. 2005). How it acts to accomplish the control on nematode remains unclear. In *P. syringae*, however, the AprA homolog was able to antagonize the immunity inducer of the eukaryote host and evade the host immune responses (Pel et al. 2014).

In the Pf-5 genome the PFL_2980-87 gene cluster is predicted to produce the proteinaceous toxin Fit (*P. fluorescens* insecticidal toxin) (Pechy-Tarr et al. 2008). This product extends the biocontrol range to insects, as it has been found to kill the tobacco hornworm *Manduca sexta* and the greater wax moth *Galleria mellonella* when the strain was injected into them (Pechy-Tarr et al. 2008).

1.3.4 Bacteriocins

The Pf-5 genome has two bacteriocin homologs PFL_1229 and PFL_2127 that produces LlpA bacteriocin (Parret et al. 2005). This bacteriocin has suppression on the cultivated mushroom pathogen *Pseudomonas tolaasii* as well as *P.fluorescens* strains correlated to Pf-5 (Parret et al. 2005).

1.3.5 volatile organic compounds

Some pseudomonads including Pf-5, produce the volatile organic compound hydrogen cyanide (HCN). In Pf-5 the HCN synthase encoded by the *hcnABC* biosynthetic genes PFL_2577-82 genes (Blumer and Haas 2000). HCN has a range of targeted plant pathogens, including the fungus *Thielaviopsis basicola* which causes black root rot of tobacco (Voisard et al. 1989) and *Phytium* species which cause damping-off in cucumber (Ramette et al. 2003).

1.4 Rhizosphere colonisation

Apart from production of anti-pathogen products, root colonisation is another critical factor and an essential capability for the bacteria to achieve *in situ* biocontrol effectiveness. When the bacteria colonize biotic surfaces, they may remain localized and form a multicellular community known as a biofilm, or instead, they can be motile to migrate towards more favourable niches to occupy. Motility and biofilm formation are two key bacterial behaviours influencing surface colonizing competency.

1.4.1 Bacterial motility

Motility is bacterial surface translocation behaviour recognized as one of the most important traits required for rhizosphere colonisation. Mutants with defective motility in plant-associated bacteria such as *P. fluorescens* and *Agrobacterium tumefaciens* are severely impaired in competitive root colonisation (Shaw 1991, de Weert et al. 2002). In the biocontrol strain *P. fluorescens* SBW25, motile cells are more efficient at attaching to the plant root compared to the isogenic nonmotile cells (Turnbull et al. 2001). Motile populations of bacteria have an ecological advantage that they can rapidly reach favourable niches for ideal colonisation (Rather 2005). There are six forms of surface motility identified: swimming, swarming, gliding, twitching, sliding, and darting (Harshey 2003). Among them, the swimming and swarming motility are the two categories which involve the assembly and rotation of propeller-like flagella (Harshey 1994) and are frequently investigated in biocontrol pseudomonads (Figure 1.4).

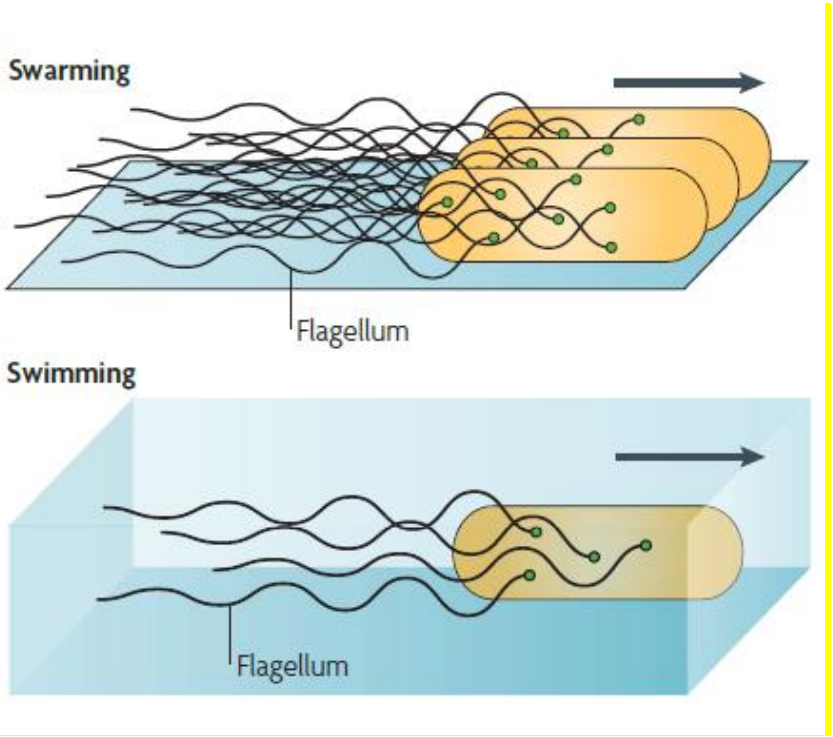

Figure 1.4: swimming and swarming motility are the two forms of bacterial movement mediated by flagellar rotation: swarming is group behaviour on solid surface, while swimming is an individual behaviour in an aqueous environment. Adapted from (Kearns 2010).

Most bacteria swim in an aqueous environment by rotation of their flagella (Larsen et al. 1974). Counterclockwise rotation of flagella results in a smooth swimming mode, and clockwise rotation leads to tumbling movement (Larsen et al. 1974). The chemotaxis system of bacteria in response to exogenous stimuli together with the CheY protein has a central regulation in switching between counterclockwise and clockwise rotation of the flagella. When the CheY protein via the chemotaxis system senses an environmental attractant or repellent and becomes phosphorylated, it will interact with the flagellar switch proteins (FliG, FliM and FliN) and regulates flagellum rotation (Bourret et al. 2002, Mariconda et al. 2006). In pseudomonads, in similarity to many Gram-negative bacteria, several plant-associated species including *P. aeruginosa*, *P. syringae* and *P. protegens* have polar flagella for their mobility while interacting with their eukaryote hosts (Jackson, 2009). As the majority of pseudomonads are traditionally recognized as monotrichous and propel their movement by a single flagellum (Lugtenberg *et al*., 2001), many details of their flagellum-driven pathway for swimming have homology to the well-described *E. coli* model system (Sampedro I et al. 2014). One instance is the soil inhabiting strain *P. fluorescens* SBW25 which swims by right-handed rotation of flagellum (Ping et al. 2013).

Swarming motility is the coordinated flagellum-driven movement by a group of bacteria across a moist solid surface (Henrichsen 1972), in comparison to the individual behaviour in swimming motility. The swarming bacterium usually requires biosynthesis of additional flagellum to move on the solid surface. For example, *P. aeruginosa* which is able to swim with a single polar flagellum will produce additional flagella during swarming (Kohler et al. 2000, Rashid and Kornberg 2000). Genomic mutations in bacteria overexpressing flagella genes will increase flagella number and promote swarming motility, while suppressed expression of flagella genes will reduce flagella number and reduce or abolish swarming motility (Kearns and Losick 2005, Wang et al. 2005). Apart from the parameter of flagellum-driven force, the secretion of surfactant by some swarming bacteria also promotes the surface wetness and facilitates motile movement. For some bacteria such as *B. subtilis* and *Serratia*

liquefaciens, the surfactant is essential for swarming, since mutations abolishing surfactant production also abolishes swarming (Lindum et al. 1998, Kearns and Losick 2003). In *P. aeruginosa*, the biosurfactant produced to facilitate swarming motility has been identified mainly as rhamnolipid (Soberón-Chávez et al. 2005).

1.4.2 Formation of biofilm

Biofilms are a sessile form of bacterial multicellular aggregates bound by a matrix of extracellular polymers and often attached to a surface (O'Toole et al. 2000). The matrix generally consists of a complex mixture of extracellular polysaccharide (EPS), protein, and DNA to form infrastructure skeleton which not only allows the bacteria to cohere to one another but also adhere to solid surfaces (Flemming and Wingender 2010). The bacterial population in biofilms have competitive survival advantages over their planktonic counterparts, such as increased antibiotic resistance (Hoyle and Costerton 1991) and diversity in genetic and metabolic features (Boles et al. 2004).

Biofilm development occurs in three main stages: (1) initial surface attachment; (2) proliferation and maturation; and (3) detachment or dispersal (O'Toole et al. 2000) (Figure 1.5). Among the pseudomonads, biofilm development and its molecular mechanism have been intensively studied in the human pathogen *P. aeruginosa* as well as some biocontrol *P. fluorescens* strains.

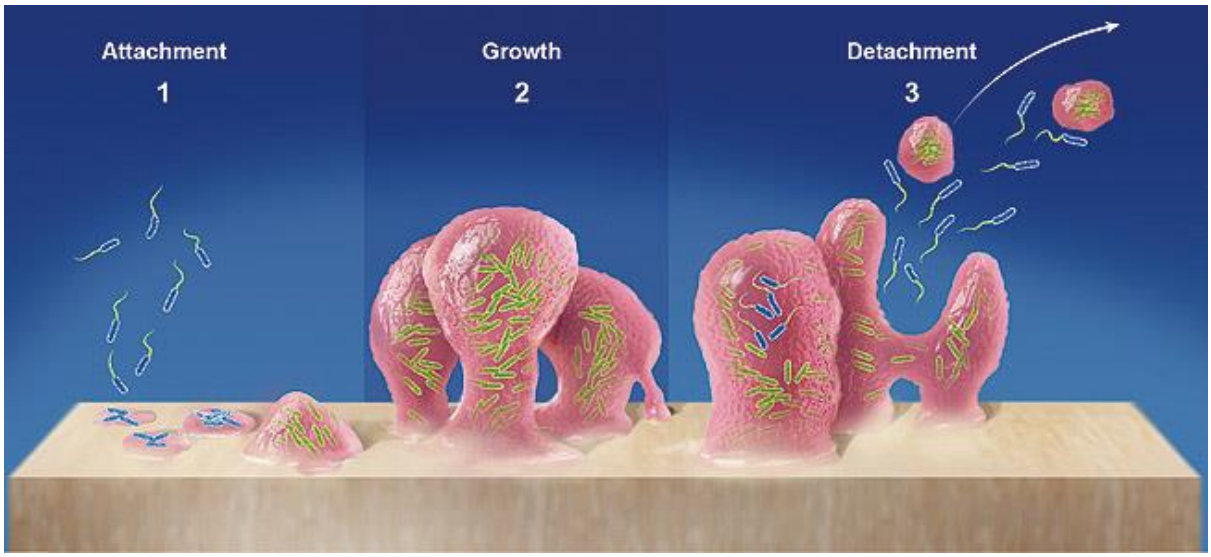

Figure 1.5: the stages in biofilm development including initial attachment, colonization, and detachment. Taken from Montana State University Center for Biofilm Engineering.

In *P. aeruginosa*, the extracellular DNA (eDNA) (Whitchurch et al. 2002) and the exopolysaccharides including Psl, Pel and alginate (Boyd and Chakrabarty 1995, Ma et al. 2009) and the outer membrane adhesion protein CdrA (Borlee et al. 2010) are integral parts of the intercellular matrix and mediate the biofilm surface attachment. In *P. fluorescens*, LapA protein, as the mediator for cell surface adhesion, appears an important matrix component for biofilm formation since its mutation gives rise to severe defects in biofilm (Hinsa et al. 2003).

1.4.3 Transition between biofilm and motility

Biofilm formation and motility are interconnected and are influenced by several factors. It has been demonstrated in *P. aeruginosa* that flagella and motility are the major parameters for initial attachment and subsequent biofilm differentiation (O'Toole et al. 2000), but unregulated motility during biofilm formation destabilizes the biofilm structure and induce the bacterial dispersal into planktonic counterparts (O'Toole and Kolter 1998). Once the biofilm formation initiates, flagellar gene expression and motility decrease as the biofilm matures. Consistent with the notion that motility antagonizing biofilms, *P. aeruginosa* colony morphology variants forming more robust biofilms are reduced in motility compared with their parental strains (Kirisits et al. 2005).

The small cytoplasmic secondary messenger molecule bis- (3_-5_)-cyclic dimeric-guanosine-monophosphate (c-di-GMP) plays a critical role in regulating the motility-to-biofilm transition (Figure 1.6). Generally the elevated level of intercellular c-di-GMP inhibits motility while inducing biofilm formation in *P. aeruginosa* (Romling et al. 2005). The reduction of motility by high c-di-GMP is due to its binding to its protein effector FleQ, a master regulator of flagellum biosynthesis that represses the expression of flagellum biosynthesis genes (Baraquet and Harwood 2013). Meantime, a high internal level of c-di-GMP in *P. aeruginosa* also induces the production of adhesin CdrA and extracellular biofilm matrix components alginate, exopolysaccharides Pel and Psl. The c-di-GMP in combination with FleQ positively regulate the production of CdrA (Rybtke et al. 2012). c-di-GMP also binds to the Alg44 receptor protein and modulates its activity to increase alginate production (Merighi et al. 2007), and positively regulates the operons of *psl* and *pel* (Hickman et al. 2005). In *P. fluorescens* Pf0-1, c-di-GMP binds to the transmembrane receptor LapD to promote biofilm-surface adherence. The c-di-GMP/ LapD complex will sequester the

periplasmic protease LapG to induce biofilm dispersal (Newell et al. 2011). Without sequestration, LapG will cleave the biofilm adhesion protein LapA and cause destabilisation of the biofilm structure.

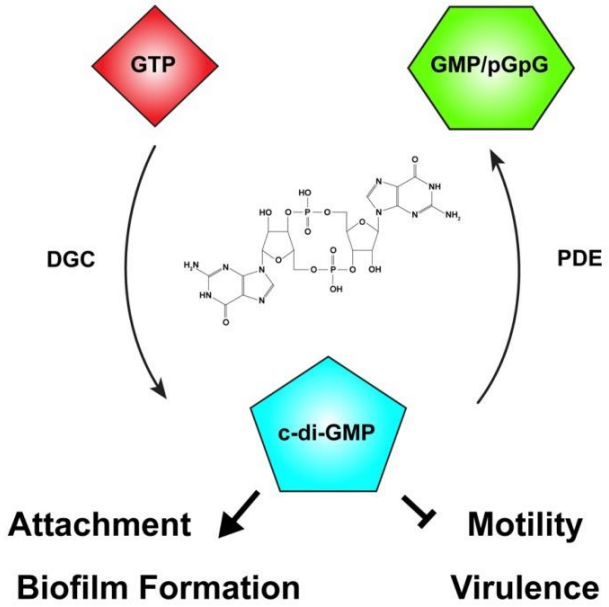

Figure 1.6. The secondary messenger c-di-GMP represses motility and virulence while it positively regulates biofilm formation and attachment. GTP, Guanosine-5'-triphosphate; GMP, Guanosine monophosphate; DGC, diguanylate cyclase; PDE, phosphodiesterase. Taken from (Heindl et al. 2014).

In pseudomonads, apart from the regulation by c-di-GMP, another mechanism to regulate the biofilm formation and the swarming motility is the bacterial communication procedure termed quorum sensing (QS). In *P.aeruginosa* the production of the biosurfactant rhamnolipids which are important for motility and biofilm formation is controlled by the QS signal N-acyl-homoserine lactones (AHLs) (Daniels et al. 2004). QS plays an important role in regulating the expression of biofilm-related genes in *P. aeruginosa* (Chugani et al. 2001). In *P. fluorescens*, while the regulation by QS on production of cyclic lipopeptides can facilitate swarming motility on solid surface, it also has a positive effect on biofilm formation (de Bruijn et al. 2007, de Bruijn et al. 2008).

In pseudomonads, the sRNA regulation is the third critical parameter involving biofilm formation and motility. The sRNAs *rsmZ* and *rsmY* in *P.aeruginosa* play their regulatory roles via binding to the biofilm inhibitor RsmA protein to relieve the translational repression of the associated gene including *psl* and other QS-related genes (Brencic and Lory 2009, Irie et al. 2010). In *P. fluorescens* F113, the sRNAs *rsmZ* and *rsmY* are involved in the regulatory cascade by the two component transduction system GacAS which represses motility and

promotes biofilm formation (Martinez-Granero et al. 2012). In the plant-beneficial strain *P. protegens* CHA0, the sRNAs RsmX-Z form a triad to antagonize ribosome-binding proteins RsmA and RsmE for derepression of their downregulated genes (Kay et al. 2005). The RsmA protein in *P. aeruginosa* and its homolog CsrA in *E. coli* serves as repressors for their biofilm formation (Jackson et al. 2002), conceivably in CHA0 the regulatory effects exerted on it by RsmX-Z would modulate the bacterial biofilm formation.

1.5 Regulation by quorum sensing

1.5.1 quorum sensing

Similar to cell–cell communication via hormones in eukaryotic cells that enables information to be passed from cell to cell even over considerable distances, bacteria exploit a cell density-dependant regulation called quorum sensing (QS) to communicate intercellularly and coordinate gene expression at the population level. Bacteria generate signalling molecules termed autoinducers, whose extracellular concentration rises in response to increasing cell density and auto-induce its own biosynthesis. When the amount of autoinducer increases to a threshold stimulatory concentration, they will be detected either directly or via sensor kinases to alter the expression of a large collection of genes from bacteria of various species (Waters and Bassler 2005). In pseudomonads, QS has been well characterised in *P. aeruginosa*. There are two sets of QS systems up-to-date discovered and intensively investigated in *P.aeruginosa*.

1.5.2 the LuxR-I circuit

Similar to a number of other Gram-negative bacteria, *P. aeruginosa* has two paired LuxI/R signal circuits: LasI/R and RhlI/R,that are involved in AHL-driven QS. LuxI is the AHL synthase, while LuxR is a regulatory protein that binds AHLs and forms a complex. The complex binds to specific promoter sequences called lux-boxes in the upstream of target genes controlling the transcription of them (Fuqua and Greenberg 2002) including those genes encoding for the production of pyoverdine (Stintzi et al. 1998), rhamnolipid biosurfactants (Ochsner and Reiser 1995) and other virulence factors (Pearson et al. 1997). In some *P. fluorescens* strains, for instance 2P24, there is the PcoI/R signalling cascade as

homolog to LuxI/R which regulates biofilm formation and biocontrol abilities (Wei and Zhang 2006).

1.5.3 LuxR 'solo' proteins

A number of QS-related LuxR sensors have been discovered by bioinformatic analysis in many sequenced proteobacterial genomes without their paired cognate homologous LuxI synthase (Case et al. 2008). Subsequently the unpaired LuxR-family proteins are designated as LuxR solos (Subramoni and Venturi 2009).

LuxR solo proteins may exist in AHL-producing or non-AHL producing bacteria. The QscR protein is a LuxR solo discovered from a *P. aeruginosa* genome, and it is suggested to expand the regulatory repertoire of the typical LuxI/R QS systems by accepting endogenously produced AHLs (Fuqua 2006). Alternatively, the LuxR solo SdiA of *Salmonella enterica* and *Escherichia coli* senses exogenous AHLs released from neighbouring bacteria (Ahmer 2004).

Apart from the LuxR solos that sense AHLs, there is a newly discovered class of LuxR solos in plant-associated bacteria that appear to respond to signalling molecules released from plants instead of bacterial AHLs. One typical example is PsoR in the biocontrol strains *P. protegens* Pf-5 and CHA0, which has been shown to activate a *lux*-box containing promoter in response to unidentified plant compound(s) rather than AHL (Subramoni et al. 2011). This class of LuxR solos can also exist in plant pathogens, such as XccR in *Xanthomonas campestris*, which also senses unidentified plant compound(s) (Zhang et al. 2007). This group of LuxR solos has been found in plant-associated bacteria and is suggested to mediate the communication between plants and microbes termed 'interkingdom signaling'.

1.5.4 The *Pseudomonas* quinolone signal (PQS) mediated QS system

In addition to the LuxR-I circuit, there is another set of QS system that recognise the *Pseudomonas* quinolone signal (PQS) discovered in *P. aeruginosa* (Pesci et al. 1999). PQS mediated regulation is linked to the *las* and *rhl* QS systems of *P. aeruginosa* because the *las* system was involved in PQS production and the *rhl* system in its biological effects (Pesci et

al. 1999, McKnight et al. 2000). In this system, the *pqs* operon including *pqsABCDE* with the transcriptional regulator gene *pqsR* are responsible for the biosynthesis and sensing of PQS (Cao et al. 2001). This system positively regulates the production of some virulence factors such as pyocyanin (Gallagher et al. 2002), lectin and rhamnolipids (Diggle et al. 2003). There have been attempts to find a similar system in other species including *P. fluorescens*, *P. syringae* and *P. fragi* however without success (Lepine et al. 2004).

1.5.5 Interkingdom signalling and quorum sensing

As prokaryotes and eukaryotes coexist and have coevolved together over hundreds of millions of years, it seems likely that the two kingdoms communicate with each other via signalling molecules. Deciphering the chemical signals that mediate extensive communication between bacteria and their eukaryote hosts has become a new emerging field.

Interkingdom signalling involves communication in bi-directional manner. The plant-associated bacteria can generate bioactive compounds as the signal to induce responses in the plant host. It has been reported the QS signal AHLs from the symbiotic *Sinorhizobium meliloti* and pathogenic *P. aeruginosa* bacteria stimulate a change in the proteomic profile of the host plant legume *Medicago truncatula* and induce its production of QS antagonistic compounds (Mathesius et al. 2003). Meanwhile, the plant host can release various exudates which may alter the gene expression in the bacteria. For example, the *picA* (for Plant-Inducible Chromosomal) gene in *Agrobacterium. tumefaciens* was first identified as being induced by the polygalacturonic acid fraction of polysaccharides released by carrot (Rong et al. 1991).

Interkingdom signalling is complicatedly interconnected with bacterial QS. As mentioned above, while a specific class of LuxR solos respond to plant-released exudates, the AHLs as QS mediator can affect gene expression in the host and induce its antagonism against pathogens. Conceivably interkingdom signalling will be a potential hotspot for future research.

1.6 The small RNA *phrS*

1.6.1 Small RNAs in Pseudomonads

More than 150 small RNAs (sRNA) have been found in prokaryotes, as most of them do not encode protein products, but instead mediate specific and critical regulatory functions on bacterial gene expression (Waters and Storz 2009). Bacterial sRNAs range in size from 50 to 500 nucleotides (Altuvia 2007). Classically a number of investigations on sRNA started intensively on *E. coli* and discovered close to 100 sRNAs (Waters and Storz 2009). As the majority of pseudomonads have genomes of larger sizes, they are predicted to include more sRNAs. Bioinformatic analysis of pseudomonad genomes attempted to identify candidate sRNA sequences and led to functional characterisation of specific sRNAs (Livny et al. 2006, Gonzalez et al. 2008). Generally most small RNAs exert their regulatory effects on gene expression at a post-transcriptional level by two modes: regulatory antisense sRNAs basepair with a stretch on the target mRNAs and block transcription (Waters and Storz 2009), or several other regulatory sRNAs including the *rsm* family mentioned above can sequester the RNA-binding proteins which repress or activate translation (Babitzke and Romeo 2007, Beisel and Storz 2010). The following sections describe a few sRNAs discovered in pseudomonads.

6S RNA

The 6S RNA was directly isolated by purification from *P.aeruginosa* as it is expressed in abundant copies (Vogel et al. 1987). This piece of RNA has been mainly studied in *E. coli*. As the predicted secondary structure of it in *P. aeruginosa* is very similar to it in *E. coli*, together with the 60% sequence identity between them (Vogel et al. 1987), the features characterised of it in *E.coli* are extrapolated to its homologue in pseudomonads. The secondary structure of 6S RNA resembles that of open DNA promoters, hence it can bind to RNA polymerase and interfere with transcription of a variety of genes (Steuten et al. 2014).

4.5S RNA

The 4.5S RNA was initially studied for its similarity to its homologue in *E. coli* (Toschka et al. 1989). This piece of RNA is the product of *ffs* gene. The function of this RNA is not determined in *P. aeruginosa*, but is possibly predictable from its homologue in *E. coli*, as the *ffs* genes have 75% sequence identity between the two organisms. The 4.5S RNA is one component of the signal recognition particle, the ribonucleoprotein which directs proteins

containing signal sequences that are being synthesised at the ribosome to the cytoplasmic protein translocation machinery (Keenan et al. 2001).

PrrF sRNAs connect iron concentration and quorum sensing

The tandemly aligned genes *prrF1* and *prrF2* in the *P. aeruginosa* genome encode two highly similar sRNAs of about 110 nucleotides each (Wilderman et al. 2004). The expression of the two genes is responsive to the availability of the essential micronutrient iron in the environment (Wilderman et al. 2004). These RNAs inhibit the translation initiation of the iron-containing enzyme superoxide dismutase (*sodB*) by the base pairing mechanism (Wilderman et al. 2004). When iron concentrations are low, the sRNAs are produced to block *sodB* translation. When the iron is abundant, by contrast, the sRNAs will not be expressed and *sodB* can be translated.

In *P.aeruginosa* the *prrF* sRNAs become the intermediate between iron availability and quorum sensing (QS) system. As anthranilate is the precursor molecules for *Pseudomonas* quinolone signal (PQS) production, these RNAs repress the expression of *antABC* genes which give rise to anthranilate degradation and therefore reduce PQS production (Oglesby et al. 2008). As PQS acts as a QS signal (Heeb et al. 2011), the reduction of it will affect the QS process.

Rsm sRNAs

As mentioned above, the *rsmZ* and *rsmY* sRNAs in *P. aeruginosa* are under positive control by phosphorylated GacA and affect biofilm formation via their effector protein RsmA (Brencic and Lory 2009). In *P. fluorescens*, this family of sRNAs affect biofilm formation and motility because they sequester the RsmA and RsmE proteins to liberate the sigma factor AlgU (Martinez-Granero et al. 2012). AlgU is the upstream regulator for the AmrZ, which represses the flagellum biosysnthesis regulator FleQ and thereby influences biofilm formation and flagellum-mediated motility.

1.6.2 *phrS* modulates the *Pseudomonas* quinolone signal (PQS) mediated quorum sensing system

Bioinformatic analysis shows that the small RNA *phrS* exists in the genomes of *P. aeruginosa* PAO1, *P. fluorescens* A506 and *P. protegens* Pf-5. *phrS* has been identified in *P.*

aeruginosa by copurification of the sRNA with its binding protein Hfg (Sonnleitner et al. 2008). Unlike most of other sRNA, *phrS* contains an open reading frame of 37 codons and has its translated product in *P. aeruginosa*, although the function of the peptide is not determined (Sonnleitner et al. 2008). The transcription of *phrS* has been shown to be under the control of the oxygen-responsive regulator ANR, and itself regulates the transcriptional regulator *pqsR* positively and subsequently the synthesis of pseudomonas quinolone signal (PQS), as PqsR is a positive regulator for PQS production (Sonnleitner et al. 2011). Since PQS serves as a class of signalling molecules in quorum sensing (Pesci et al. 1999), *phrS* therefore is predicted to influence the QS and expression of a range of correlated genes.

1.7 Scope of thesis

This study investigated the regulatory functions of the LuxR solo regulator PFL_3627 and small RNA *phrS* on the root-colonisation associated phenotypes of biocontrol strain *P. protegens* Pf-5. The phenotypes relevant to colonisation on eukaryote host by bacteria include biofilm formation and flagellum-mediated motility. In a broad spectrum of bacteria these microbial physiological activities have been investigated in details and are conceived to be regulated at the molecular level by the intertwined network of LuxR-I circuits in QS, sRNAs and other molecular pathways. While in some pathogenic pseudomonads LuxR-I QS system or LuxR solos and sRNAs have been reported to play dominant roles in microbe-host interactions, colonisation on eukaryote hosts and pathogenesis, the function of LuxR solos and sRNAs in plant-beneficial bacteria *P. protegens* strains which do not produce endogenous AHLs are largely unknown. In biocontrol strain *P. protegens* Pf-5, the LuxR solo PFL_3627 and sRNA PhrS are hypothesized to be involved in biofilm formation and bacterial motility. In our study, to investigate their regulatory roles, gene knockouts of these two genes were constructed and the phenotypic effects of the mutations were examined using biofilm formation and motility assay. Microarray analysis of the transcriptomic effects of the PFL_3627 knockout mutant were undertaken. Additionally, the ability of PFL_3627 to respond to N-aceyl-homoserine-lactones (AHLs) was examined through the use of biosensor strains and the exogenous addition of AHLs.

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Chapter 2. Mutation of LuxR solo PFL_3627 in the biocontrol strain *Pseudomonas protegens* Pf-5 facilitates early-phase biofilm formation by promoting the initial attachment and motility

Abstract

Rhizosphere colonisation is a key trait for plant-beneficial bacteria, such as *Pseudomonas protegens* Pf-5, to achieve their biocontrol effectiveness. The correlated phenotypes biofilm formation and motility are often regulated by LuxR family protein-associated quorum sensing (QS). This study investigated the regulatory effects mediated by the LuxR protein PFL_3627 in *P. protegens* Pf-5 on its bacterial proliferation, biofilm formation and motility as well as gene expression by constructing a gene knockout mutant strain. Quantitative real-time PCR (qRT-PCR) demonstrated the expression level of PFL_3627 remained constant across different growth phases under various culture conditions. Mutation of this gene resulted in defective bacterial growth, hyperswarming motility, a 'smooth' pattern of swimming motility, and promoted biofilm formation in earlier growth phases conferred by substantially increased initial surface attachment. Complementation of the mutant restored these phenotypes as wild-type. A genome-scale microarray revealed that the expression profile of more than a hundred of genes was affected by the PFL_3627 gene knockout, the majority of which are for synthesis and regulation of flagellum and biofilm matrix. Consistent with the finding that no acyl-homoserine lactone (AHL) synthase gene has been discovered in the Pf-5 genome, the use of biosensor bacteria detected no AHL production from Pf-5. An assay using exogenous addition of AHL indicated the LuxR regulator interacts with none of the short- or long-chain AHLs used in the study. This suggests that the LuxR solo PFL_3627 may sense undefined signalling molecule(s) possibly of plant origin and has a positive impact on the phenotypes associated with rhizosphere colonisation.

Introduction

Pseudomonas protegens Pf-5, previously known as *P.fluorescens* Pf-5 (Ramette et al. 2011), was originally isolated from soil in Texas and shown to mediate suppression of the cotton disease caused by *Rhizoctonia solani* (Howell and Stipanovic, 1979; 1980). Pf-5 was subsequently shown to have significant potential for biocontrol application against plant pathogenic bacteria, fungi and oomycetes because it synthesizes at least seven bioactive secondary metabolites, including pyrrolnitrin, pyoluteorin, hydrogen cyanide, rhizoxin analogs, 2,4-diacetylphloroglucinol (DAPG), and cyclic lipopeptide orfamide A, that antagonize the plant pathogens (Howell and Stipanovic, 1979; 1980; Nowak-Thompson and Gould 1994, Whistler et al. 1998, Gross et al. 2007, Loper et al. 2008). In addition, its production of insecticide against tobacco hornworm has been identified (Pechy-Tarr et al. 2008).

In addition to the production of anti-pathogen compounds, aggressive rhizosphere colonisation is another essential parameter that determines biocontrol capabilities. To colonize the plant roots, the grouped bacteria cooperatively adhere to their selected specific ecological niches and form a multicellular community *in situ* known as a biofilm (Danhorn and Fuqua 2007). Biofilm formation implies the strategy to assemble a mass of bacteria protected by their self-secreted extracellular polymeric substance (EPS) to initiate more evolutionarily advantageous interaction with host plants and form sessile aggregates in contrast to their planktonic counterparts. The extracellular matrix generally consists of exopolysaccharides, extracellular DNA (eDNA) and proteins to construct its integrated structure (Branda et al. 2005). Biofilm formation is a multi-phase process that is well studied in the *Pseudomonas* species *P. aeruginosa*. The phases include initial surface attachment, biofilm differentiation and maturation, and biofilm dispersal (Klausen et al. 2003). The extracellular polysaccharide components Pel (involved in pellicle formation) (Friedman and Kolter 2004) and Psl encoded by *psl* (polysaccharide synthesis locus) in the biofilm skeleton of *P. aeruginosa* mediate the surface attachment and contribute to structural stability and architecture (Colvin et al. 2012). In *Pseudomonas fluorescens*, the LapA protein embedded in the extracellular matrix components is the fundamental adhesin which allows planktonic bacteria to adhere to and proliferate on surfaces during the various stages of biofilm formation (Hinsa et al. 2003). The capability of biofilm formation and EPS production by microorganisms are generally recognized as essential for their colonisation on various surfaces (Sutherland 2001).

Apart from biofilm formation, motility is another trait critical for successful colonisation on plant surfaces by *Pseudomonas spp* (Turnbull et al. 2001). In pseudomonads there are two types of widely investigated motility mediated by rotated flagellum. One is the swimming movement on interstitial liquid that is normally tested in soft agar liquid. Swimming motility in *E.coli* has been featured as flagellum-mediated behaviour that the counterclockwise rotation of flagellum generates bacterial smooth swimming, while the clockwisewise rotation leads to a tumbled pattern (Harshey 2003). The other is the termed swarming motility as the cooperative bacterial movement in a group over half-solid surface which is determined by flagellum combined with surfactant. This feature describes the bacterial flagellum-mediated movement by motile populations to reach their preferred niches for colonisation (Eberl et al. 1999). It has been widely concluded that the non-motile strains or strains with reduced motility result in largely impaired competitive root colonisation (Simons et al. 1996, Capdevila et al. 2004). Motility function is determined by several factors. In pseudomonads, such as *P. aeruginosa*, the motile cells in swarming movement on half-solid medium have two differentiated flagella instead of one in their vegetative state (Kohler et al. 2000). Besides using the driving force generated by the flagella, some swarmers can also secrete surfactants to reduce the surface friction and facilitate bacterial translocation, resulting in promoted motility (Kearns and Losick 2003). As a consequence of active motility, the bacteria appear to have an advantage in initial attachment during surface colonisation (Turnbull et al. 2001). Evidently motility plays a key role in active biofilm dispersal after matured colonisation, which is essential for bacterial spreading and inhabitation on new sites (Sauer et al. 2002).

Biofilm formation and motility are both cooperative group behaviours regulated by a mechanism named quorum sensing (QS). QS is essentially bacterial cell-to-cell communication. It uses signalling molecules to coordinate gene expression at the population level. As the cell density increases, the concentration of signalling molecules accumulates to a threshold level, regulatory proteins sense them and form complexes with them, then the complexes activate or repress the expression of the quorum spectrum of genes (Waters and Bassler 2005). QS regulates biofilm differentiation and maturation by affecting the production of polysaccharides and eDNA in the matrix, as demonstrated in *P. aeruginosa* (Davies et al. 1998, Allesen-Holm et al. 2006). QS also influences the production of biosurfactants and swarming motility, and therefore affects biofilm initiation, as swarming is correlated with initial surface attachment (Ochsner and Reiser 1995). The well-characterised QS paradigm in pseudomonads is the LuxR-I circuit mediated by acyl-homoserine lactone

(AHL). This two-component system includes LuxI type synthase for AHL synthesis and LuxR type protein receptors to sense the AHL signal. AHLs interact with LuxR-family receptors to induce a conformational change that allows LuxR to bind to short DNA sequences controlling transcription initiation called DNA *lux* box and thus regulate the transcription of target genes.

In contrast to the paired LuxR-I systems, there are a number of unpaired LuxR regulators identified without their cognate LuxI synthase in bacterial genomes. These LuxR family proteins have been defined recently as "LuxR solos" (Subramoni and Venturi 2009). The LuxR solos are predicted to share similar key residues to other LuxR receptors, and they are approximately 250 amino acids long and are modular with two functional domains: an amino-terminal ligand-binding domain and a carboxy-terminal domain containing a helix-turn-helix DNA-binding motif (Nasser and Reverchon 2007). One class of LuxR solos has been shown to respond to endogenous or exogenous AHLs and amplify the QS regulon. An example of this class is the QscR protein of *P. aeruginosa* which responds to endogenous AHL synthesized by the LasI AHL synthase and regulates the expression of a range of genes distinct from those controlled by the inherent LasI/R or RhlI/R systems (Chugani et al. 2001). In non-AHL producing bacteria, the LuxR type protein accomplishes its regulation by 'eavesdropping' exogenous AHLs released from neighbouring bacteria. Another example of this subclass is SdiA in *E. coli* which senses AHLs diffused from adjacent AHL-producing bacterial communities (Kanamaru et al. 2000).

Apart from AHL-binding LuxR type proteins, there has been a subclass of LuxR-solo regulators recently found to respond to unidentified plant compounds rather than the AHLs released by bacteria. One instance is XccR of *Xanthomonas campestris* pv. Campestris, that responds to compounds derived from cabbage leaves and seeds (Zhang et al. 2007, Wang et al. 2011). With an increasing number of bacterial genome sequences available the application of bioinformatic tools has helped to identify homologues of XccR in several bacterial genomes, including some pseudomonads (Zhang et al. 2007).

In a recent study of Pf-5 and its closely related rhizosphere colonizing strain *P. protegens* CHA0, two open reading frames *psoR* (PFL-5298) and PFL-3627 were identified as putative LuxR homologs by *in silico* analysis. Functional analyses demonstrated that *psoR* falls into the class of LuxR-solo responding to unknown plant compound rather than AHL (Subramoni

et al. 2011). Across the fully sequenced genome of Pf-5, it is suggested there are no annotated *luxI* homologs for the biosynthesis of AHLs (Subramoni et al. 2011), and all *luxR* family genes exist as *luxR*-solos. Although sequence alignment analysis reveals that LuxR family proteins conserve low identity (18 to 25%), 95% of LuxR family regulators including PsoR share 9 highly conserved amino acid residues: tryptophan 57 (W57), tyrosine 61 (Y61), asparagine 70 (D70), proline 71 (P71), tryptophan 85 (W85), and glycine 113 (G113) in the signal molecule-binding domain, and glutamine 178 (E178), leucine 182 (L182), and glycine 188 in the DNA binding domain (Gonzalez and Venturi 2013). Irrespective of the sequence data on this class of LuxR solos, there is a lack of information regarding their regulated phenotypes, especially the ones related to biocontrol features in plant-associated bacteria, as well as the range of genes under their control. While *psoR* in Pf-5 has been characterised, this study hypothesized the other LuxR solo, encoded by PFL_3627, accepts some unidentified signalling molecule to activate a global regulatory effect on the transcriptome and results in modulation of phenotypes relevant to root colonization. Therefore we used a targeted gene deletion to give insight into the biocontrol-related phenotypes and genes regulated by PFL_3627 in the model strain *P. protegens* Pf-5.

Methods

Bacterial strain and growth conditions

The bacteria strains and plasmids used are listed in Table 2.1. The *P. protegens* Pf-5 strain was cultured in Luria Bertani (LB) medium at 27 °C with the shaking speed of 200 rpm. *Escherichia coli* TOP10 and *E. coli* S17-1 were used respectively for routine cloning and conjugation procedures and grew in Luria Bertani (LB) medium at 37 °C under the shaking speed of 200 rpm. Biparental mating was performed on LB plates supplemented with 1 % (W/V) glycerol.

Table 2.1. Bacteria and plasmids used in site-directed mutagenesis

Strains or plasmids	**Characteristics**	**Sources**
Strains		
P. protegens Pf-5	Wild-type	Howell and Stipanovic (1979)
Pf-5ΔPFL_3627	*P. protegens* Pf-5 ΔPFL_3627	This study
E. coli S17-1	*recA thi pro, tra* genes (from plasmid RP4) integrated into chromosome	Simon et al. (1983)

E. coli TOP10	F- *mcrA* (*mrr-hsdRMS-mcrBC*) *80lacZM15 lacX74 recA1 ara139* (*ara-leu*)*7697 galU galK rpsL* (StrR) *endA1 nupG*	Invitrogen
Plasmids		
pEX18Tc	Broad-host-range suicide plasmid vector; TC^R	(Hoang et al. 1998)
pEX18Tc-PFL_3627	1.5 kb SOE-PCR fragment containing truncated PFL_3627 gene cloned into *Hin*dIII-*Bam*HI sites of pEX18Tc; used to construct Pf-5ΔPFL_3627	This study
pBBR1-MCS2	5.1-kb broad-host range plasmid; KmR *lacZ*	Kovach et al. (1995)
pBBR1-PFL_3627	PCR product containing contiguous PFL_3627 gene with native promoter and terminator regions cloned into pBBR1-MCS2 at *Hin*dIII-*BamHI* sites.	This study

Targeted gene deletions

To construct the mutant, the target gene PFL_3627 was deleted from the chromosome via an allelic exchange method according to a previous protocol with some modifications (Hoang et al. 1998). The up and downstream fragments of approximately 550 bps in the flanking regions of genes for homologous recombination with the chromosome were constructed using either KOD Hot Start DNA Polymerase (Merck) or GC-RICH PCR System (Roche), and spliced together with the splicing overlap extension (SOE) PCR technique (Choi and Schweizer 2005). The primers for amplification of flanking regions are listed in Table 2.2. The spliced DNA product was propagated in pGEMT-Easy cloning vector for TA cloning via re-amplification with GC-RICH PCR System (Roche).

Table 2.2. Primers for amplification of the flanking regions of PFL_3627

PFL_3627 Up Forward	gtattgatatGGATCCcttggacagcacgaacaagg (capital letter depicts *Hind*III digested site)
PFL_3627 Up Reverse	gcgatgtggtagtcgcttgaactctatctcgggcg
PFL_3627 Dn Forward	gagatagagttcaagcgactaccacatcgccaaga
PFL_3627 Dn Reverse	attgcatttaTCTAGAggtggccggcacctacctgg (capital letter depicts *Bam*HI digested site)

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Cloning of the truncated gene into the suicide vector pEX18Tc was achieved by restriction enzyme digestion at the *Hin*dIII and *Bam*HI sites and T4 ligation, and resulted in recombinant vectors with the truncated genes. The recombinant suicide vector carrying the DNA product was transformed into *E. coli* S17-1 under chemical transformation as S17-1 carries helper genes to catalyse the subsequent conjugation in the chromosome. Then the plasmid was conjugated from S17-1 to wild-type (WT) Pf-5 by biparental mating. For biparental mating, cells for each bacteria were mixed in 1:1 ratio (based on OD_{600} of 0.5 for each bacteria) and plated onto LB agar containing 1 % glycerol (w/v). The plate was incubated without spreading for 6 hours at 37°C. After the incubation, cells were harvested from the surface of the plate and resuspended in 5 mL 0.1 M $MgSO_4$. The resuspended solution was spread on LB agar medium containing 200 μg/mL tetracycline and 100 μg/mL streptomycin to select for Pf-5 bacteria carrying the recombinant plasmids (tetracycline was used for selection of the pEX18Tc vector while streptomycin provided the selection for *P. protegens* Pf-5 which displays innate streptomycin resistance). The surviving colonies were grown in 1 mL of non-selective LB media with shaking at 200 rpm, at 25°C for 2-3 hours and then plated onto LB agar containing 5 % sucrose. The high sucrose content selected against bacteria that carry pEX18Tc due to the presence of *sacB* gene in the plasmid (Hoang *et al.*, 1998). Colonies that were selected by sucrose plating were patched in parallel onto both LB agar containing 5 % sucrose and LB agar containing 200 μg/mL tetracycline, the latter to confirm the absence of the recombinant pEX18Tc plasmid in the bacteria. The colonies were then screened by colony PCR with FastStart Taq DNA polymerase (Roche) and gene-specific primers for mutants that had truncated genes in the chromosomes. The PCR product was sequenced to verify the gene deletion.

The detection of growth defect of PFL_3627 mutant

Overnight cultures in LB at 27 °C of Pf-5 and Pf-5Δpfl-3627 were diluted in the same medium to OD_{600} = 0.04. The growth of both strains was monitored at 27°C with shaking speed of 200 rpm. The culture of wild-type and mutant were sampled for OD_{600} absorbance measurement constantly at each hour for a 12-hour period to determine their growth curve.

RNA extraction, cDNA synthesis and labelling

For RNA extraction, 10 ml of Pf-5 and Pf-5ΔPFL_3627 cultures at OD_{600} 1.0 were harvested by centrifugation at 4000 rpm for 15 min at 4 °C. Trizol (Invitrogen) and the RNAeasy kit (Qiagen) were used to extract the RNA according to the provided protocols. Off-column

RNAse-free DNAse (Ambion) treatments of the RNA samples were used to remove any residual DNA contamination. The concentrations of RNA samples were measured by Nanodrop ND1000 spectrophotometer (NanoDrop Technologies). Approximately 10 µg of extracted RNA per sample was reverse-transcribed with random hexamers using the SuperScript Plus Indirect cDNA Labeling System (Invitrogen). The cDNA was synthesized in the presence of either cyanine 3 (Cy 3) or cyanine 5 (Cy 5)-coupled nucleoside triphosphate analogues. For each indirect labelling reaction, approximately 20 µg of cDNA containing at least 200 pmol of Cy 3 or Cy 5 dye per µg were produced. Synthesized cDNA was labeled with either Alexa Fluor 555 or Alexa Fluor 647 (Invitrogen) as per the manufacturer's instructions. The cDNA concentration and labeling efficiency were determined using a Nanodrop 384 ND1000 spectrophotometer (NanoDrop Technologies).

qRT-PCR quantification of PFL_3627 expression across different growth phases in liquid culture and biofilms

To determine the time point for characterisation of the PFL_3627 regulated transcriptome of Pf-5, levels of the PFL_3627 transcript were monitored throughout the growth cycle of Pf-5. Wild-type strain Pf-5 was grown in LB and RNA was isolated and processed from triplicate cultures at OD_{600} = 0.5, 1.0, 2.0, 3.0 and 4.0. The cDNA was reverse-transcribed from RNA using Superscript II and random hexamers (Invitrogen). Quantitative reverse-transcriptase PCR was performed on the cDNA using SYBR Green on an Eppendorf mastercycler (Eppendorf AG, Hamburg, US). Cycle threshold (CT) values were measured by Eppendorf Mastercycler ep Realplex 2.2 software. The ΔΔCT method was used to calculate the relative template abundance in control versus experimental samples (Livak and Schmittgen 2001). The PFL_4054 gene encoding a homolog of a *P. aeruginosa* housekeeping gene involved in cell wall recycling was utilized as an internal control (Jacobs, Joris et al. 1995). The pairs of primers used for qRT-PCR quantification pf PFL_3627 expression and reference gene were designed by primer 3 software to generate a product of 164 bp and 192 bp in length respectively (table 2.3).

The qRT-PCR quantification was reapplied to measure the expression of PFL_3627 in biofilm and the counterpart planktonic cells in the supernatant throughout biofilm development over a 24 h period in 6-well plates. The biofilm and its supernatant were separated and RNA extracted after 8, 16 and 24 h incubation respectively. The reverse transcription and qRT-PCR were performed as described above.

Table 2.3. Primers for qRT-PCR in quantification of PFL_3627 expression.

Target genes	Primer name	Primer sequence
PFL_3627	PFL_3627-qF	GACCGAGCTGGAACGAGAAA
	PFL_3627-qR	TCTTCAGGATCGGGTCCACT
Reference gene	Ref-qF	CACACAACCTTGACCGGACT
PFL_4054	Ref-qR	GTAATGTCGCTTGCCGTTGG

DNA Microarray experiments

A *P. fluorescens* Pf-5 genomic microarray composed of 70mer DNA oligonucleotides representing each of the 6147 annotated protein-coding genes spotted in triplicate was used for the microarray experiments. The slides used were spotted with 70-mer DNA oligonucleotides that represent 6147 open reading frames in Pf-5 genome (Hassan et al. 2010). Three biological replicates of fluorescently labelled cDNA were hybridized onto microarray slides according to previous protocols (Peterson et al. 2004) and subsequently scanned with Axon 4000B scanner empowered by GenePix 6.0 software. The data was analysed with Spotfinder (TIGR) software and normalized by the LOWESS algorithm with block mode and a smoothing parameter value of 0.33 with the TIGR-MIDAS package (Saeed et al. 2003). The gene expression was analysed statistically by using the Statistical Analysis of Microarrays (SAM) algorithm with a threshold of false discovery rate (FDR) of less than 1 % where a change of 2 fold or greater was considered as significant (Tusher et al. 2001).

qRT-PCR validation of microarray results

To validate the microarray data, quantitative reverse-transcriptase PCR (qRT-PCR) was used to analyse a subset of genes. In the qRT-PCR experiment, 2.5 µg of extracted RNA samples used for the microarray experiments were reverse-transcribed to cDNA using SuperScript VILO cDNA Synthesis Kit (Invitrogen). The synthesized cDNAs were used as the template for qRT-PCR reactions. The reaction consisted of 5µl solutions in total, including GoTaq® qPCR Master Mix and gene-specific primers designed online by primer 3 software. The genes PFL_5586 and PFL_5587 encoding ribosomal protein S7 and S12 were chosen as internal reference genes. The reactions were processed in a Mastercycler ep Realplex4 S (Eppendorf). Three technical replicates were performed for each sample. Cycle threshold (CT) values were measured by Eppendorf Mastercycler ep Realplex 2.2 software. The ΔΔCT

method was used to calculate the relative template abundance in control versus experimental samples (Livak and Schmittgen 2001).

Swimming and swarming Motility assay

Swarming motility and swimming motility of Pf-5 and Pf-5ΔPFL_3627 were assessed respectively on LB media supplemented with 0.6% and 0.3% agar (Invitrogen). The wild type and mutant strains were each grown overnight at 27°C and then diluted to OD_{600}= 0.2 in LB, and 5µl of the culture was spotted onto the centre of agar plates. Plates were incubated at room temperature for two days prior to imaging. For each assay, three biological replicates with 5 plates for each replicate were tested.

Biofilm formation and rapid attachment assays

Biofilm formation was assayed in 96-well microtitre plates at 27°C according to a previous protocol with some modifications (O'Toole and Kolter 1998). Three biological replicates were performed for each test. Briefly, the wild type and mutant strains were grown at 27°C overnight and diluted to OD_{600}= 0.04 in LB. 100µl aliquots of culture were transferred into each well of the microtiter plates and incubated for 8, 16, and 24 hours at 27°C respectively without agitation. After incubation samples were rinsed by double-distilled H_2O (ddH_2O) twice and stained with 1% crystal violet. Biofilm staining was performed at room temperature for 20 min, and then the plates were washed twice with ddH_2O to remove any unbound stain. Ethanol was added to the wells for 20 min to extract the crystal violet dye. Then the absorbance of the dissolved dye was read at 600 nm. The values were corrected for the negative controls, which are the microtitre dishes with LB and washed as above.

Rapid attachment of bacterial cells to a surface was analyzed as described previously with modifications (O'Toole and Kolter 1998). Briefly, overnight cultures grown in LB were diluted in the same medium to an OD_{600} of 1.0. In each well of the 96-well microtiter plate, 100 µl culture was dispensed. Cells were allowed to adhere for 30 min at 27°C prior to crystal violet staining. The following washing steps were as described in biofilm formation assay. Three biological replicates were tested in the experiment. The results were analyzed statistically by a student t-test.

Complementation test

PFL_3627 with its putative promoter region was amplified by PCR using Pwo DNA polymerase (Roche) for complementation of the mutant Pf-5ΔPFL_3627. To construct the

complementation plasmid pBBR1-PFL_3627 for the mutant, the amplified fragment was cloned into pBBR1-MCS2 at *Hind*III and *Bam*HI sites. The resulting complementation plasmids were transformed into *E. coli* S17-1 and then transferred into the mutant strains via biparental mating as described above. The Pf-5 wild type and Pf-5ΔPFL_3627 carrying the pBBR1-MCS2 plasmid were used as the control. Strains carrying these vectors conferred kanamycin resistance so they were selected by incorporating kanamycin (50 μg/mL) into the substrate medium.

Detection of acyl-homoserine lactones (AHL) using biosensor strains

The method for AHL detection is based on previous protocols (Ravn et al. 2001). There were two biosensor strains used here. *A. tumefaciens* strain A136 has a *lac*Z fusion to the *tra*I gene and produces a blue colour in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and AHLs. The *C.* v*iolaceum* strain CV026 encodes CviR which regulates the production of a purple pigment when induced by short chain AHLs. The biosensor strains were grown on LB agar plates supplemented with antibiotics (*A. tumefaciens* A136: Tetracycline 4.5 mg/ml, Spectinomycin 50 mg/ml; *C.* v*iolaceum* CV026: Kanamycin 20 mg/ml). *P. aeruginosa* PAO1 was used as the positive control. The strain to be tested for induction of *C. violaceum* CV026 was streaked on a LB agar plate in parallel to the monitor strain with a distance of approximate 2cm. Detection of AHL production by *A. tumefaciens* A136 was done in a similar assay with substrate agar supplemented with 50 mg/ml X-gal.

Exogenous AHL assay

The exogenous AHLs were added into the substrate medium for Pf-5 wild-type and mutant in biofilm formation and motility assays in order to test if the luxR regulator PFL_3627 senses these signalling molecules. The AHLs were added at the final concentration 20μM. Four AHLs representing long- and short-chain respectively were used in the assays: N-butanoyl-homoserine lactone (BHL or C4-HSL), N-(β-ketocaproyl)-L-homoserine lactone (KHL or 3-O-C6-(L)-HSL), *N*-(3-hydroxy-decanoyl)-L-homoserine lactone (HDHL or 3-OH-C10-HSL), and *N*-(3-oxo-decanoyl)-L-homoserine lactone (ODHL or 3-oxo-C12-HSL).

Result

The putative LuxR type regulator PFL_3627

A previous study identified two putative LuxR family protein genes PFL_3627 and PsoR, PFL_5298, in Pf-5 and its closely related strain CHA0 by *in silico* analysis (Subramoni et al. 2011). The former LuxR type regulator, PFL_3627, encodes a hypothetical protein product of 240 amino acid residues.

The construction of Pf-5ΔPFL_3627

To elucidate the regulatory role of the LuxR family protein, PFL_3627, this gene was deleted from the Pf-5 chromosome by allelic exchange. The unmarked mutant Pf-5ΔPFL_3627 was constructed by successful truncation of the target gene and the deletion was verified by PCR (Figure 2.1). The constructed mutant has about 0.6kbp sequence truncated from the gene PFL_3627 which is approximately 0.7kbp in length. For complementation tests, the PFL_3627 with its upstream putative promoter was cloned into the plasmid pBBR1-MCS2 to create the pBBR1-PFL_3627 and mobilised into the mutant. The wild-type Pf-5 and PFL_3627 mutant carrying the blank plasmid pBBR1-MCS2 were used as the control.

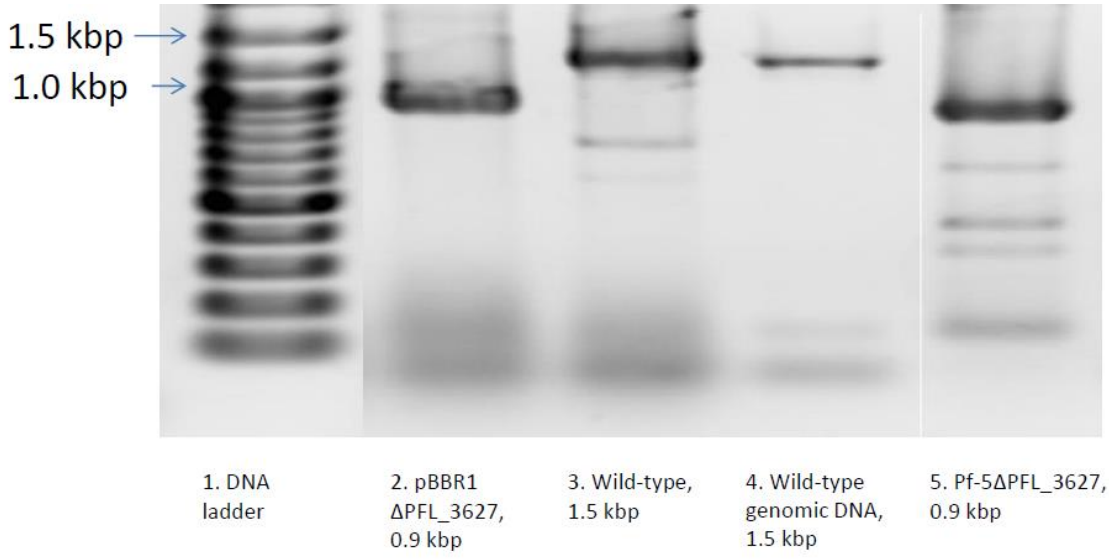
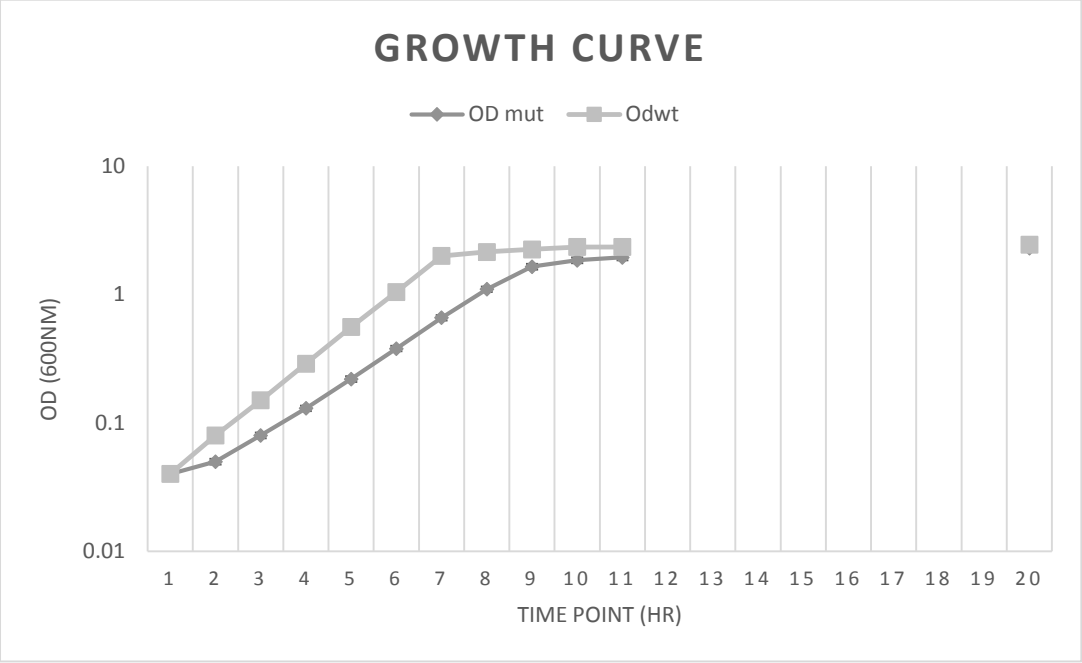


Figure 2.1. The PCR verification of the mutation on the luxR regulatory protein PFL_3627. From left to right: lane 1, DNA ladder; lane 2, product from pBBR1ΔPFL_3627; lane 3, product from Pf-5 wild-type; lane 4, product from wild-type genomic DNA; lane 5, product from Pf-5ΔPFL_3627. The amplified DNA product from the wild-type genomic DNA of Pf-5 was 1.5 Kbp, whist 0.9 Kbp from the mutant with 0.6kbp truncated.

Deletion of PFL_3627 causes a growth defect in Pf-5

To determine the effects of the PFL_3627 deletion on the phenotype of Pf-5, we first investigated if there was any impact on bacterial fitness. Some mutations can cause impaired cellular physiological functions. Therefore, growth of the wild-type and mutant strains was monitored during lag, exponential and stationary phases (Figure 2.2). The growth curves indicate the mutant had an increased lag time and a reduced cell replication rate during the exponential phase compared with the wild-type strain, as the generation time was approximately 60 mins for wild-type and 84 mins for mutant (Figure 2.2a). The growth defect was rescued by introducing pBBR1-PFL_3627, a plasmid expressing PFL_3627 into the mutant for complementation (Figure 2.2b). The growth of the mutant carrying the pBBR1-PFL_3627 was identical to the wild-type containing the blank pBBR1-MCS2 plasmid, while comparatively the PFL_3627 mutant carrying the empty vector showed defective growth. Together, these results demonstrate that the loss of PFL_3627 leads to a reduction in cellular fitness.

a).



b).

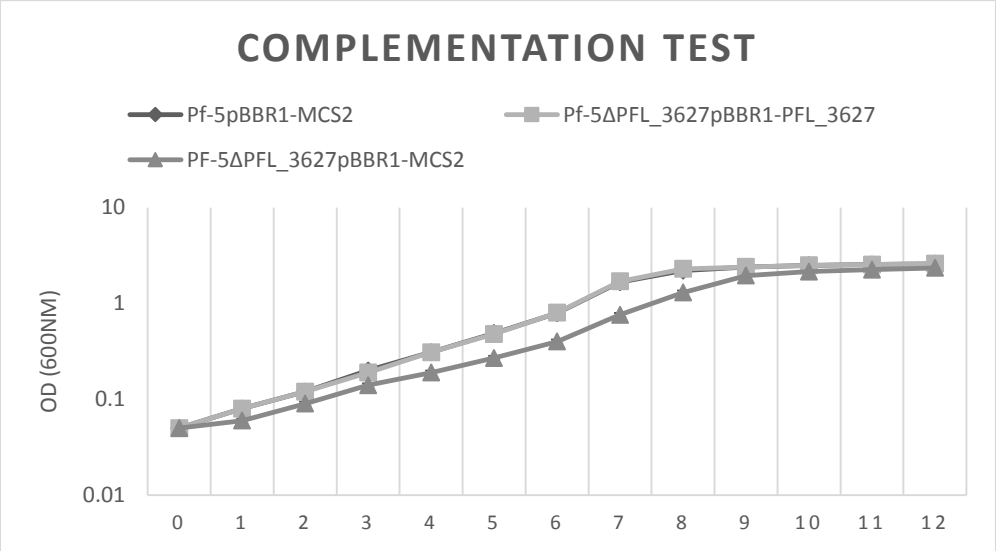


Figure 2.2. The Pf-5ΔPFL_3627 has a defect in exponential growth phase. a) Growth curves of Pf-5 wild-type and Pf-5ΔPFL_3627 at 27°C with shaking speed 200 rpm. b) Complementation test: the growth curves of Pf-5pBBR1MCS2, Pf-5ΔPFL_3627pBBR1PFL_3627 and Pf-5ΔPFL_3627pBBR1MCS2 at 27ºC shaking at 200 rpm.

Transcription of PFL_3627 is not affected by bacterial cell density or growth phase

As LuxR regulated QS is a cell density dependant process affecting the sessile-planktonic growth transition and biofilm formation, we monitored the transcription level of the LuxR type regulator PFL_3627 in various cell densities across the planktonic growth phases and static biofilm development of Pf-5, respectively, with the purpose of choosing an optimal sampling point for the subsequent transcriptomic analysis and to detect any difference in its expression in sessile vs. planktonic growth. To extract the RNA from planktonic culture, an overnight culture of Pf-5 was inoculated into LB medium at 27°C with a shaking speed of 200 rpm and harvested at OD_{600} values of 0.5, 1.0, 2.0, 3.0 and 4.0 respectively. Similarly, the overnight culture was inoculated into LB medium in 6-well plates for biofilm formation, and at time points of 8, 16 and 24-hr incubation, the RNA was extracted from both the biofilm and static planktonic counterpart in the supernatant. The RNA extracted from the samples was reverse transcribed into cDNA and the relative abundance of PFL_3627 transcripts was determined by qRT-PCR. The relative transcription levels of PFL_3627 stayed unchanged throughout the growth phases, indicating its expression is cell density independent (Figure 2.3). Its transcription in biofilm and supernatant at each time point again remained constant, suggesting the expression of this gene is not affected by growth phase (table 2.4). For each experiment, three biological replicates were assessed and the values were averaged.

Table 2.4. The relative transcript levels of PFL_3627 in biofilm and supernatant detected at 8h, 16h and 24h by qRT-PCR are not statistically different.

	8hr	16hr	24hr
biofilm	1.00±0.031	0.97±0.025	1.03±0.059
supernatant	1.07±0.032	0.97±0.049	0.95±0.028

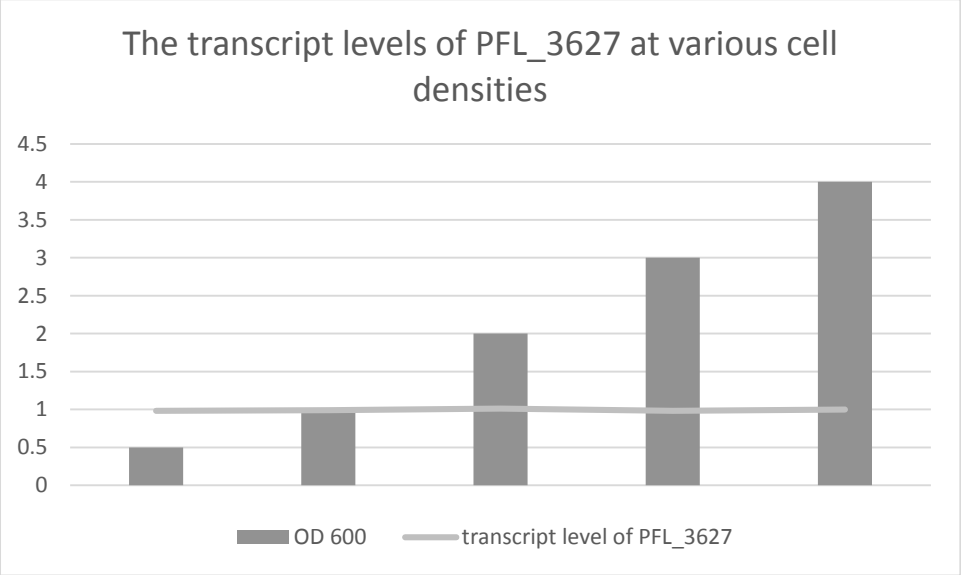


Figure 2.3. The expression levels of PFL_3627 transcripts in liquid culture growth at 27°C across bacterial growth measured by OD600 values of 0.5, 1.0, 2.0, 3.0 and 4.0. The quantity of the transcripts per cell remained constant and independent on growth phase.

Deletion of PFL_3627 promotes initial surface attachment and early-phase biofilm formation in Pf-5

P. protegens Pf-5 can transfer from planktonic to a sessile growth form stabile on surfaces and subsequently form multi-cellular communities called biofilms. Biofilm formation involves sequential stages including the initial attachment of cells to a surface, development of microbial colonies and differentiation of extracellular matrix, biofilm maturation, and dispersal. As LuxR-associated QS is often recognized to control biofilm formation, we investigated the effects of PFL_3627 on initial surface attachment and biofilm development. With the crystal violet assay, the biofilm formed by wild-type and mutant was tested after 8, 16 and 24 hours depicting the phases of biofilm differentiation, maturation and dispersal

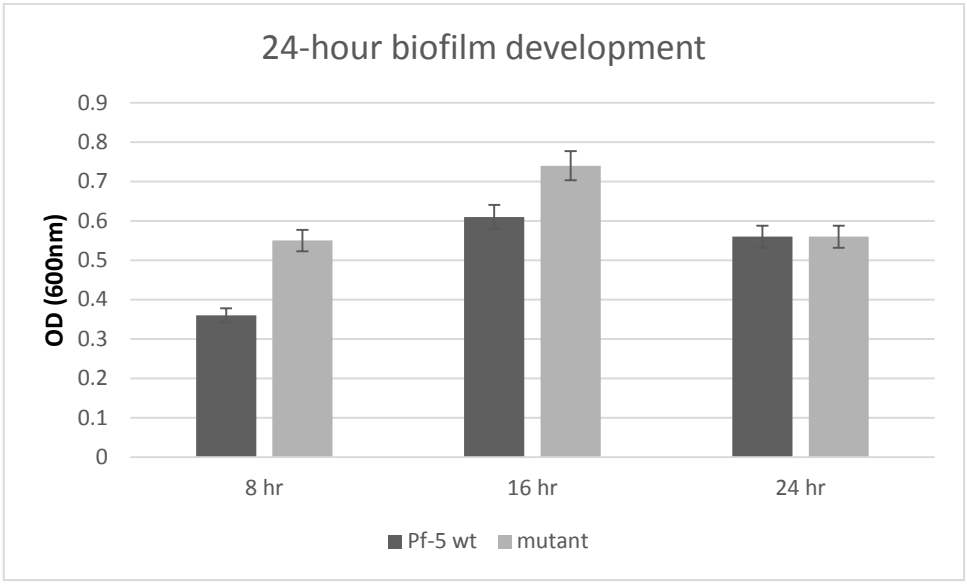
(Figure 2.4). A t-test calculation on the results suggested that the mutant exhibited augmented biofilm formation at 8 and 16 hours incubation. However after 24 hours, when the development progressed into the dispersal phase, the biofilms formed by wild-type and mutant were not statistically different. The elevated biofilm formed by the mutant compared with the wild-type at the 16-hour point decreased to an equal level as wild-type at the 24-hour point suggesting the dispersal of the mutant biofilm is possibly faster than wild-type one. A complementation test confirmed that when PFL_3627 was expressed in the mutant *in trans*, its biofilm formation at early phases was suppressed and restored to wild-type levels (Figure 2.4).

As the gene PFL_3627 represses the early biofilm formation in Pf-5, we investigated if its suppression applied to bacteria in other species. The pBBR1-PFL_3627 was transformed into *E. coli* top10 and its biofilm formation for the period of 12 hours was tested, using the bacteria containing the blank plasmid pBBR1MCS2 as the control. The result suggests the biofilm formed was not statistically different and thus PFL_3627 does not influence the biofilm formation of *E. coli* (Figure 2.5).

Biofilm formation can be affected by active initial attachment or efficient differentiation during development. To test the efficiency of the initial attachment of the wild-type and mutant strains, a rapid attachment assay was performed (O'Toole and Kolter 1998). The results suggest the mutant has an augmented surface-attaching capability, as its population during initial attachment was double that of the wild-type (Figure 2.6). The same test on the complemented strains additionally verifies the increased capability of surface attachment by the mutant: when the plasmid pBBR1-PFL_3627 was introduced into the mutant, its initial attachment was repressed and restored to the wild-type.

Overall, these data suggest that the PFL_3627 negatively regulates early biofilm formation in Pf-5 by repressing its initial attachment.

a).



b).

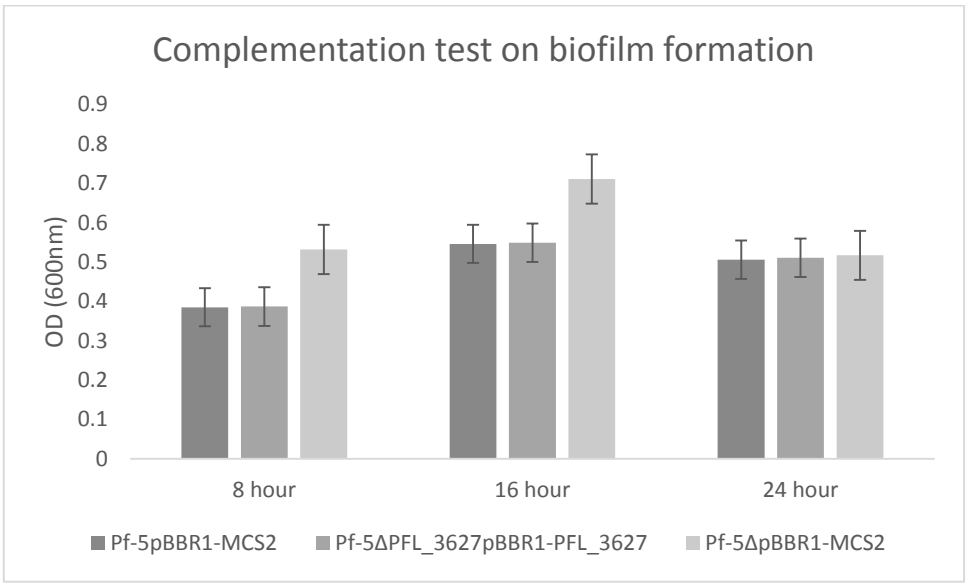


Figure 2.4. Static biofilm assay in 96-well titre plates sampled at time points of 8, 16 and 24 hours incubation, tested for 3 biological replicates in each experiment: a).The biofilm formed by Pf-5 wild-type and mutant using LB medium in 96-well plates after 8, 16 and 24 hours. b). Complementation test on biofilm formation by Pf-5ΔPFL_3627pBBR1-PFL_3627, Pf-5pBBR1-MCS2 and Pf-5ΔPFL_3627pBBR1-MCS2.

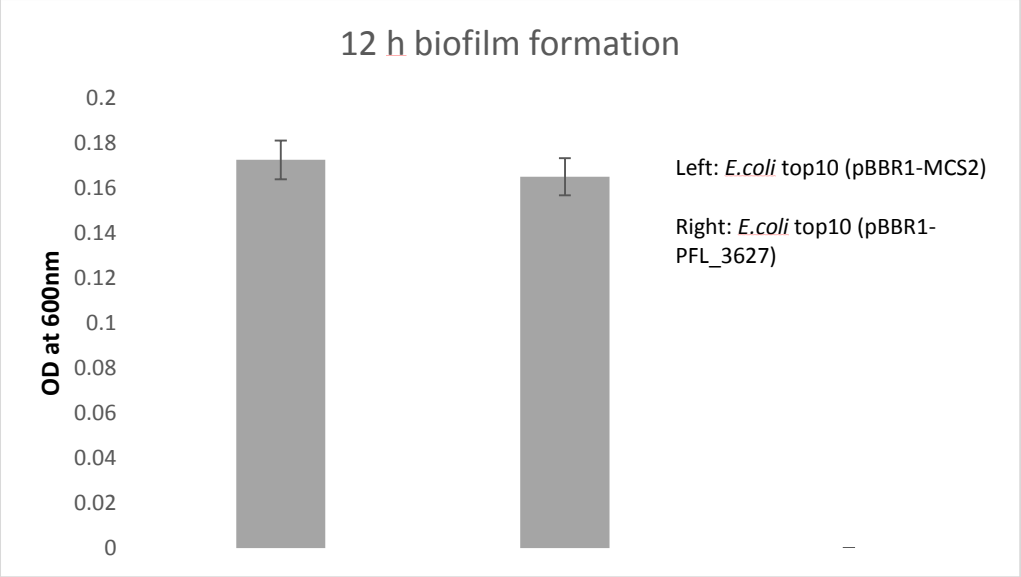


Figure 2.5. Static biofilm assay of *E. coli* Top10 (pBBR1-PFL_3627) vs. *E. coli* Top10 (pBBR1-MCS2). The cloned gene PFL_3627 did not induce a difference in biofilm formation in *E. coli* Top10.

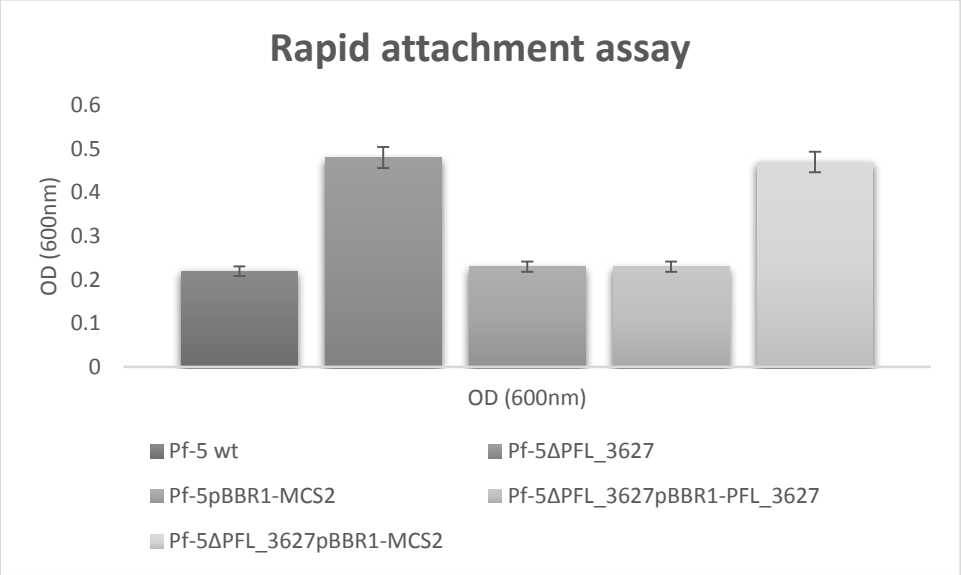


Figure 2.6. Rapid attachment assay on Pf-5 wild-type, PFL_3627 mutant and the complementation strains wild-type Pf-5 pBBR1-MCS2, Pf-5ΔPFL_3627 pBBR1-MCS2 and Pf-5ΔPFL_3627 pBBR1-PFL_3627. The bacteria were dispensed in 96-well microtiter plate filled with 100µl LB media per well. Cells were allowed to adhere for 30 min at 27°C and stained by crystal violet for quantification on stabilized biomass.

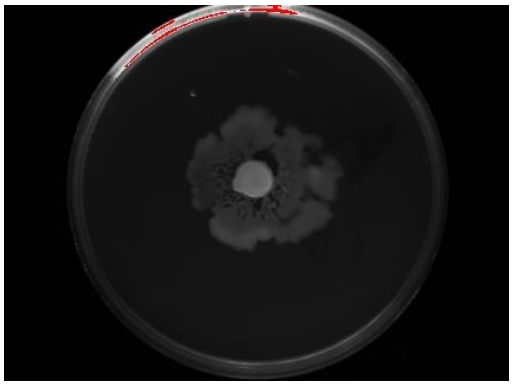
Mutation of PFL_3627 gives rise to more active swarming and 'smooth' swimming motility

LuxR regulated QS is known to affect the initial attachment for biofilm by regulating bacterial flagellum-mediated motility (Ochsner et al. 1995). As the LuxR type protein PFL_3627 has been found to repress the initial attachment to surfaces, a motility assay was performed to investigate its effect on the swimming and swarming capability of Pf-5. In the swarming motility assay on 0.6% agar plates, the Pf-5ΔPFL_3627 performed a

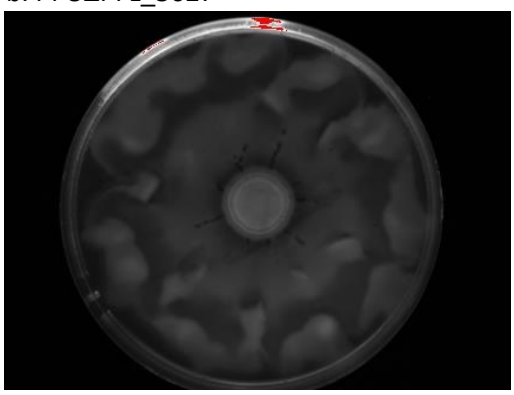
hyperswarming phenotype in comparison to the wild-type (Figure 2.7). As swarming motility involves factors including the synthesis of flagellum and biosurfactant, the hyperswarming motility from deletion of PFL_3627 suggests the regulator possibly supresses the flagellum differentiation or biosurfactant production. Complementation of the mutant reduced its swarming motility to wild-type (Figure 2.7). When the plasmid containing PFL_3627 was introduced into the mutant strain, it swarmed in the pattern identical to the wild-type strain carrying the blank plasmid. The movement of cells was suppressed by the transformation of the plasmid and the cell density appeared to be higher.

Swimming motility is another type of bacterial translocation driven by flagellum rotation. In the swimming motility assay on 0.3% agar LB plates, the mutant translocated in a smooth manner while the wild-type was in 'tumbled' swimming (Figure 2.8). The expression of PFL_3627 in the complemented mutant reversed this phenotype to wild-type. It is known in *E.coli* that the counterclockwise rotation of flagellum results in smooth swimming, while the clockwisewise rotation leads to tumbled pattern (Harshey 2003). If this is true also of Pf-5, PFL_3627 may regulate the directional change of flagellum rotation in swimming movement.

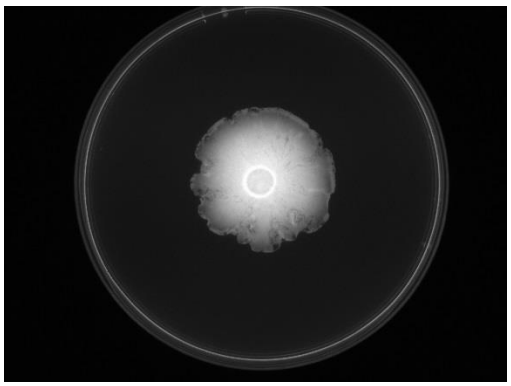
a. Pf-5 wt



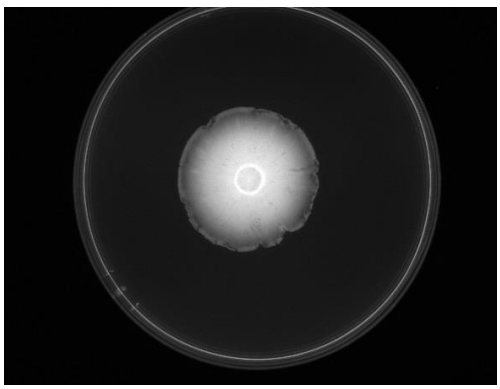
b. Pf-5ΔPFL_3627



c. Pf-5pBBR1MCS2



d. Pf-5ΔPFL_3627pBBR1PFL_3627



E. Pf-5ΔPFL_3627pBBR1MCS2

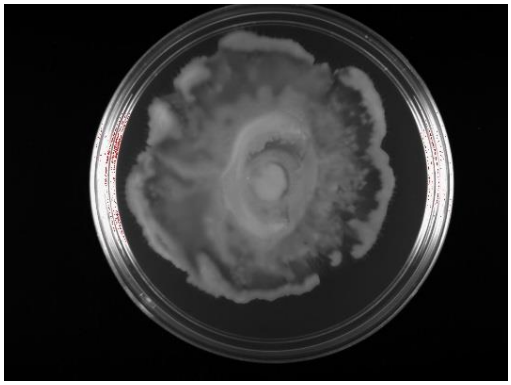
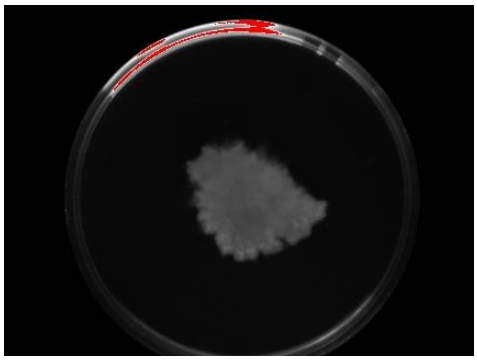
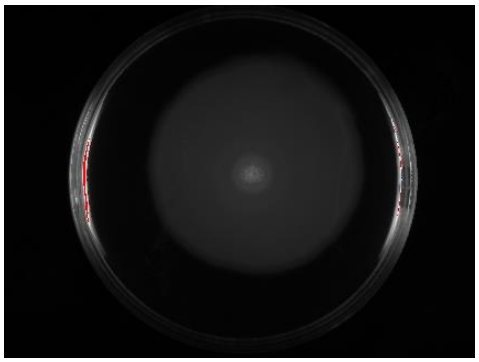


Figure 2.7. Swarming motility assay on LB 0.6% agar plates of Pf-5 wild-type, Pf-5ΔPFL_3627 and complementation strains Pf-5 wild-type pBBR1-MCS2, Pf-5ΔPFL_3627 pBBR1-MCS2 and Pf-5ΔPFL_3627 pBBR1-PFL_3627 after 48 h incubation at room temperature. The PFL_3627 mutant performed a hyperswarming motility and spread on the whole area on the plate.

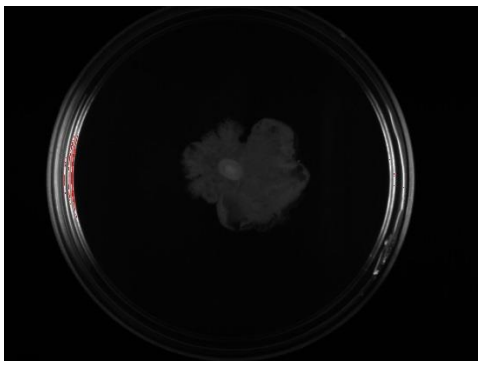
a. Pf-5 wt



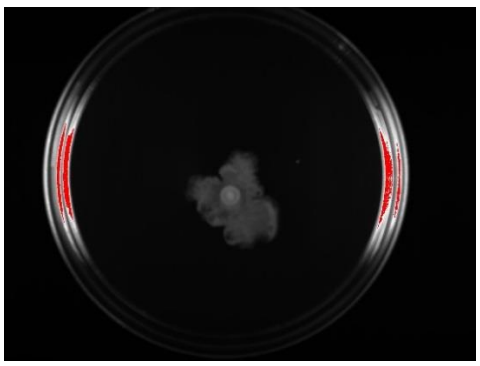
b. Pf-5ΔPFL_3627



c. Pf-5 pBBR1MCS2



d. Pf-5ΔPFL_3627pBBR1PFL_3627



e. Pf-5ΔPFL_3627pBBR1MCS2

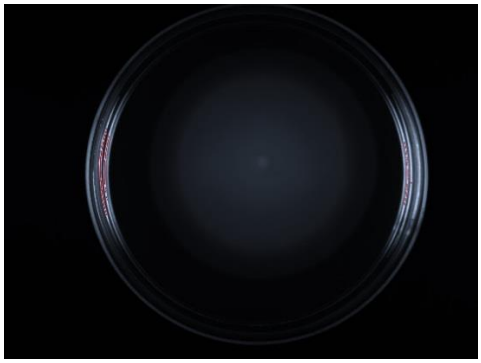


Figure 2.8. Swimming motility assay on LB 0.3%agar plates of Pf-5 wild-type, Pf-5ΔPFL_3627 and complementation strains Pf-5 wild-type pBBR1-MCS2, Pf-5ΔPFL_3627 pBBR1-MCS2 and Pf-5ΔPFL_3627 pBBR1-PFL_3627 after 48 h incubation at room temperature. The PFL_3627 mutant translocated in a smooth manner, while the wild-type was in a tumbled manner. On the plates a moderate portion of bacteria moved on the surface of media which might be in swarming manner, while the rest population were swimming within the media.

Together, the impacts of PFL_3627 deletion on the motility behaviours of Pf-5 allow speculation that it controls the direction of bacterial flagellum rotation, and negatively regulates flagellum differentiation or surfactant production.

The LuxR type regulatory protein PFL_3627 regulates the expression of a range of genes

In order to gain insight into the spectrum of the regulation by the LuxR family protein PFL_3627, a whole-genome oligonucleotide microarray of *P. protegens* Pf-5 was used to identify differentially expressed genes in the mutant in comparison to the wild-type (Figure 2.9). As the qRT-PCR results monitoring the transcripts level of PFL_3627 across the growth phases of Pf-5 indicates its expression remains constant at various cell densities and independent on growth phase, it was decided to harvest the culture of wild-type and mutant at OD_{600} of 1.0 for the whole transcriptome study as this was within the time point of early exponential phase. During the early exponential phase, the wild-type reached the sampling OD in less than 6 hours while the mutant was cultured for 8 hours before harvesting.
Pf-5 has three gene clusters encoding extracellular polysaccharides, which are verified to be components of the biofilm matrix in *P. aeruginosa*: Pel (Pellicle formation), Psl (polysaccharide synthesis locus) and alginate (Ryder, Byrd et al. 2007, Ghafoor, Hay et al. 2011). Genes of the *pslABDEFGHIJ* cluster (PFL_4208– 4216) for Psl production were significantly downregulated more than two fold in the PFL_3627 mutant (Table 2.5). The more than two fold downregulation also occurred to the entire *pelABCDEFG* operon (PFL_2972–2978), which is the gene cluster for Pel synthesis (Table 2.5). Four genes:

PFL_1013, FPL_1014, PFL_1015 and PFL_1016 in the gene cluster (FPL_1013-1024) were downregulated more than 2 fold. For the rest of the alginate biosynthetic gene cluster (PFL_1017–1024), no gene was significantly differentially regulated. Apart from polysaccharide, protein is another structural component in the biofilm matrix of the pseudomonads (Mann and Wozniak 2012). In *P. fluorescens*, the LapA protein is the adhesin for the extracellular matrix formation and surface attachment (Hinsa et al. 2003). The transcription of the two genes PFL_0133 and PFL_0134 for LapA synthesis and secretion was downregulated in the PFL_3627 mutant. All these results suggest that PFL_3627 functions as a positive regulator for the genes to synthesize biofilm extracellular matrix.

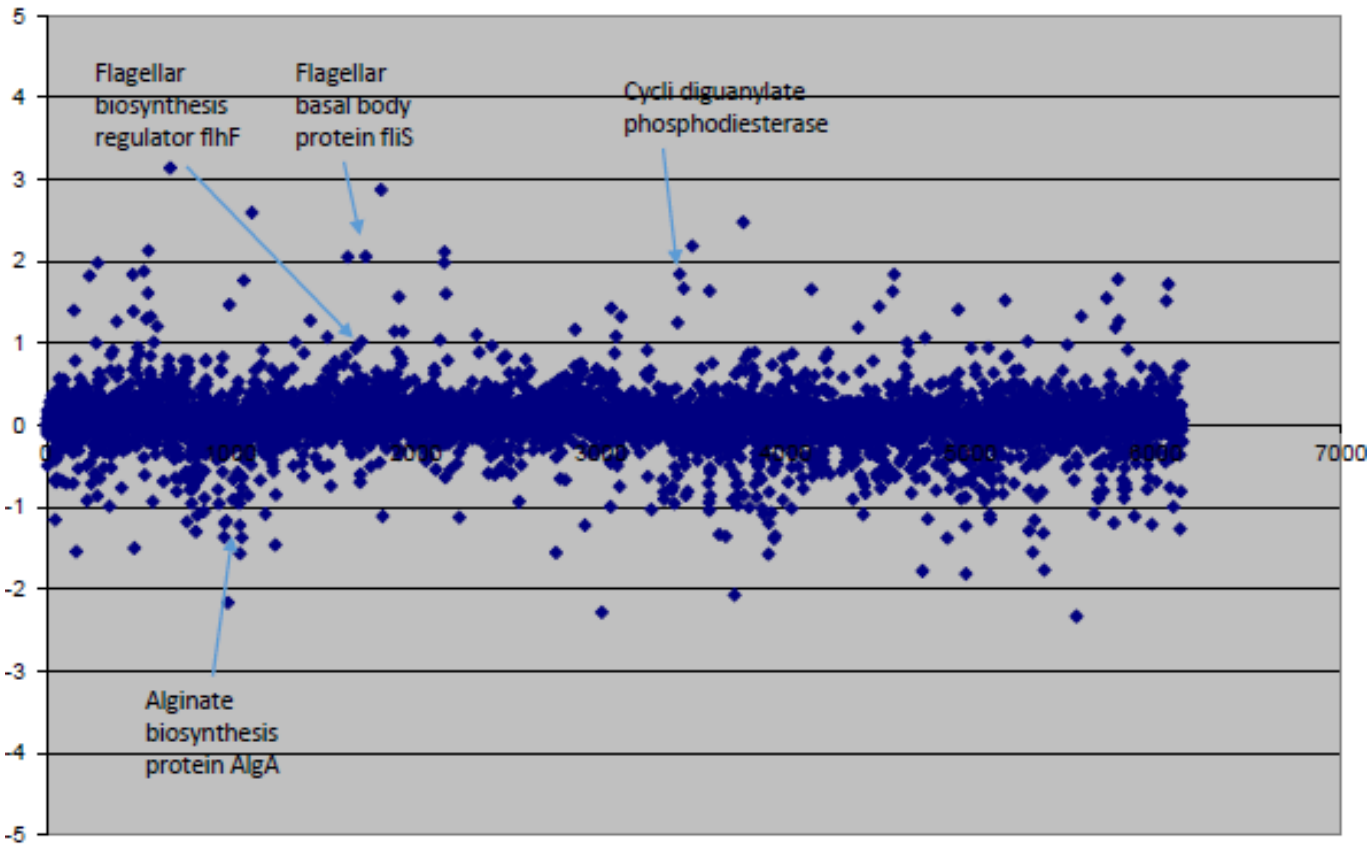


Figure 2.9. Differential gene transcription between wild-type *P. protegens* Pf-5 and PFL_3627 mutant assessed by genome-scale microarray. Each point is one of the 6147 annotated genes in the Pf-5 genome. The *x*-axis shows gene order (the origin of replication from 0 to 6147), and the *y*-axis shows the log2 value of transcript abundance of each gene in the PFL_3627 mutant relative to the wild-type strain Pf-5. Gene clusters whose members are regulated in a like manner by the mutation are seen as spikes in the array data.

Table 2.5. The list of genes overexpressed A) or underexpressed B) more than 2 fold in the PFL_3627 mutant in comparison to wild-type.

A)

Gene ID	Function	log_2 fold change
PFL1539	Protein of unknown function, DUF482 superfamily	3.13
PFL4674	helicase, SNF2 family	2.47
PFL1977	hypothetical protein	2.59
PFL2146	Non-ribosomal peptide synthetase ofaB	2.87
PFL4387	ABC transporter, periplasmic substrate-binding protein	2.18
PFL3035	aldehyde dehydrogenase family protein	2.11
PFL1419	hypothetical protein	2.12
PFL1632	flagellar protein fliS	2.05
PFL4479	Flagellar basal body rod protein FlgC	2.05
PFL3819	cyclic diguanylate phosphodiesterase	1.98
PFL4477	Flagellar hook protein FlgE	1.97
PFL1394	lipoprotein, putative	1.87
PFL5523	conserved hypothetical protein TIGR00244	1.84
PFL0089	cyclic diguanylate phosphodiesterase	1.77
PFL1934	conserved hypothetical protein	1.76
PFL0808	transcriptional regulator, AraC family	1.71
PFL1098	hypothetical protein	1.82
PFL4340	sulfatase family protein	1.66
PFL1614	FlgN protein superfamily	1.63
PFL4318	phosphoglycolate phosphatase	1.84
PFL5515	conserved hypothetical protein	1.63
PFL6140	L-asparaginase I	1.52
PFL0468	membrane protein, putative	1.55
PFL4478	Flagellar basal body rod modification protein FlgD	1.61
PFL1665	Flagellar biosynthesis regulator flhF	1.46
PFL5439	leucyl-tRNA synthetase	1.44
PFL3940	transcriptional regulator, Cro/CI family	1.42
PFL1666	Flagellar synthesis regulator FleN	1.41
PFL1338	conserved hypothetical protein	1.38
PFL0330	formate dehydrogenase, beta subunit	1.32
PFL3996	membrane protein, TerC family	1.32
PFL3390	cyclic diguanylate phosphodiesterase	1.83
PFL1433	lipoprotein, putative	1.32
PFL1667	Flagellar biosynthesis sigma factor	1.29
PFL0534	transporter, small multidrug resistance (SMR) family	1.27
PFL2787	probable alkylhalidase (EC 3.8.1.1) pltA	1.56
PFL3043	flaA2 protein (flaA2)	1.60
PFL4308	ribosomal protein S1	1.25
PFL3745	prophage LambdaSo, tail assembly protein I	1.16
PFL2808	hypothetical protein	1.14
PFL1246	acetyltransferase, GNAT family	1.26
PFL5324	ribosomal protein S20	1.19
PFL0798	conserved hypothetical protein	1.51
PFL0515	lipopolysaccharide core biosynthesis protein, putative	1.19
PFL2762	conserved hypothetical protein	1.14

PFL1616	flagellar basal body P-ring biosynthesis protein flgA	1.20
PFL3966	2-phosphonoacetaldehyde hydrolase	1.08
PFL5054	GGDEF domain protein	1.65
PFL5706	outer membrane ferrichrome-iron receptor	1.06
PFL1615	Flagellar basal body L-ring protein flgH	1.03
PFL3209	protease inhibitor Inh	1.10
PFL0032	transcriptional regulator, LysR family	1.42
PFL1449	sigma factor algU negative regulatory protein MucA	1.40
PFL1614	flagellar basal body rod protein FlgG	1.39
PFL2585	SIS domain protein	1.02
PFL5607	tyrosyl-tRNA synthetase	1.01
PFL1665	flagellar biosynthesis regulator flhF	1.01

B)

Gene ID	Gene function	Log2 fold change (-)
PFL0303	dTDP-4-dehydrorhamnose 3,5-epimerase	-2.33
PFL2972	Biofilm formation protein PelA	-2.28
PFL1846	hypothetical protein	-2.18
PFL2973	Biofilm formation protein PelB	-2.07
PFL5927	aminotransferase, class III	-1.81
PFL5693	biotin synthetase	-1.78
PFL0082	DNA polymerase I polA	-1.77
PFL4824	conserved hypothetical protein	-1.57
PFL1913	membrane protein, putative	-1.57
PFL3641	ornithine cyclodeaminase	-1.56
PFL2974	Biofilm formation protein PelC	-1.55
PFL1027	PhoH-like protein	-1.54
PFL1343	conserved hypothetical protein	-1.49
PFL2975	Biofilm formation protein PelD	-1.46
PFL2976	biofilm formation protein PelE	-1.38
PFL2977	Biofilm formation protein PelF	-1.37
PFL1923	cytochrome c oxidase, cbb3-type, CcoQ subunit	-1.37
PFL1825	conserved hypothetical protein	-1.36
PFL4577	transporter, LysE family	-1.35
PFL4859	bacterioferritin	-1.35
PFL2978	biofilm formation protein PelG	-1.33
PFL0134	TolC family type I secretion outer membrane protein	-1.37
PFL0133	lapA large adhesive protein	-1.29
PFL0042	bacterial luciferase family protein	-1.28
PFL1659	bacterial transferase	-1.26
PFL1013	alginate biosynthesis protein AlgA	-1.23
PFL1911	conserved hypothetical protein	-1.22
PFL3799	hypothetical protein	-1.22
PFL1014	alginate biosynthesis protein AlgF	-1.21
PFL1829	conserved hypothetical protein	-1.19
PFL4825	transporter, LysE family	-1.19

PFL1015	alginate biosynthesis protein AlgJ	-1.19
PFL1016	alginate biosynthesis protein AlgI	-1.17
PFL1837	glutamine amidotransferase, class I	-1.16
PFL4212	Glycosyltransferase PslE	-1.16
PFL4211	Glycosyltransferase PslD	-1.15
PFL6059	ribonuclease PH	-1.15
PFL4210	Glycosyltransferase PslC	-1.14
PFL3115	methyl-accepting chemotaxis protein	-1.12
PFL0621	hemolysin activator protein, HlyB family	-1.11
PFL2698	IS66 family transposase protein	-1.11
PFL4208	Glycosyltransferase PslA	-1.09
PFL1674	ParA family protein	-1.09
PFL4209	Glycosyltransferase PslB	-1.09
PFL4795	conserved hypothetical protein	-1.08
PFL2058	thioesterase family protein domain protein	-1.08
PFL4213	Glycosyltransferase PslF	-1.07
PFL4839	chaperonin, 10 kDa	-1.06
PFL4216	Glycosyltransferase PslH	-1.05
PFL4215	Glycosyltransferase PslG	-1.04
PFL4165	lipoprotein, NLPA family	-1.03
PFL4946	2-isopropylmalate synthase	-1.01
PFL4788	exodeoxyribonuclease I	-1.01
PFL4214	Glycosyltransferase PflF	-1.00
PFL1135	response regulator WspR	-1.00

Generally motile and sessile bacteria in biofilms are recognized as two opposing styles in a mutually exclusive relationship. Swarming and swimming motility are forms of flagellum-mediated movement and in rhizosphere-associated pseudomonads they appears as an essential feature for root colonisation (Barahona et al. 2010). In the PFL_3627 mutant the genes directing the synthesis of structural protein components of flagella (PFL_4477-PFL_4479) as well as the flagellar assembly and regulation (PFL_1614-1616, PFL_1632, PFL_1665) were moderately upregulated. The production of surfactants can enhance motility as these compounds reduce the surface tension for bacterial translocation. In Pf-5 there is a triple-cistronic operon consisting of genes *ofaA-C* (PFL_2145-2147) encoding for the production of the cyclic lipopeptide orfamide A as the surfactant to facilitate its motility (Gross et al. 2007). From the transcriptome analysis, the transcription of *ofaB* has been upregulated in the PFL_3627 mutant, suggesting production of orfamide A may be higher in the mutant and contribute to increased motility.

It has been widely acknowledged that in a range of bacteria the transition between biofilm and motile life stages is regulated by the secondary messenger cyclic diguanylate (c-di-GMP)

(Hengge 2009). In principle an increased level of c-di-GMP will induce biofilm formation and restrain the motility, while a lower level of it reverses the regulation. C-di-GMP is synthesized by diguanylate cyclases (DGCs) and degraded by phosphodiesterases (PDEs). The genome of Pf-5 includes 47 genes annotated as DGCs or PDEs or containing domains associated with DGC (GGDEF domain) or PDE (EAL domain) activity or both (Paulsen et al. 2005). In the transcriptome of the PFL_3627 mutant, the response regulator *wspR* (PFL_1135) which is a DGC encoding gene whose expression has been reduced more than 2 fold relative to the wild-type. Additionally, there are three genes PFL_0089, PFL_3819 and PFL_3390 encoding cyclic diguanylate phosphodiesterases (PDEs) which are upregulated in the PFL_3627 mutant, suggesting negatively regulated production and active cleavage of c-di-GMP, as well as potentially enhanced motility and inhibited biofilm differentiation.

Validation of microarray data by qRT-PCR

To validate the results of the microarray, qPT-PCR was performed with a set of gene specific primers targeting 8 highly regulated genes (Table 2.6). The results correlated strongly with the microarray data (Figure 2.10).

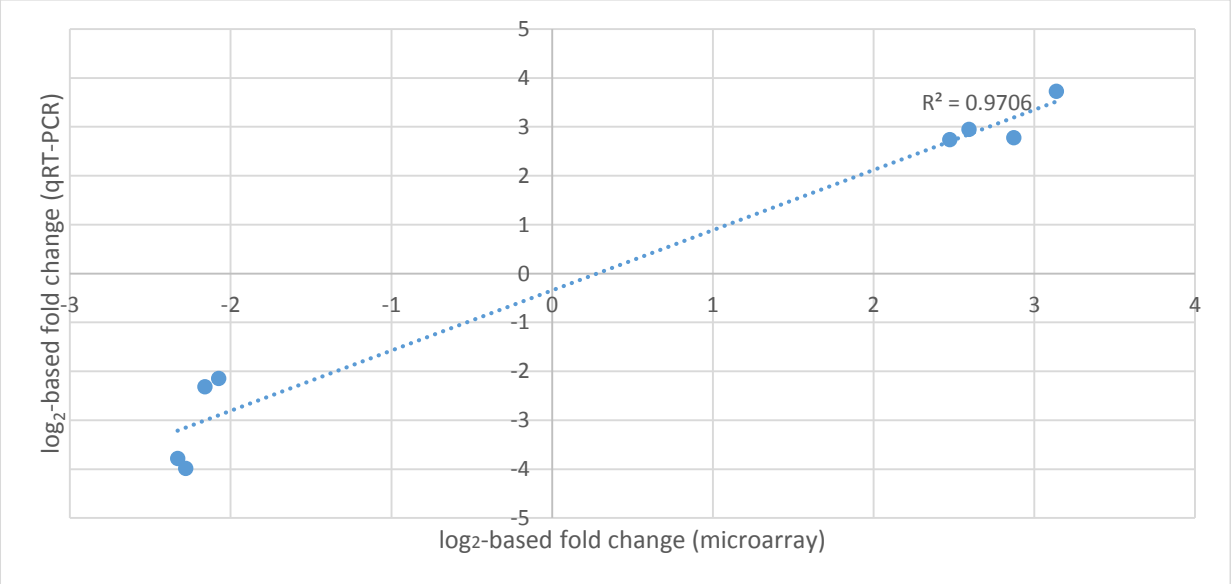


Figure 2.10. Validation of microarray result by qRT-PCR. The chart shows the correlations of log2-based fold-changes for 8 genes (PFL_0303, PFL_2972, PFL_1846, PFL_2973, PFL_1539, PFL_4674, PFL_1977 and PFL_2146) derived from microarray study and qRT-PCR with a strong correlation of coefficient (R2) of 0.9706.

Table 2.6. Primers used in qRT-PCR.

Target gene	Primer (forward)	Primer (reverse)
PFL_0303	CTTTCTGCGAGCGTGAATGG	ACGAGCGTGGCTTCTTCTAC
PFL_2972	TACGGCTTTCGCGATGTGAT	CTGCTTGGTGCCCGAATAGA
PFL_1846	TTTTGCACGCTCTGTCTCCA	GGCAAGGATAGCGTTCCAGT
PFL_2973	CGTGACCAGTTGTCCTGGAA	AACTGCAAGGTGTGGTCGAA
PFL_1539	TCGAAGGACTTTCCAGTGCC	CACGGTTCTGCCAGTGAAAC
PFL_4674	AACAAGGTGCTACTCCGTCG	AGGTCGAGCTCGCTGTTAAG
PFL_1977	TTTCAGCTCAACTTGCCCCA	CAGACATGCGATGAACTGCG
PFL_2146	GCTGTTCCATTCCTTCGCCTTC	AGCAGTCATCCGGCGAACG

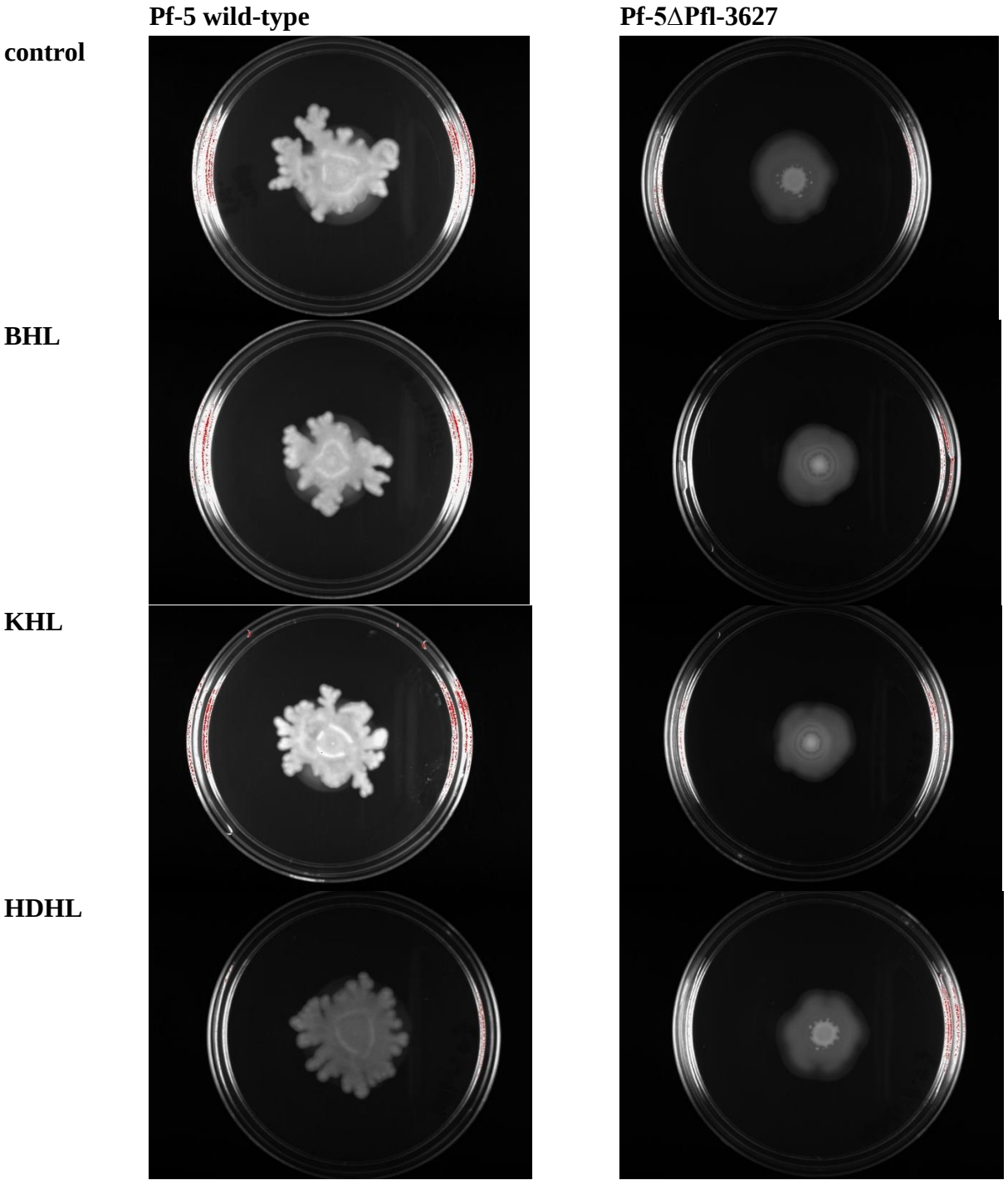
Biosensor strains detected no AHL synthesis from Pf-5

Endogenous production of AHLs can usually be detected by a colour indication using biosensor bacteria (Ravn et al. 2001). As LuxR family proteins have been widely shown to sense AHLs for activation of their regulation, the biosensor strains *A. tumefaciens* A136 and *C. violaceum* CV026 were used to screen for any endogenous AHL production by Pf-5 and Pf-5ΔPFL_3627 with the protocols from previous method (Ravn et al. 2001). The well-known AHL-producer *P. aeruginosa* PAO1 was used as the positive control. Consistent with the observation that the fully sequenced genome of Pf-5 has no annotated LuxI ortholog encoding synthase for AHL production (Subramoni et al. 2011), the biosensor strains did not detect any AHL released from Pf-5 or Pf-5ΔPFL_3627.

The LuxR regulator PFL_3627 does not appear to sense exogenous AHLs

To test if PFL_3627 responds to any exogenous AHLs, N-butanoyl-homoserine lactone (BHL or C4-HSL), N-(β-ketocaproyl)-L-homoserine lactone (KHL or 3-O-C6-(L)-HSL), *N*-(3-hydroxy-decanoyl)-L-homoserine lactone (HDHL or 3-OH-C10-HSL), and *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (OdDHL or 3-oxo-C12-HSL), representing long- and short-chained AHLs were dissolved in 10µl of ethanol and added into the LB medium at the final concentration of 20µM for the biofilm formation and motility assay. KHL mediates QS in the orginal model *Vibrio fischeri* (Engebrecht & Silverman 1984). HDHL was identified previously as one of the signalling AHLs in *P. fluorescens* (Shaw et al. 1997), while BHL and OdDHL were discovered in *P. aeruginosa* (Pearson et al. 1995, Winson et al. 1995). For the control, 10µl ethanol was added into the medium without any AHLs. If any of the exogenously added AHLs affected the phenotypes of wild-type but not the mutant, it would provide evidence that these compounds can be sensed by the LuxR protein receptor PFL_3627. None of the exogenously added AHLs have any impact on the swimming or swarming motility of these strains (Figure 2.11, 12), so it seems that PFL_3627 does not

detect AHLs as signalling molecules to induce its regulatory function. The results of the biofilm assay after 8 hours suggested no AHLs affected the biofilm formation by Pf-5 wild-type or the PFL_3627 mutant (Figure 2.13).



OdDHL

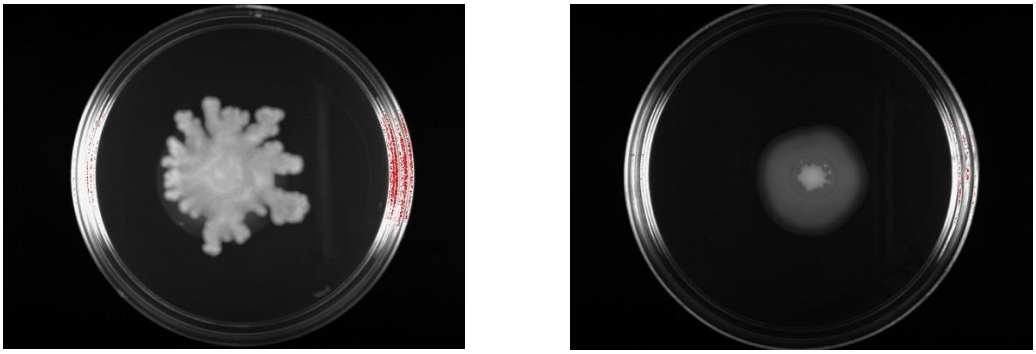
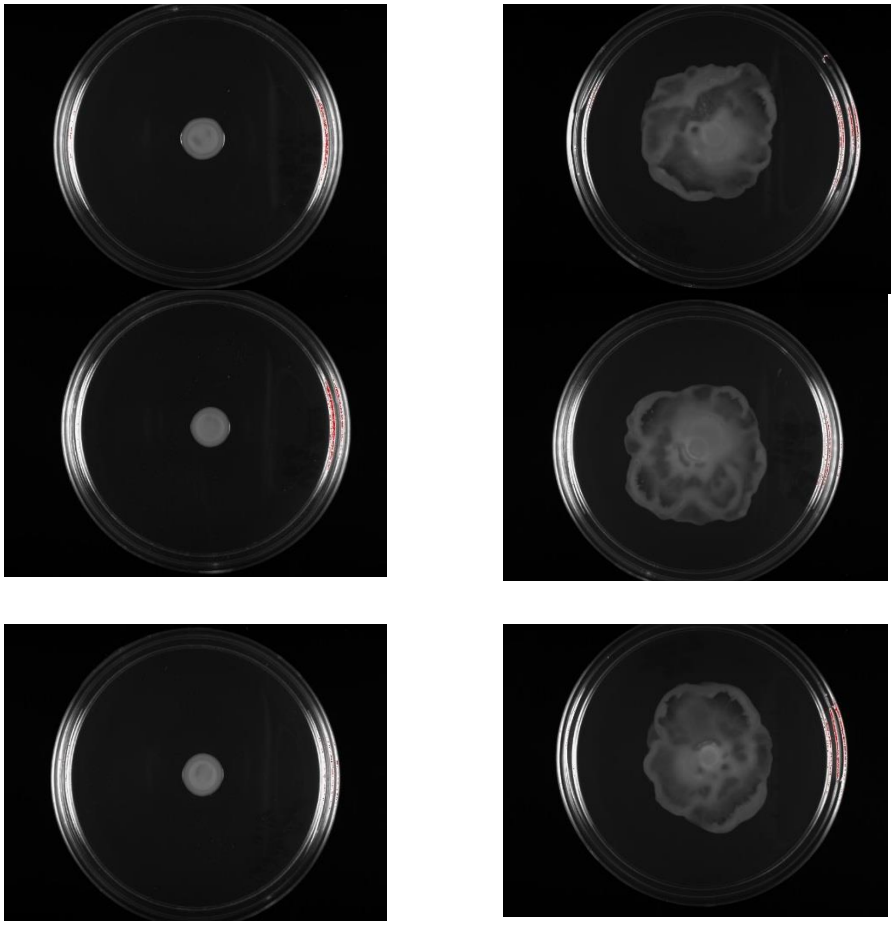


Figure 2.11. Swimming motility assay of Pf-5 wild-type and Pf-5ΔPFL_3627 with the addition of exogenous AHLs at the final concentration of 20μM in the substrate medium. The test was allowed to proceed for 48 hours at room temperature. None of the AHLs added caused any change in motility. A relatively large amount of bacteria swarmed on top of each plates, while a few of the bacteria were swimming with the agar media.

Pf-5 wt **Pf-5ΔPFL_3627**

Control

KHL



HDHL

OdDHL

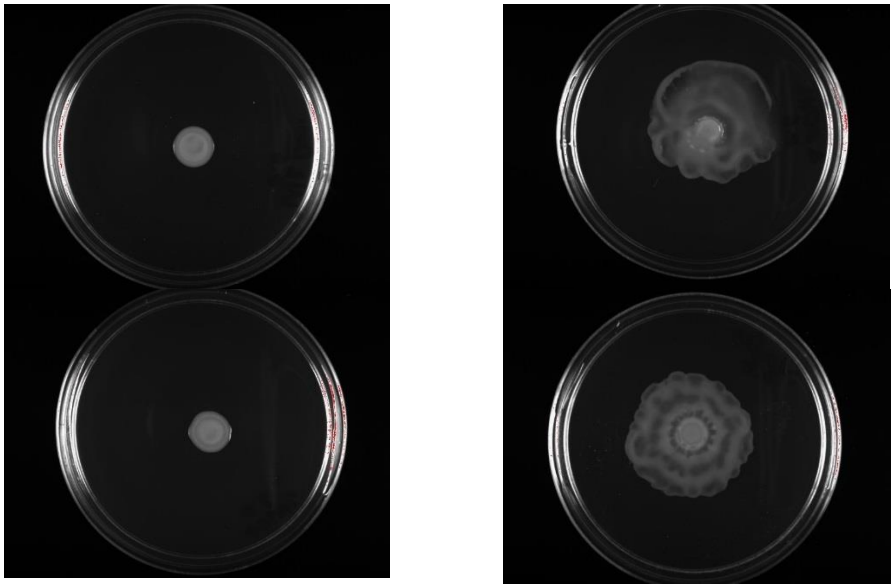


Figure 2.12. Swarming motility assay of Pf-5 wild-type and Pf-5ΔPFL_3627 with added exogenous AHLs at the final concentration of 20 µM in the substrate medium. The test was allowed for 48 hours at room temperature. None of the AHLs added caused any change in motility.

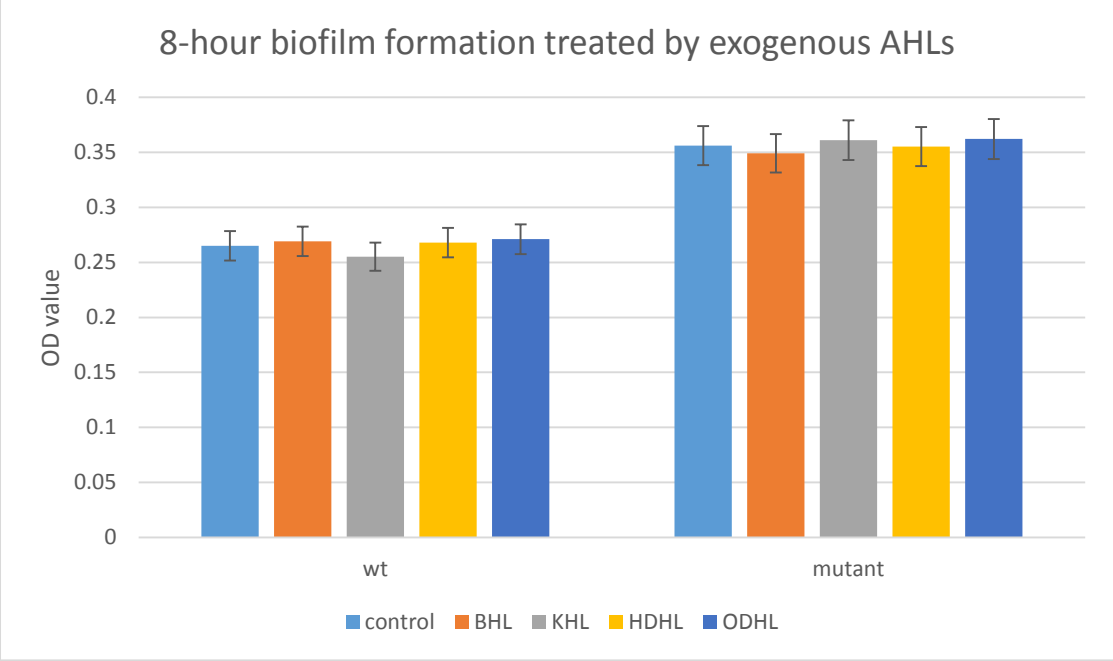


Figure 2.13. Biofilm formation assay at 8 h of Pf-5 wild-type and Pf-5ΔPFL_3627 with the addition of exogenous AHLs at the final concentration of 20 µM in the substrate medium. The exogenous addition of AHLs did not affect the biofilm formation by Pf-5 and Pf-5ΔPFL_3627.

Discussion

The interplay of gene expression in bacteria regulates their various phenotypes including bacterial interactions with the eukaryotic hosts. Bacterial motility facilitates the individual movement of these organisms across the various surfaces of their hosts and allows them to find favourable niches that promote the formation a multicellular community termed biofilm. The majority of pseudomonads are traditionally recognized as monotrichous and propel their movement by a single flagellum in similarity to the well-described *E. coli* model system (Sampedro I et al. 2014). Apart from bacterial flagellum, type IV pilus is another bacterial cellular appendage which mediates twitching as well as gliding motility in a broad spectrum of species of bacteria (Harshley 2003).

LuxR-family proteins can be associated with quorum sensing (QS) to synchronize bacterial gene expression at the population level and thereby affect motility and biofilm formation. Among the isolated plant-associated pseudomonads, there are a small proportion that encode an intact AHL mediated LuxR-I QS circuit (DeAngelis et al. 2007) while many others carry LuxR solos without a paired LuxI ortholog. In our studies, we used the model strain *P. protegens* Pf-5. The fully sequenced genome of this strain suggests it has no annotated LuxI ortholog for AHL synthesis (Paulsen et al. 2005) but 11 LuxR solos divided into three subclades upon the Pfam HMM search: PFL_0965, PFL_1107, PFL_2150, PFL_3220, PFL_3627, PFL_4238, PFL_4282, FPL_4852, PFL_5114, PFL_5298 (*psoR*) and PFL_5640 (Figure 2.14), and the current study investigated into the LuxR solo PFL_3627.

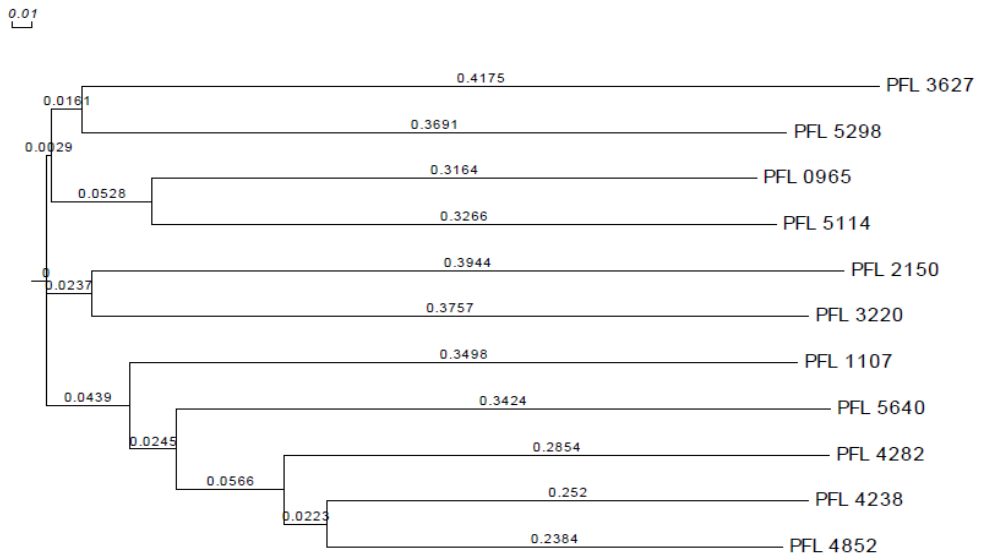


Figure 2.14. Phylogenetic tree of LuxR family regulators in Pf-5 genome. Overall 11 LuxR regulators have been predicted. The line segments with numbers indicate the lengths of branches representing

the amounts of genetic changes. The numbers next to each nodes represent credibility values for the node. The tree was generated from ClustalW2 by the neighbour joining method (Larkin et al. 2007).

The LuxR type regulator PFL_3627 contains all the nine amino acid residues conserved in the AHL-binding domain which are conserved in the majority of LuxR family proteins (Nasser and Reverchon 2007), but it did not respond to any of the long and short-chained AHLs tested in our study: N-butanoyl-homoserine lactone (BHL or C4-HSL), N-(β-ketocaproyl)-L-homoserine lactone (KHL or 3-O-C6-(L)-HSL), *N*-(3-hydroxy-decanoyl)-L-homoserine lactone (HDHL or 3-OH-C10-HSL), and *N*-(3-oxo-decanoyl)-L-homoserine lactone (OdDHL or 3-oxo-C12-HSL). The BHL and OdDHL are the signalling molecules triggering the QS in two distinct LuxR-I cascades (*rhl* and *las*) in *P. aeruginosa* (Deziel et al. 2004). As they were not sensed by PFL_3627 in Pf-5, neither did they induce any difference in biofilm formation or motility by Pf-5, it indicates a systematic difference in LuxR associated regulation between AHL producer pseudomonads and Pf-5. The well-conserved nine amino acid residues in the AHL-binding domains from both PFL_3627 and LuxR homologs from *P. aeruginosa* might indicate this structure is not the only requirement to determine utilisation of AHLs as the signalling molecule. PFL_3627 has an ortholog in the closely related strain *P. protegens* CHA0 and both strains are suggested as non-AHL producers (Subramoni et al. 2011). Genome analysis has shown that PFL_3627 differs from the previously characterised LuxR type regulator *PsoR* in Pf-5 and other LuxR solos which sense plant signal molecules as it has no proline imino peptidase (pip) encoding gene flanking it. Overall, the signalling molecule utilized by this LuxR family receptor to regulate gene expression in its regulon remains unidentified.

The Pf-5ΔPFL_3627 knockout mutant was used in a genome-scale microarray to identify the spectrum of genes under the regulation of PFL_3627, since the exact mechanism of its function and induction is not known. The expression profiles of 58 genes was upregulated and 54 downregulated as significant change (>2 fold), suggesting that PFL_3627 is a global regulator controlling the expression of genes involving in a range of cellular function categories. The gene clusters for synthesis of flagellum and biofilm extracellular matrix were especially abundant in the set of genes that are under the regulation of PFL_3627, suggesting one of its predominant regulatory roles is controlling flagellum-mediated motility and biofilm formation by affecting the assembly of the functional unit flagellum and the biofilm extracellular matrix. The microarray transcriptomic data correlated with the phenotypic

analysis of the PFL_3627 mutant in planktonic growth form which displayed a more active swarming motility and efficient initial surface attachment.

In the transcriptome of the PFL_3627 mutant, the response regulator/diguanylate cyclase encoding the gene PFL_1135 *wspR* (wrinkly spreader phenotype regulator) was significantly repressed relative to the wild-type strain. The WspR regulator is conserved across various pseudomonads including *P.aeruginosa, P. putida* and *P. fluorescens*. Its mutation reduces the level of c-di-GMP and thereby generates promoted swimming and swarming motility while abolishing or weakening biofilm formation in *P. aeruginosa* and *P. fluorescens* (Navazo et al. 2009). In combination to the upregulated three genes PFL_0089, PFL_3819 and PFL_3390 encoding the cyclic diguanylate phosphodiesterase which cleaves the c-di-GMP, it is appropriate to predict that in the PFL_3627 mutant the level of c-di-GMP would be reduced, and correspondingly the expression of genes involved in motility would be induced, whereas those involved in biofilm formation would be suppressed. Therefore, the regulation by the LuxR family protein PFL_3627 is potentially interconnected to the secondary messenger c-di-GMP controlled pathway: the LuxR regulator might regulate expression of some of the genes associated with the biofilm formation and motility via an indirect manner by interfering with the c-di-GMP pathway.

The microarray data suggests that PFL_3627 represses expression of genes for flagellum biosynthesis and regulation in planktonic growth, since in the PFL_3627 mutant in Pf-5, genes encoding the structural protein components of flagella (PFL_4477-PFL_4479) and genes encoding for its assembly and regulation (PFL_1614-1616, PFL_1632, PFL_1665) were overexpressed. The expression data is consistent with the observed changes in swimming and swarming motility seen in the mutant.

Highly motile bacterial populations are often recognized as advantageous for biofilm initiation as they can rapidly reach their favourable niches to colonize, and may enable a more competitive initial surface attachment before biofilm development (Eberl et al. 1999, Rather 2005). In agreement with this principle, the deletion of PFL_3627 confers an advantageous initial adherence as the PFL_3627 that was double that of the wild type bacteria. However, as time passed the surface-stabilized population ratio of mutant/wild-type decreased in subsequent biofilm development, possibly implying either less active biofilm differentiation or faster dispersal (Figure 2.15).

The transcriptional suppression of genes encoding the extracellular biofilm matrix components in the PFL_3627 mutant supported the possibility that biofilm development is hindered in this mutant: the genes PFL_0133 and PFL_0134 encoding the protein adhesin LapA and a protein for its secretion were downregulated, and the whole operons encoding for extracellular polysaccharide biosynthesis, *pslABDEFGHIJ* cluster (PFL_4208– 4216) for Psl production and *pelABCDEFG* operon (PFL_2972–2978) for Pel synthesis, as well as three genes: PFL_1013, FPL_1014 and PFL_1015 in the gene cluster (FPL_1013-1024) which synthesizes alginate were downregulated. It is appropriate to speculate that suppression of these genes encoding extracellular biofilm matrix would inhibit biofilm development.

At the late stage of biofilm development, the PFL_3627 mutant may have a faster dispersal than wild-type. This could be a consequence of the "hyperswarming" motility, as swarming motility has been suggested to be involved in the dispersal stage of biofilms (O'Toole and Kolter 1998). The production of various cyclic lipopeptides in pseudomonads is another potential parameter involved in biofilm dispersal (Boles et al. 2005, Dubern et al. 2006).

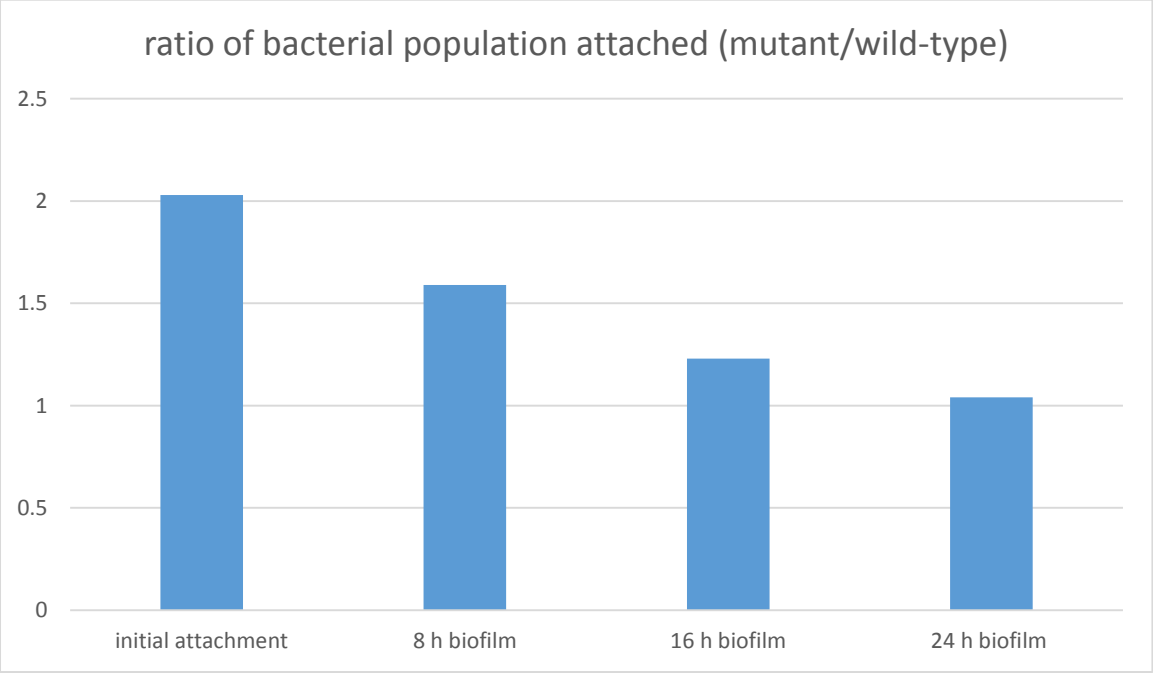


Figure 2.15. The ratio of bacterial population (mutant/wild-type) attached on surface at the initial attachment and during different time points of biofilm development and dispersal.

In the fully sequenced genome of Pf-5, there is a gene cluster orfA-C (PFL_2145-2147) which synthesizes the surfactant cyclic lipopeptide orfamide A in Pf-5 (Gross et al. 2007). Interestingly, in the gene cluster for production and regulation of orfamide A (PFL_2142 to

2150) there is another reversely transcribed LuxR family regulator PFL_2150 (Paulsen et al. 2005). In the transcriptome of the PFL_3627 mutant, the *orfB* gene (PFL_2146) was overexpressed relative to wild-type. This overexpression could result in higher levels of the cyclic lipopeptide orfamide A in Pf-5ΔPFL_3627. As a surfactant, orfamide A serves to reduce the surface tension for bacterial translocation and induce biofilm dispersal, so it might result in facilitating the hyperswarming motility and active biofilm dispersal in the PFL_3627 mutant (Figure 2.4 & 2.7). Apart from its effects on biofilm and motility, the diverse cyclic lipopeptides (CLPs) produced by pseudomonads have various antibiotic properties and a number of them exhibit antifungal, hemolytic, antiviral, phytotoxic, or antibacterial activities (Raaijmakers, de Bruijn et al. 2006). Particularly, orfamide A has been found to lyse the zoospores of *Phytophthora ramorum* (Gross et al. 2007), therefore its potential overproduction in PFL_3627 mutant might suggest the mutation provides plants with beneficial antagonistic activities against plant pathogens.

Apart from the cyclic lipopeptide orfamide A, the siderophores and antibiotics produced by Pf-5 are also important pathogen antagonists which play critical roles in biocontrol so the regulation on their production would have significant impact on the biocontrol properties of Pf-5. However, the transcriptomic profile suggests no gene or gene clusters for the production of the antimicrobial compounds including 2, 4-Diacetylphloroglucinol, pyochelin, pyoluteorin, pyrrolnitrin, and hydrogen cyanide were significantly differentially expressed in the PFL_3627 mutant. Notably, PFL_3627 in Pf-5 is located in the gene cluster (PFL_3599 to 3627) for pyrrolnitrin production by *prnABCD* (PFL_3604 to 3607) (Paulsen et al. 2005, Loper, Kobayashi et al. 2007). As heterologous expression of *prnABCD* in *E. coli* alone is able to produce pyrrolnitrin (Hammer et al. 1997), the flanking genes of them including PFL_3627 are not strictly essential for pyrrolnitrin biosynthesis while the mutation on PFL_3627 does not affect the transcription of them. Direct assaying for pyrrolnitrin production and proteomic profiling to exclude the possibility of posttranscriptional regulation of pyrrolnitrin production by PFL_3627 would clarify whether PFL_3627 has any role in pyrrolnitrin regulation.

In addition to its regulatory effects on phenotypes relevant to root colonisation and biocontrol, the PFL_3627 mutant displayed a growth defect from the lag to exponential phases indicating reduced bacterial fitness in laboratory monoculture growth . This suppression on bacterial growth starts from early stage at relatively low cell densities. At that stage, it is less likely for the bacteria to reach quorum density and for signalling molecule to

accumulate to the threshold concentration, so it is appropriate to hypothesize the activation of QS has not started. Therefore this growth defect is presumably independent of QS. At present, several known LuxR solos activated by unidentified plant compounds are not associated with AHL-mediated QS and their signalling molecules remain to be revealed.

Conclusion

LuxR family proteins have been shown to regulate quorum sensing, which controls phenotypes critical for biocontrol. Some of these regulators also mediate interkingdom signalling between plants and bacteria. However, limited studies have been conducted on LuxR regulators in plant beneficial rhizosphere pseudomonads. In this study, we targeted a LuxR type protein in the biocontrol strain *P. protegens* Pf-5 and investigated its regulatory effects on a several important phenotypes. Our data shows the LuxR family regulator PFL_3627 plays a role in the transcriptional regulation of a range of biological functions within *P. fluorescens* Pf-5, including bacterial growth, swimming and swarming motility, as well as biofilm formation. Deletion of PFL_3627 from the Pf-5 genome results in a reduced fitness and growth defect in the lag and exponential phases. This mutation alters the swimming motility phenotype of Pf-5, changing from smooth translocation in the wild-type to a tumbling phenotype, and induces hyperswarming motility. The hyperswarming PFL_3627 mutant is more efficient in initial surface attachment than wild-type in the initiation of the biofilm formation. These results suggest PFL_3627 has significant influence on the efficiency of biocontrol by Pf-5.

The genome-scale transcriptomic microarray provides the clues of the regulation by PFL_3627 on these phenotypes at the molecular level. More than one hundred genes were differentially expressed in the PFL_3627 mutant. The genes in clusters encoding components of the extracellular biofilm matrix, exopolysaccharides Psl, Pel and alginate, and lapA protein were downregulated by the mutation on PFL_3627, while the genes for biosynthesis and regulation of flagellum and its components were upregulated. The expression of genes for the synthesis of the secondary messenger c-di-GMP, which is widely recognized as the effector to suppress motility and promote biofilm, were suppressed, while the ones for its degradation were induced, thereby suggests a reduced level of c-di-GMP and correspondingly the increased motility. Overall, the data from transcriptomic microarray suggests the PFL_3627 influences the biofilm formation and motility in Pf-5 by directly regulating the genes

encoding the components of the extracellular biofilm matrix and flagellum or indirectly by coordinating the intracellular level of the secondary messenger c-di-GMP.
LuxR family regulators are recognized to control gene expression in its regulated operon by binding to signalling molecules, such as AHL or undefined plant compounds. The LuxR protein PFL_3627 has all conserved amino acid residues as the majority of AHL-responding LuxR homologs. However it did not sense the long- and short-chained AHL in our assay. In agreement with the absence of LuxI encoding AHL synthase in genome sequence of Pf-5, the biosensor strains did not detect AHL production by it. The signalling molecule recognised by PFL_3627 remains undefined.
There has been a growing interest in LuxR solos discovered in plant-associated bacteria, however, in rhizosphere inhabitant pseudomonads there have been limited number of studies on this category of regulator. The LuxR solos are suggested to mediate the bi-directional signalling between the bacteria and their eukaryote hosts. The in-depth knowledge of its regulatory mechanism will help the manipulation of the bacterial biocontrol properties.

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Chapter 3. The small regulatory RNA PhrS interferes with motility and biofilm formation in the biocontrol strain *Pseudomonas protegens* Pf-5

Abstract

In the past two decades, small regulatory RNAs have been intensively investigated on their roles in bacteria. The regulation imposed by small RNAs (sRNA) intervenes in various aspects of bacterial physiology by the mechanisms of basepairing with mRNA or modulating protein activities. However in pseudomonads like most bacterial lineages, although the fully sequenced genomes of a few strains reveal the existence of a number of small RNAs, only a small portion of these have been experimentally characterised and the majority are yet to be elucidated for their regulatory pathways and effects. In the biocontrol strain *Pseudomonas protegens* Pf-5, the complete genome was searched for predicted sRNAs by RNAspace and the sRNA PhrS was targeted in this study. The inactivation of *phrS* in Pf-5 abolished its swarming and swimming motility, and resulted in an abnormal biofilm development across a 24-hour culture period. When the *phrS* mutant grows on solid agar medium, the bacteria penetrate and proliferate within the solid substrate and differentiate into colonies of rhizoid morphology rather than forming round colonies on top of the surface. However, in liquid culture the growth rate of the *phrS* mutant did not show a significant difference compared to the wild-type. Consequently, multiple lines of evidence suggest that the sRNA PhrS regulates the phenotypes correlated to rhizosphere colonisation competency of Pf-5.

Introduction

The rhizosphere inhabitant bacterial species *Pseudomonas protegens* Pf-5 (previously known as *Pseudomonas fluorescens* Pf-5) evokes its biocontrol activity to suppress plant pathogens by colonizing the rhizosphere and secreting a range of anti-microbial compounds. A range of studies have identified its production of pathogen-antagonistic compounds and the identified the genes responsible (Table 3.1). However, the rhizosphere colonisation abilities of Pf-5 which are equally essential for effective biocontrol *in situ* have rarely been investigated.

Table 3.1. The synthesized anti-pathogen compounds by Pf-5 and encoding gene clusters.

Anti-pathogen compound produced by Pf-5	Gene locus	Sources
Pyoverdine	PFL_4079-98, PFL_4169-79 and PFL_4189-93	(Paulsen et al. 2005)
Enantio-pyochelin	PFL_3473-504	(Gross and Loper 2009)
2,4-Diacetylphloroglucinol (DAPG)	PFL_5951-58	(Bangera and Thomashow 1999)
Pyoluteorin	PFL_2784-801	(Paulsen et al. 2005)
Pyrrolnitrin	PFL_3599-609	(Choi and Schweizer 2005)
Rhizoxin analogs	PFL_2980-97	(Brendel et al. 2007, Loper et al. 2008)
orfamides A, B, and C	PFL_2142-51	(Gross et al. 2007)

In laboratory assays, bacteria in groups form colonies that vary in morphology when cultured on agar surface plates. The different extracellular components including exopolysaccharides and proteins often lead to changed colony morphology (Yoshida et al. 1985, Arias et al. 2003). The morphological types of colonies on agar media has been well exemplified in the pathogen *Flavobacterium columnare* (the causative agent for columnaris disease) as rhizoid and flat, non-rhizoid and hard, round and soft as well as irregular and soft (Kunttu et al. 2009). The various phenotypes of colonies imply diversity in terms of motility, virulence and surface adherence (Kunttu et al. 2009). In pseudomonads, the morphotypes of the rugose small-colony variants and wrinkly spreaders have been proofed for their relevance to biofilm-facilitated environmental adaptation (Mann and Wozniak 2012). In rhizosphere inhabiting *P.*

fluorescens strain F113, variants showing modulated colony morphology have been connected with competitive root colonization (Martinez-Granero et al. 2005). In fact, the colony variation may imply more modulation in their metabolic and ecological features than root inhabitation. In a later study, the *gac*/*rsm* transduction system controlling the production of a range of secondary metabolite compounds has been revealed to be inactivated in the morphological variants in *P. protegens* CHA0 (Workentine et al. 2010).

During bacterial colonisation of eukaryotic hosts, motile populations can efficiently reach novel niches where they can colonize (Eberl et al. 1999, Rather 2005). The colonizing bacteria usually aggregate in a sessile multicellular community assembled by self-produced intercellular matrix termed biofilm (Turnbull et al. 2001, de Weert et al. 2002, Danhorn and Fuqua 2007). This suggests that flagellum-driven motility on surfaces or within liquids and sessile biofilm formation are particularly decisive to achieve aggressive rhizosphere colonisation. Defects in the motility of rhizospheric bacteria, such as *P. fluorescens* and *Agrobacterium tumefaciens,* result in impaired competitive rhizosphere colonisation (Shaw 1991, de Weert et al. 2002). Among the patterns of motile translocation utilized by the pseudomonads, swimming and swarming motility mediated by rotational flagellum has been frequently investigated. It has been described in *P. fluorescens* SBW25 that the motile population is more competitive for their initial surface attachment (Turnbull et al. 2001). Attachment is the first stage in the initiation of bacterial biofilm development. Once the attached bacterial population stabilizes on the surface, they start to proliferate and secrete the extracellular matrix substances that stabilize the biofilm structure. The development of the motile population into a prosperous bacterial community colonized on a plant host is frequently regulated by the regulatory cascade termed quorum sensing (QS), which promotes synchronized gene expression at the population level. Some of the regulated genes are relevant to the synthesis and differentiation of the appendages including flagellum and extracellular matrix components which are critical for motility, surface attachment and biofilm formation. *P. aeruginosa* is a well-investigated model for QS. In addition to the widely known LuxR-I circuit mediated by the signalling molecule acyl-homoserine lactone (AHL) (see Chapter 2), a recent study revealed the presence of another set of QS hierarchy that exploits *Pseudomonas* quinolone signal (PQS) (Pesci et al. 1999).
PQS (2-heptyl-3-hydroxy-4-quinolong) belongs to the family of compounds termed 4-hydroxy-2-alkylquinolines. The PQS biosynthesis genes *pqsABCDE, pqsR, pqsH* and *pqsL* in *P. aeruginosa* were identified by screening a transposon-generated mutant library (D'Argenio

et al. 2002, Gallagher et al. 2002, Lepine et al. 2004). The precursor molecule for PQS synthesis is anthranilate, which is provided by conversion of chorismate or degradation of tryptophan (Farrow and Pesci 2007). Similar to the self-controlled production of AHL, PQS is another autoinducer which regulates its own synthesis by inducing the operon *pqsABCDE* (Gallagher et al. 2002). The PQS-mediated quorum sensing system is interconnected with the two sets of LuxR-I circuits (*las* and *rhl* systems) in *P. aeruginosa* for PQS biosynthesis and bioactivity (Pesci et al. 1999, McKnight et al. 2000, Diggle et al. 2003). To date, it has been shown in *P. aeruginosa* that PQS is involved in the regulation of physiological functions including biofilm formation (Diggle, Winzer et al. 2003), siderophore-mediated ion transport (Diggle et al. 2007) and production of the virulence factor pyocyanin (PYO) (Xiao et al. 2006). Apart from its own autoinduction, another stimulus regulating the production of PQS in *P. aeruginosa* is the small RNA PhrS (Sonnleitner et al. 2011). Consequently, PhrS interferes with the AHL-mediated QS signalling system indirectly.

As a novel class of regulators controlling gene expression in prokaryotes and eukaryotes, sRNAs have been investigated in numerous studies on their functional roles in bacterial lifestyles. In prokaryotic bacteria, sRNAs are usually 50 to 300 nucleotides in length and are usually not translated into peptides, although some exceptions including PhrS in *P. aeruginosa* have been found to be translated into polypeptides (Altuvia 2007, Sonnleitner et al. 2008). More than 150 sRNAs have been discovered based on bioinformatic prediction and experimental validation in prokaryotes, including 100 in *E. coli*, although only a small number of them have been experimentally characterized(Voss et al. 2009, Waters and Storz 2009). The approaches to predict sRNA candidates include comparative genomics, and machine-learning which predicts targets by their sequence features (Li et al. 2012). The genomics comparison approach detects sRNA by sequence conservation and secondary structure in closely-related genomes. Using a comparative genomics approach, the BLAST program was applied to *Escherichia. coli* searching for the sRNA genes in the intergenic regions by comparing potential sRNA regions against the genomes of *Salmonella typhi, S. paratyphi* and *S. typhimurium* and identified 24 putative sRNA genes (Argaman et al. 2001). The machine-learning approach sorts out features of known sRNAs and uses these features to build up models to predict candidates. By this method, more than 100 sRNA candidates were predicted in *E. coli* and subsequent experimental verification suggested a relatively high rate of them were actual sRNA transcripts (Saetrom et al. 2005). In pseudomonads, transcriptome sequencing and comparative analyses identified sRNA candidates in *P. aeruginosa* PAO1

and subsequent experiments revealed the Phr family RNAs from these candidate sequences (Sonnleitner et al. 2008).

The sRNAs are generally defined into several categories according to their mechanisms of regulatory interplay. The most extensively studied class are the *trans*-encoded sRNAs, that regulate mRNAs by a short region of base-pairing interactions (Waters and Storz 2009). Many sRNAs falling into this class interact with the ribosome binding site (RBS) of the target mRNA and thereby block translation. The other members belonging to this family of sRNAs function by interfering with ribosome-mRNA interaction by other mechanisms such as prevention of secondary structure formation or modification of mRNA stability. In contrast to *trans*-encoding sRNAs, there is another class of sRNAs affecting gene expression by basepairing with target RNA in a *cis*-encoding manner, denoted as antisense RNAs (asRNAs). This class of regulatory RNAs also interfere with translation or mRNA stability of the complementary sense genes themselves, or their transcribed products (Thomason and Storz 2010, Georg and Hess 2011). Apart from RNA-RNA interactions, another class of sRNAs achieve their regulatory effects by modifying protein activity through RNA-protein binding, without base-pairing to mRNA. This class of small regulatory RNA is exemplified by the highly conserved 6S RNA characterized in *E.coli* (Willkomm and Hartmann 2005). The cellular processes regulated by sRNAs so far encompass bacterial nutrient metabolism, quorum sensing (QS) and biofilm formation, environmental adaptation, and pathogenesis (Michaux et al. 2014). One typical instance of sRNA regulating QS and biofilm formation is the series of sRNAs *qrr1-5* in *V. cholera* (Figure 3.1) which interact with the global regulator Hfq protein, an RNA binding protein conserved in a broad range of species, which promotes translation of the genes required for the virulence and biofilm formation (Hammer and Bassler 2007). The central player Hfq has been probably one of the most intensively studied RNA-binding regulatory proteins. The *hfq* gene is conserved in more than 50% of sequenced bacterial genomes (Sun et al. 2002) and it was first identified as a host factor for phage Qβ in *E. coli* (Franze de Fernandez et al. 1972). The assistance of Hfq is essential to achieve the regulatory roles of a large collection of trans-acting sRNAs in addition to *qrr1-5*, such as DsrA (Soper and Woodson 2008) and OxyS (Figure 3.1) (Salim and Feig 2010). The conformational features of Hfq are a critical factor contributing to its pleiotropic regulatory effects, as it has a distal site for binding target mRNA (Soper and Woodson 2008, Link et al. 2009), and a proximal site as well as a lateral site to bind sRNAs (Schumacher et al. 2002, Brennan and Link 2007, Sauer et al. 2012, Sauer 2013). With this structure, the Hfq recruits sRNAs and target mRNA to form sRNA-Hfq-mRNA complex, facilitating the basepairing of

the sRNA with the mRNA. Apart from *qrr1-5*, other examples of verified sRNAs regulating QS include RNAIII in *S. aureus* which represses the expression of genes encoding the biofilm adhesin (Korem et al. 2005), and the *E. coli* sRNA McaS which induces the expression of genes encoding flagellum and negatively regulates the ones for biofilm matrix synthesis (Figure 3.1) (Jorgensen et al. 2013). A few sRNAs exert their regulatory effects on the transition of bacterial growth between aerobic and anaerobic conditions. This class of sRNAs is exemplified by ArcZ in *E.coli* and *S. enterica* which is upregulated under aerobic growth and repressed under anaerobic growth (Figure 3.1) (Mandin and Gottesman 2010), regulates their bacterial morphotypes and controls the shift between sessility and mobility (Monteiro et al. 2012).

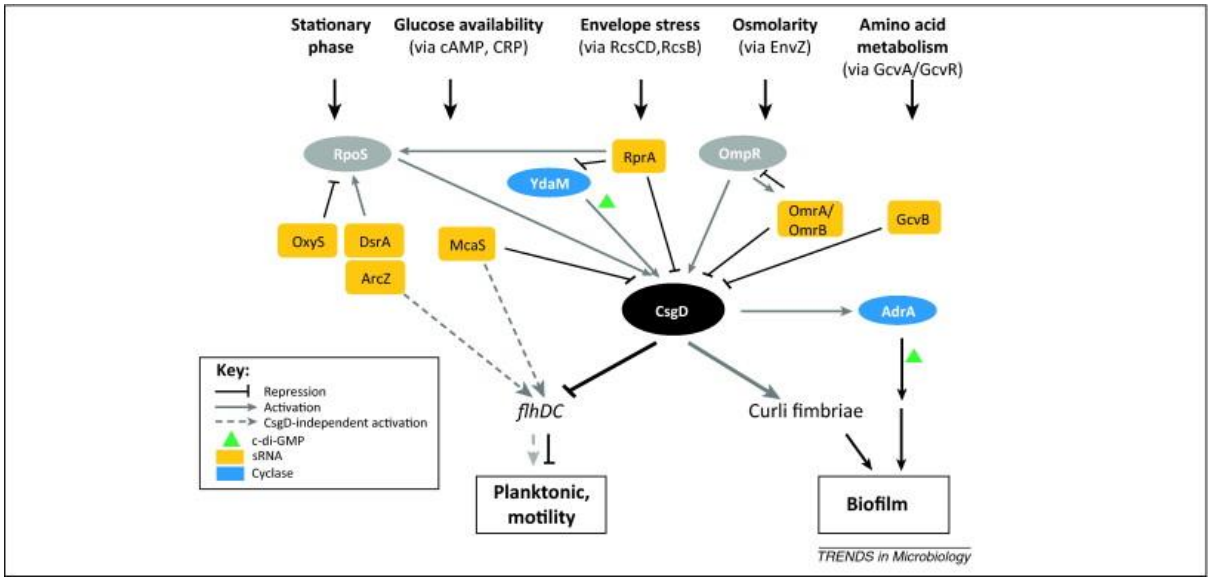

Figure 3.1. Regulatory pathway of sRNAs and their effect on biofilm formation and motility. The *oxyS*, *dsrA*, *arcZ* and *mcaS* positively regulate the expression of master regulator *flhDC* and induce the biosynthesis of flagellum for motility, while *mcaS* represses the transcriptional regulator *csgD* to inhibit biofilm formation. Taken from (Chambers and Sauer 2013).

In pseudomonads, sRNAs were detected early in this bacterial genus. The classical ones 6S and 4.5S sRNAs were detected in abundance from the lysates of *P. aeruginosa*, however their functions were not unravelled at that stage (Vogel et al. 1987, Toschka et al. 1989). In our knowledge, to date there is merely a trace number of sRNAs identified that have been functionally characterised in *Pseudomonas. spp* with details. As the number of studies on sRNAs from pseudomonads has been growing, the approaches to identify sRNAs are generally outlined as: direct isolation of the sRNAs in abundance and identification by sequencing (Vogel et al. 1987), extrapolation by sequence similarity to known sRNA in other genus (Toschka et al. 1989), prediction of sRNA by computational tools (Valverde et al.

2003), co-purification of sRNA with its binding protein effector (Sonnleitner et al. 2008) and genetic screening (Aarons et al. 2000). Among the limited number of sRNAs with known or predictable functions in pseudomonads, the classical one, 6S RNA, in *P. aeruginosa* PAO1 was suggested to have similar function to its homolog in *E. coli* due to high identity (Vogel et al. 1987). In *E. coli*, the 6S RNA inhibits the transcriptional expression of certain σ^{70} promoters in stationary phase by interacting with its RNA polymerase (Wassarman 2007). The other classical one, 4.5S RNA, from PAO1 was also not experimentally verified but extrapolated for its biological role from *E. coli* as the encoding genes *ffs* have 75% identity in its nucleic acid sequence from the two organisms (Sonnleitner et al. 2008). In *E. coli*, the 4.5S RNA forms the bacterial signal recognition particle with *ffh* encoded protein and serves essentially to target proteins to the cytoplasmic membrane (Keenan et al. 2001). Apart from the classical regulatory RNAs, the RsmY and RsmZ are the two sRNAs present in a range of pseudomonads strains across several species (Heurlier et al. 2004, Kay et al. 2005, Kay et al. 2006). The two sRNAs in *P. aeruginosa* interfere with the two component transduction system GacS/GacA cascade and sequester the regulatory protein RsmA (Pessi et al. 2001). As the RsmA is a repressor for the translation of several virulence genes, by sequestration of it, RsmY and RsmZ indirectly stimulate the expression of these virulence factors. Somewhat different in Pf-5 and its closely related strain *P. protegens* CHA0, in addition to RsmY and RsmZ, there is a third GacA-dependent RNA RsmX in this family (Kay et al. 2005). The three sRNAs jointly sequester regulatory proteins RsmA and its paralogue RsmE to relieve their inhibitory effects on expression of a set of genes and allow synthesis of biocontrol factors (Reimmann et al. 2005). By a similar pattern of protein sequestration, the sRNA CrcZ in PAO1 interacts with catabolite repression regulatory protein Crc and thereby regulates the expression of catabolite genes (Sonnleitner et al. 2009). Beside the sRNAs interacting with proteins, in *P. aeruginosa* there are a pair of highly similar sRNAs PrrF1 and PrrF2, which base-pair with the ribosome binding site of *sodB* mRNA and thereby occlude its translation initiation (Wilderman et al. 2004). The *sodB* gene encodes the iron-containing enzyme superoxide dismutase, and by blocking its translation, the PrrF1 and PrrF2 RNAs interfere the bacterial adaptation to iron-starving conditions.

In addition to the sRNAs described above, there is a recently identified small regulatory bifunctional RNA named PhrS belonging to the Phr (pseudomonas hfq-binding RNAs) family in multi-species of pseudomonads including *P. aeruginosa, P. protegens, and P. fluorescens*. A few bifunctional RNAs which act by basepairing with target mRNA and simultaneously encoding a peptide product have been discovered in several bacterial species

including the RNAIII in *Staphylococcus aureus* (Boisset et al. 2007) and *SgrS* in *E. coli* (Wadler and Vanderpool 2007). The *phrS* gene encoding a small regulatory RNA of 213 nucleotides in length was detected in three genomic surveys in *P. aeruginosa* (Livny et al. 2006, Gonzalez et al. 2008, Sonnleitner et al. 2008). Within the *phrS* gene of *P. aeruginosa* there is an open reading frame (ORF) of 37 codons (Sonnleitner et al. 2008). Unlike the majority of sRNAs, it appears to be translated into a 37 amino acid peptide and thus is featured the first translated bifunctional sRNA discovered in pseudomonads, however the biological function and acting mechanism of the polypeptide product are to be identified. It is produced in abundance in batch culture of high cell density under scarce oxygen availability, reflecting a potential regulatory role during anaerobic growth or high density culture. As a matter of fact, the recent study reveals its transcriptional activation by the oxygen-responsive regulator ANR under low oxygen concentration upon the physiological desire to activate the transcription of *pqsR* and thereby stimulate the production of PQS in *P. aeruginosa* (Figure 3.2) (Sonnleitner et al. 2011). The oxygen concentration is an essential factor for the biosynthesis of PQS in *P. aeruginosa*. Under a reduced oxygen concentration, to avoid abrogating PQS production, the ANR will upregulate the expression of *phrS* to induce the *pqsR*, thereby balance the bacterial cellular PQS level. Significantly, this regulatory pathway stabilizes the PQS level in pseudomonads under fluctuating availability oxygen source.

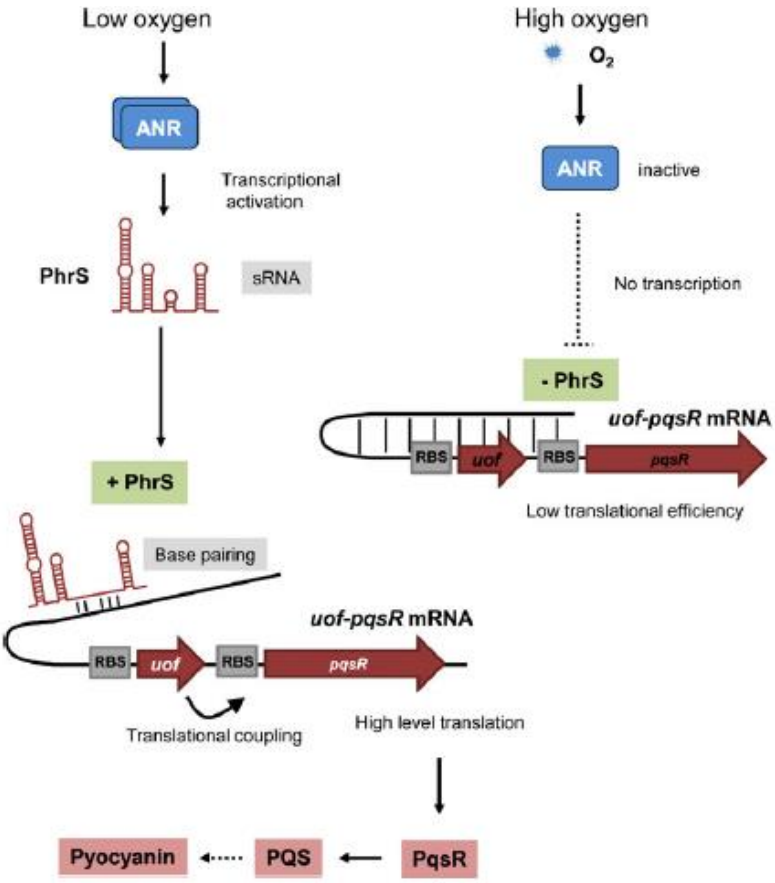

Figure 3.2. The current model of regulation on *pqsR* expression by PhrS in reaction to oxygen availability (Sonnleitner and Haas 2011). In response to the fluctuating oxygen concentration, the ANR will bridge the oxygen level and activity of PhrS which controls the translation of *uof-pqsR* mRNA. When the mRNA is translated at relatively high level, the PqsR protein will positively regulate PQS production and induce pyocyanin biosynthesis.

The *phrS* gene PFL_6286 annotated in *P. protegens* Pf-5 genome is 207 nucleotides in length (Paulsen et al. 2005). Based on BLAST, it has 71% nucleotide sequence identity to *phrS* in *P. aeruginosa* PAO1, and shares 100% sequence identity with homologs in *P. protegens* CHA0 and *P. protegens* Cab57 DNA . The genome of *P. protegens* Pf-5 is significantly different from that of *P. aeruginosa* and lacks the *pqsR* regulator, so the regulatory role of *phrS* in Pf-5 remains unclear. As a member of the Phr family of sRNAs, it putatively exerts its function by interacting with the Hfq protein (see above). In this study, the *phrS* gene was deleted from the Pf-5 genome using the allelic exchange approach, and subsequent tests on the phenotypes including bacterial fitness, swarming and swimming motility, and biofilm development were performed. This study hypothesized that PhrS is involved in the global regulatory network that controls phenotypes associated with root colonisation by Pf-5, and aimed to investigate the effects of sRNA regulation by PhrS particularly on these phenotypes.

Method

Bacterial strains, plasmid, and growth conditions

The bacterial strains and plasmids used in this study are in the list of Table 3.2. The *P. protegens* Pf-5 strain was the biocontrol strain investigated in our study. Pf-5 was routinely cultured in Luria Bertani (LB) medium supplemented with 100µg/ml streptomycin at 27°C and shaking at 200rpm. *E. coli* TOP10 was used for routine cloning. Biparental mating was performed on LB plates supplemented with 1 % (W/V) glycerol, using *E. coli* S17-1 strain as a donor. *E.coli* strains were routinely grown in LB medium at 37°C with a shaking speed of 200 rpm.

Identification of highly-expressed sRNAs in Pf-5 biofilms

To identify highly expressed sRNAs, the transcriptome from Pf-5 biofilm was extracted for RNA sequencing. For RNA extraction, 30 ml of Pf-5 were incubated in 6-well plates for 8 hours. Then the supernatant was removed followed by washing 3 times with phosphate buffer saline (PBS), and the biofilm were harvested by dispersing 150µl Trizol (Invitrogen) per well. The RNAeasy kit (Qiagen) was used to extract the RNA according to the provided protocols. Off-column RNAse-free DNAse (Ambion) treatments of the RNA samples were used to remove any residual DNA contamination. The concentrations of RNA samples were measured by Nanodrop ND1000 spectrophotometer (NanoDrop Technologies). The rRNA was removed from the sample by Ribo-Zero™ rRNA Removal Kits according to manufacturer's manual (Epicentre). Then the rRNA-depleted sample was sent to Ramaciotti Centre in University of New South Wales for RNA sequencing. The whole sequencing on the RNA samples was subject to Illumina's GAII sequencing system. The sequenced transcriptome was aligned by bowtie 2 to Pf-5 genome for quantification of the transcripts (Langmead and Salzberg 2012). The putative sRNAs transcribed at the most abundant level were selected.

Prediction of sRNA candidates in Pf-5 genome

To survey for putative sRNAs in the Pf-5 genome, the Pf-5 genome sequence was screened by RNAspace for prediction of any distributed sRNA candidates (Cros et al. 2011). RNAspace is an online platform integrating programs for homology searches, comparative analysis, and ab initio prediction for the discovery of sRNAs. The sRNA candidates in the range of 50-500 nt located in intergenic regions or antisense to open reading frames (ORF) were selected for further examination. Fragments of rRNA and tRNA were excluded.

Site-directed deletion of the target gene *phrS*

Deletion of the *phrS* gene PFL_6286 was performed via an allelic exchange procedure adapted from Hoang *et al.* (1998). The truncated genes used to generate deletions within the chromosomal genes were constructed using either KOD Hot Start DNA Polymerase (Merck) or GC-RICH PCR System (Roche), according to the splicing overlap extension (SOE) PCR technique with some adaptation from previous protocols (Choi and Schweizer 2005). Primers used for mutagenesis are listed in Table 3.3. Briefly, the regions around the N- and C-termini of the genes of interest were amplified by PCR. Amplified PCR products were spliced together to form the truncated gene construct using the SOE PCR method. The spliced product was propagated in pGEMT-Easy cloning vector where A-tails were added for TA cloning via re-amplification with the GC-RICH PCR System (Roche). Cloning of the truncated gene into the *Hin*dIII and *Bam*HI sites of the suicide vector pEX18Tc was performed through restriction digestion and ligation, thus creating the recombinant vectors with the truncated gene fragments.

Table 3.2. The list of bacterial strains and plasmid

Strains or plasmids	**Genotype or characteristics**	**Sources**
Strains		
P. protegens Pf-5	Wild-type	(Howell and Stipanovic. 1979)
Δ*phrS*	*P. protegens* Pf-5 ΔPFL_6286	This study
E. coli S17-1	*recA thi pro, tra* genes (from plasmid RP4) integrated into chromosome	(Simon et al. 1983)
E. coli TOP10	*F- mcrA (mrr-hsdRMS-mcrBC) 80lacZM15 lacX74 recA1 ara139 (ara-leu)7697 galU galK rpsL (StrR) endA1 nupG*	Invitrogen
Plasmids		
pGEM-T Easy	TA cloning plasmid vector, ApR	Promega
pEX18Tc	Broad-host-range suicide plasmid vector; Tc_R	(Hoang et al. 1998)
pEX18Tc-*phrS*	1.1 kb SOE-PCR fragment containing truncated PFL_6286 gene cloned into *Hin*dIII-*Bam*HI sites of pEX18Tc; used to construct ΔPFL_6286 strain	This study

Table 3.3. The primers used for the site-directed deletion of *phrS* and mutant screening.

*capital letters depict the restriction enzyme cutting site.

Primers	DNA sequence
Upstream Forward	tacggcttatAAGCTTacatgcggtttcctgtgggg (*Hin*dIII cutting)

Upstream Reverse	atgccttgcgtgctcgttggaaaaacatgactgg cctcc
Downstream Forward	Ggccagtcatgtttttccaacgagcacgcaaggcatt
Downstream Reverse	atttgcattcGGATCCgtgaaaaggccgacctgga (*Bam*HI cutting)
Screening Forward	aatgcggtttcctgtgggg
Screening Reverse	ggaaaaggccgacctgga

The recombinant suicide vector pEX18Tc-*phrS* carrying the truncated gene was chemically transformed into *E. coli* S17-1for subsequent conjugation. Biparental mating was performed on S17-1 carrying the recombinant plasmid and wild-type (WT) Pf-5 in order to mobilize the plasmid into Pf-5 through the procedure of conjugation. Briefly, cells were mixed in 1:1 ratio (based on OD_{600}) and plated onto LB agar containing 1 % glycerol (W/V) and incubated without spreading for 6 hours at 37°C. After the incubation, cells were harvested from the surface of the plate and resuspended in 5 mL 0.1 M MgSO4. 100 µl of suspended cells were spread on LB agar medium containing 200 µg/mL tetracycline and 100 µg/mL streptomycin to select for Pf-5 bacteria carrying the recombinant plasmids. Tetracycline was included in the medium in order to select for the presence of pEX18Tc vector in Pf-5 while streptomycin was used to select for *P. protegens* Pf-5 which is inherently streptomycin resistance). The colonies displaying resistance were propagated in 1 mL of non-selective LB media at 25°C for 2-3 hours and plated onto LB agar containing 5 % sucrose. The high concentration of sucrose content selected against bacteria that carry pEX18Tc due to the presence of *sacB* gene in the plasmid whose product is lethal to the cell in the presence of sucrose (Hoang *et al.*, 1998). Colonies that survived the sucrose selection were patched in parallel onto both LB agar containing 5 % sucrose and LB agar containing 200 µg/mL tetracycline, the latter to confirm the absence of the recombinant pEX18Tc plasmid in the bacteria. The colonies were then screened by colony PCR with FastStart Taq DNA polymerase (Roche) and a pair of gene-specific primers (Table 3.3) for mutants that had truncated genes in their genomes.

The determination of the growth curves of Pf-5 wild-type and Pf-5Δ*phrS*

To identify if the mutation on *phrS* has any effects on the fitness and bacterial proliferation of Pf-5, the growth of Pf-5 wild-type and the *phrS* mutant was monitored. The overnight culture of wild type and mutant strains in LB medium grown at 27°C with a shaking speed of 200 rpm was diluted to OD_{600} 0.04 and reinoculated into the the same medium, with identical temperature and shaking speed. The growth was monitored for 24 hours and the culture was sampled each hour to take the OD measurement at 600nm.

Biofilm formation assay

Biofilm formation by Pf-5 was assessed in 96-well microtiter plates as described in crystal violet (CV) assay according to the previous method (O'Toole and Kolter, 2002) with some modifications. Briefly, the overnight culture of Pf-5 wild-type and Pf-5Δ*phrS* was diluted with LB broth into the initial OD absorbance reading of 0.04 at 600 nm. The diluted culture was dispersed into 96-well plates in 100µl aliquots and incubated with no shaking at 27°C for 8, 16 and 24 hours respectively. After the incubation, the spent medium containing unattached cells was removed by pipetting, and the wells were washed with 150µl of phosphate buffered saline (PBS, pH 7.4) 3 times to remove loosely attached cells. One hundred microliter aliquots of a 1% (w/v) aqueous solution of crystal violet (CV) were added to each well and incubated for 20 minutes at room temperature. The dye was carefully removed by washing wells with 150 µl of PBS. One hundred microliter aliquots of 100% ethanol per well were added to the wells of microtiter plate to extract the CV as the plates were incubated at room temperature for 15 mins to allow the extraction. The ethanol extract was then diluted tenfold and the absorption was measured at 600 nm using Multiskan EX plate reader (Thermo Electron Corporation, Waltham, MA). For each experiment, three biological replicates were used respectively. Empty 96-well plates following the same washing procedure as described were used as the blank control. Statistical significance was determined by performing a t-test.

Swimming and swarming motility assay

As the regulatory pathway of *phrS* was reported to be intertwined with the LuxR-I regulatory circuitry controlled QS by stimulating the production of pseudomonas quinolone signal (PQS) in *P. aeruginosa* (Sonnleitner et al. 2011), its impact on the swimming and swarming motility of Pf-5 was investigated according to previous protocols with some modifications (Hassan et al. 2010). Briefly, the overnight growth of culture in LB broth at 27°C under shaking speed of 200 rpm including the Pf-5 wild-type and *phrS* mutant were transferred into 50ml falcon tubes and centrifuged to pellet the cells. The cells were scraped off, washed once with sterile distilled water, and resuspended in sterile water to an OD_{600} of 0.2. Five-microlitre samples were spotted onto the centre of each swimming or swarming plate with 5 plates per biological replicate. The LB 0.3% agar plates were used for the swimming assay while the LB 0.6% agar plates for swarming assays. For the mutant and wild-type, three biological replicates were tested respectively. Plates were incubated for 48 h at room temperature.

Results

Identification of overexpressed sRNAs in Pf-5 biofilms

To identify the highly expressed sRNA candidates in biofilm, we sequenced the rRNA-depleted transcripts harvested from biofilm formed by Pf-5. The most abundant sRNA identified was the 6S/SsrS RNA encoded by PFL_6234. Two other highly expressed transcripts were PFL_6294 SsrA and PFL_6307 RnpB.

The predicted sRNAs in Pf-5 genome

The Pf-5 genome was scanned for any putative sRNAs by RNAspace. All 17 ncRNA (PFL_6234, *rsm*Z PFL_6285, *phrS* PFL_6286, *rgs*A PFL_6287, *prrF2* PFL_6288, *rsm*X PFL_6289, *prrF1* PFL_6290, *rsmY* PFL_6291, *ssrA* PFL_6294, PFL_6296, *srpB* PFL_6297, PFL_6301, PFL_6302, rnpB PFL_6307, PFL_6309, PFL_6310 and PFL_6311) annotated in National Center for Biology Information (NCBI) were included in the result. Additionally, RNAspace identified 134 new putative sRNAs (appendix) which belong to more than 10 known families or undefined ones. The result suggests there may be a relatively large population of sRNAs classified in a variety of families distributed across the Pf-5 genome that play critical regulatory roles.

Mutation of *phrS* in Pf-5

The *phrS* gene PFL_6286 in *P. protegens* Pf-5 is 208 bp in length. To gain insight into the regulatory role of the sRNA PhrS in *P. protegens* Pf-5, a gene-knockout approach was opted. To delete it from the Pf-5 genome, allelic exchange mutagenesis was performed according to previous protocols (Hoang et al. 1998). The construction of mutant Pf-5Δ*PhrS* was verified by PCR screening on colonies (Figure 3.3). The amplified PCR products from the Pf-5 wild-type genomic DNA and the plasmid pEX18Tc-*phrS* were used as the control. The mutant was successfully constructed as the PCR product from its reaction was 1,150 bp, equal in length to that from the plasmid and 150bp shorter than the wild-type, so 150 bp in the target gene of 208 bp was truncated from the wild-type genome.

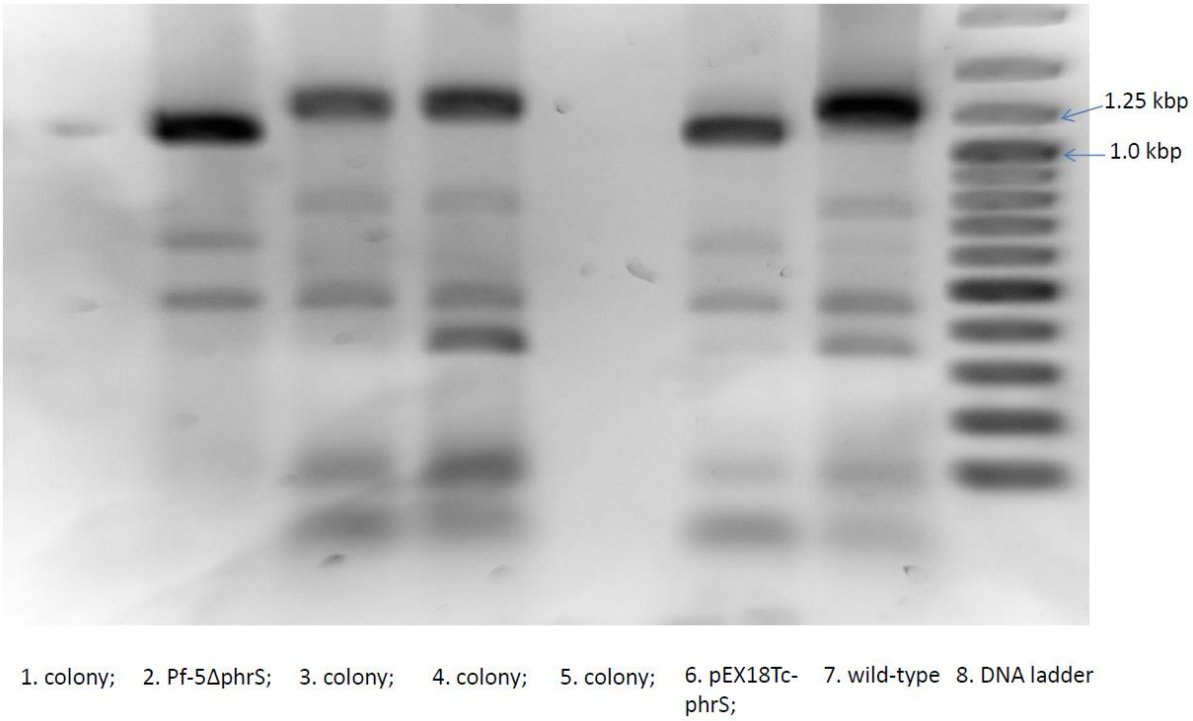

Figure 3.3. The successful truncation of *phrS* gene in Pf-5 genome for construction of Pf-5Δ*phrS*. From left to right: lane 1-5, the PCR product from screened colonies; lane 6, PCR product of pEX18Tc-*phrS* template, control; lane 7, PCR product by template of genomic DNA of Pf-5 wt, control; lane 8, DNA gel ladder. The product from lane 2 was in equal size to pEX18Tc-*PhrS* control, in length of 1,150 bp, while the product from Pf-5 wt 1,300 bp, suggesting the mutant had a truncated piece of 150 bp from the *phrS* gene in Pf-5 genome; lane 1 and 5 had no or tiny amount of PCR product and the two colonies could not be identified as wt or mutant; PCR products of lane 3 and 4 were in same length to that from wt control, suggesting the two colonies remain wt containing intact *phrS*.

The effects of *phrS* deletion on the growth of Pf-5

A recent study demonstrated that the regulatory sRNA, PhrS in *P.aeruginosa*, upregulates PQS synthesis and thereby affects QS (Sonnleitner et al. 2011). Thus, PhrS may modulate the morphological features of the bacterial colony as QS is the physiological process controlling the secretion of excellular matrix including Pel and Psl which are involved in the colony morphology. Hereby we investigated if the mutation on *phrS* in Pf-5 had any impact on the colony morphological features. By streaking on LB agar plate, while the wild-type strain formed round colonies on top of the solid surface, the *phrS* mutant proliferated into rhizoid colonies in largely reduced diameters, and during growth they penetrated into the solid medium to be embedded in the layer rather than expanding on the surface. By counting of cultivation on three independent LB plates, over 95% of the total colonies differentiated into the rhizoid form. The rhizoid morphological feature remained stable and was inherited by offspring colonies when sub-streaked onto new plates.

To detect if the Pf-5Δ*phrS* mutant had any growth defects in comparison to the wild-type, they were monitored for their growth in shaking batch culture over a 24-hour period by OD_{600} measurement. The *phrS* mutant showed essentially identical growth parameters in batch culture compared to the wild-type (Figure 3.4).

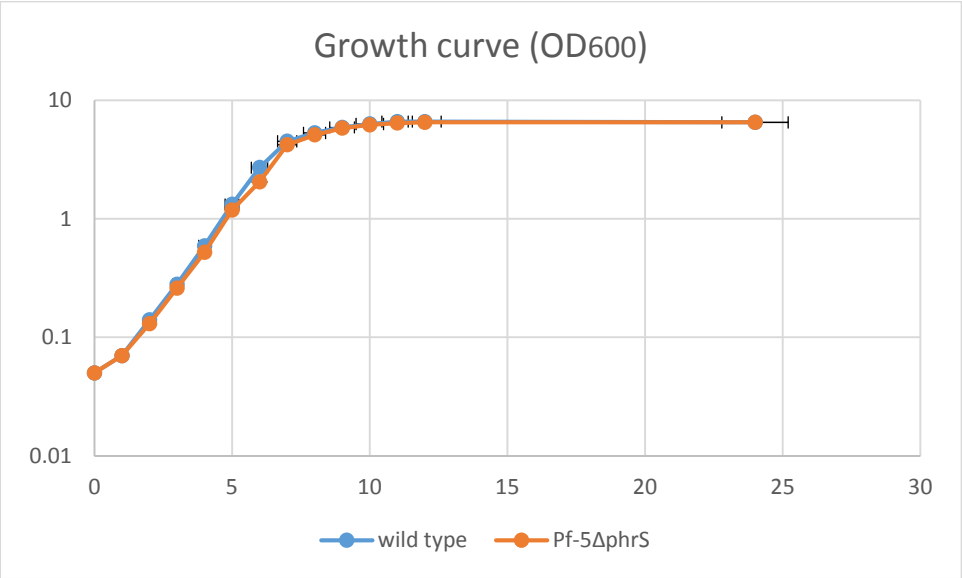

Figure 3.4. Growth of Pf-5 wild-type and *phrS* mutant measured over 24 hours. The culture course was undertaken at 27 °C with shaking speed 200rpm using LB medium.

phrS deletion affects the biofilm development

As the *phrS* mutant differentiates into colonies in a distinguishable morphotype in comparison with wild-type, it was predicted to have distinctive features in biofilm formation. Under this hypothesis, the strains were investigated on their biofilm development following the crystal violet assay protocols (O'Toole et al. 2000). The overnight culture of wild-type and mutant were diluted in LB medium to OD_{600} 0.04 and dispensed into the 96-well plates. Biofilm formation was assessed after 8, 16, and 24 h incubation. Consistant with our expectation, biofilm development in the *phrS* mutant was different from wild-type Pf-5 (Figure 3.5). The biofilm sampled at 8 h of the two strains was not statistically different. However, at the next two stages, the biofilm formed by wild-type was significant promoted in comparison to that by Pf-5Δ*PhrS* (Figure 3.5). The biofilm formed by wild-type peaked at the 16 h time point, demonstrating its maturation around this stage. The *phrS* mutant biofilm was most substantial at 8 h and dispersed across the 16 and 24 h development. At the 24 h time point, the biofilm levels were very low. This implies the biofilm formed by *phrS* mutant matured at an earlier stage than the wild-type and was almost entirely dispersed at the end of

24 h period. By comparing these results, it suggests that the small regulatory RNA PhrS affects the biofilm development and dispersal in Pf-5.

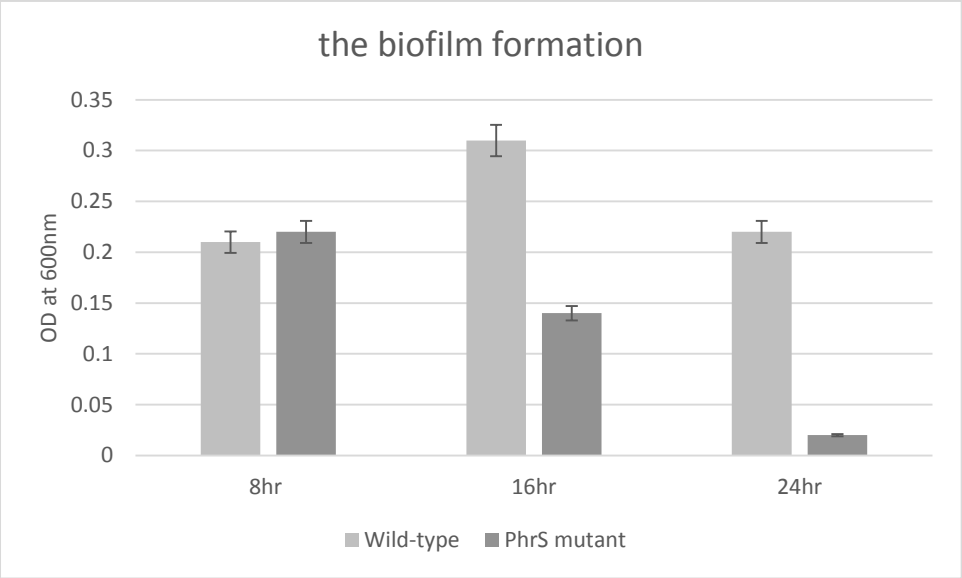

Figure 3.5. Biofilm formation of Pf-5 and Pf-5Δ*phrS* sampled after 8, 16 and 24 h incubation. The experiment used LB broth for biofilm culture in 96-well microtitre plates across the whole course and crystal violet assay for final quantification of bacterial cells attached to plates. The cultivation was maintained at temperature of 27°C for 8, 16 and 24 hours respectively without shaking.

The *phrS* deletion in Pf-5 genome abolishes swimming and swarming motility

As biofilm formation and flagellar motility are two phenotypes inherently correlated to each other, following the biofilm assay, the swarming and swimming motility of Pf-5 wild-type and *phrS* mutant were investigated. On the LB 0.6% agar plates, the *phrS* mutant was deficient in swarming motility (Figure 3.6). In the swimming assay, the Pf-5Δ*phrS* had a largely impaired swimming capability compared to the wild-type (Figure 3.6). These results suggest the small regulatory RNA PhrS is important for both swarming and swimming motility, and the deletion of *phrS* greatly diminished bacterial translocation in Pf-5.

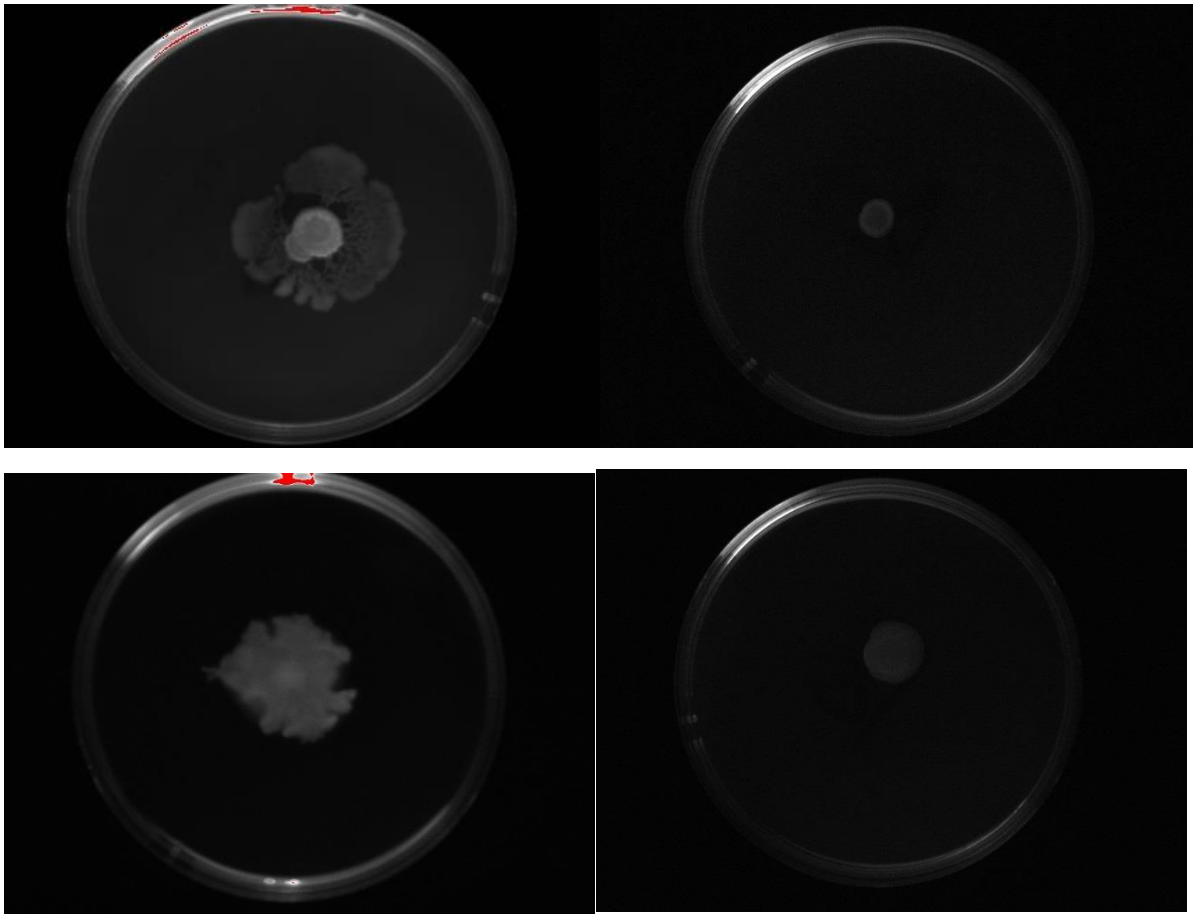

Figure 3.6. Swarming (0.6% agar in LB) and swimming (0.3% agar in LB) motility assay of Pf-5 wild-type and Pf-5Δ*phrS.* The assays continued for 48 hours at room temperature. Upper left: swarming motility of PF-5 wild-type. Upper right: swarming motility of Pf-5Δ*phrS.* Down left: swimming motility of Pf-5 wild-type. Down right: swimming motility of Pf-5Δ*phrS.*

Discussion

Recent years have seen an explosion of research on sRNA regulation. However, while more than 100 sRNA were investigated in *E.coli,* a limited number have been characterized in pseudomonads. Some sRNAs discovered in pseudomonads were extrapolated for their functions by sequence homology from *E.coli* rather than experimental evidence (Vogel et al. 1987, Toschka et al. 1989). As the majority of *Pseudomonas* strains have a significantly larger genome size than *E.coli,* it is predicted there will be more diverse sRNAs discovered from pseudomonads. Hereby, in our biological control model strain *P. protegens* Pf-5, we performed bioinformatic and laboratory work on it including sequencing the transcriptome from Pf-5 biofilm for overexpressed sRNAs, searching across the Pf-5 genome by the web-based platform RNAspace for any predicted sRNAs, and investigating the sRNA PhrS primarily on its regulatory effects on the bacterial traits correlated to rhizosphere colonisation and biocontrol effectiveness.

Recently the RNAseq has been a widely-used approach to discover sRNAs in pseudomonads including *P. aeruginosa* PAO1 and PA14 (Ferrara et al. 2012, Wurtzel et al. 2012), *P. putida* KT2440 (Frank et al. 2011) and *P. syringae* DC3000 (Filiatrault et al. 2010), although its detection may be restricted by bacterial culture condition as some sRNAs are inhibited on their transcription under specific growth conditions and may be skipped by RNAseq. Based on the transcriptomic profile of Pf-5 biofilm released by RNAseq, the 6S/SsrS RNA PFL_6234 and SsrA transfer-messenger RNA (tmRNA) PFL_6294 are the two most abundantly expressed sRNAs in early-phase biofilm (8hr). 6S RNA is a sRNA conserved across the majority of bacterial strains and investigated in details in *E. coli* and *Bacillus* (Wassarman 2007, Cavanagh and Wassarman 2014). It interacts with the RNA polymerase holoenzyme and therefore blocks the transcription of a range of genes during stationary phase in response to nutrient scarcity. Similar to its high level of expression in Pf-5 biofilms, it was originally identified by RNA fingerprinting in planktonic *E. coli* because of its high abundance (Hindley 1967). Therefore apparently it might be highly expressed in both planktonic and biofilm cultures of Pf-5. The *ssrS* gene in *E. coli* is in an operon with *ygfA* which is upregulated in biofilm development for persister cell formation (Ren et al. 2004), therefore its expression in Pf-5 biofilms might be higher than in planktonic cultures. In a recent study on *P. aeruginosa*, it was identified in higher abundance in 24 h and 48 h biofilms compared with the late exponential and stationary phases of planktonic culture (Dotsch et al. 2012). Transcriptional quantification such as qRT-PCR could be exploited to compare expression levels of the 6S RNA in biofilm vs. planktonic culture.

The RNAspace analysis identified a set of 134 putative sRNAs (Appendix) in addition to the 17 previously annotated ones from Pf-5 genome database in NCBI. The total number of 151 predicted sRNAs in Pf-5 is close to that of 150 identified in *Pseudomonas putida* DOT-T1E and *Pseudomonas extremaustralis* 14-3b each by RNAseq (Gomez-Lozano et al. 2014). Strikingly, the first genome-wide search of sRNAs using RNA-seq in *P. aeruginosa* PAO1 detected a large collection of 573 sRNAs from three different sequencing libraries (Gomez-Lozano et al. 2012). In PA14 RNAseq analysis detected 200 sRNAs (Wurtzel et al. 2012). In contrast, in the phyto-pathogenic strain *P. syringae* DC3000 only a trace number of 25 sRNAs was detected (Filiatrault et al. 2010). Seemingly the number of sRNAs may vary in different pseudomonads and Pf-5 may hold a much smaller number than pathogenic *P. aeruginosa* strains although the size of its genome is bigger. Overall the result suggests there are potentially a considerable amount of small regulatory RNAs in Pf-5 which can be

classified into various categories, and provides a spectrum of targets to examine in subsequent studies.

A number of bacterial sRNAs achieve their riboregulation by interacting with the effector protein Hfq (Zhang et al. 2003). As a member of the Phr (*Pseudomonas* Hfq-binding RNA) family of regulatory RNAs, PhrS in Pf-5 is predicted to bind the global regulator Hfq and accomplish its riboregulatory function. PhrS in *P. aeruginosa* has been elucidated for Hfq binding (Sonnleitner et al. 2008), although its binding domain remains to be determined. There are three distinct RNA-binding surfaces located on the Hfq hexamer. sRNAs are generally suggested to bind the proximal site or lateral site (Sauer et al. 2012, Murina et al. 2013). In Pf-5 genome, the PFL_0566 has been annotated as the RNA chaperone Hfq. A future wet-lab study on the interaction of its protein product with PhrS by co-immunoprecipitation is a possible chance to start mapping the molecular scheme of PhrS regulation. A transcriptomic study examining a PhrS-deletion strain could be exploited to define its downstream gene targets and thus to predict the phenotypes it is likely to control.

To date, only a tiny number of sRNAs have been shown to also encode peptides. PhrS in *P. aeruginosa* has been shown to encode a 37 aa peptide product although its function is to be determined (Sonnleitner et al. 2008). Inspection of the *phrS* PFL_6286 sequence in Pf-5 suggests it has a start codon and might contain an internal ORF of 38 amino acids. For solid evidence, further work such as the construction and analysis of translational fusion reporters could be conducted to support the translation and the dual role of PhrS as a riboregulator and sRNA.

In *P. aeruginosa*, the transcription of the regulatory RNA PhrS is under the control by the oxygen-sensing transcription factor ANR and stimulates the production of PQS by inducing the expression of the quorum sensing regulator *pqsR* as it assists the translation of upstream open reading frame (uof) (Figure 3.1) (Sonnleitner, Gonzalez et al. 2011). The positive effect on uof translation attributes to its highly conserved region of 12 nucleotides. The BLAST search suggests Pf-5 does not carry *pqsR* and its *phrS* does not contain the conserved functional region. Therefore, it seems that the posttranscriptional regulation by PhrS on *pqsR* in PAO1 does not apply to Pf-5. However, Pf-5 does carry *trpG* PFL_5623 and *trpE* PFL_5629 which direct the biosynthesis of anthranilate, the precursor molecule for PQS biosynthesis, from chorismate (Farrow and Pesci 2007). To reveal the complete picture of the regulatory pathway in Pf-5, further progress could start from identifying whether Pf-5

produces PQS or a related molecule and whether there is an interconnection with the PhrS regulatory cascade.

In order to investigate the effects of PhrS regulation on the rhizosphere colonisation capability of Pf-5, the wild-type strain and *phrS* mutant were tested for their biofilm formation and motility. The biofilm formed by Pf-*5*Δ*phrS* dispersed across the period during 8 to 24 hour time point while that formed by wild-type developed into peak value after 16 hour culture. The crystal violet assay results at time points of 16 and 24 hour indicated a significant reduction in biofilm after the inactivation of *phrS* from Pf-5. Similar to the biofilm assay result of wild-type vs. *phrS* mutant in Pf-5, in the *P. aeruginosa* mutant of ANR which has down-regulated expression of PhrS, the biofilm formed was largely impaired possibly due to the repressed activity of PhrS (Jackson et al. 2013). In the *phrS* mutant of Pf-5, the swimming and swarming motility was completely inhibited. One possible reason could be the repressed biosynthesis of flagellum or abolished production of surfactant cyclic lipopeptide orfamides as they are the two of the most prominent parameters for active flagellum-mediated motility. In our study, the effects of inactivation of PhrS on biofilm formation and motility in Pf-5 hereby suggest this sRNA is potentially a positive regulator for rhizosphere colonisation. To confirm the role of PhrS, an important next step would be cloning the *phrS* sRNA into an expression vector, and using that construct to complement the knockout mutant strain to confirm that phrS is responsible for the observed phenotypes.

In *P. aeruginosa*, a transcriptomic microarray analysis revealed that PhrS has a positive regulatory effect on a range of genes (*phzB-G, phzS*) for phenazine biosynthesis and hence modulates the production of the phenazine compound pyocyanin (Sonnleitner et al. 2008). Phenazines are represented by over fifty biologically active compounds with broad band antibiotic activity against soilborne bacterial and fungal pathogens and hence are involved in the biocontrol capability of pseudomonads (Timms-Wilson et al. 2000). In *P.aeruginosa* and *P. fluorescencens*, there are seven genes *phzA-G* involved in the biogenesis of phenazine (Mavrodi et al. 1998, Mavrodi et al. 2001). In Pf-5, in its genome there are three genes (PFL_4865, PFL_2667 and PFL_2288) encoding the PhzF family phenazine biosynthesis protein, however there is no complete phz gene cluter and its production of phenazine has not been evidenced. To further characterize the biocontrol mechanism in Pf-5, its production of phenazine and the regulatory effects of the sRNA PhrS can be investigated.

Conclusions

The bacterial sRNAs are regulatory molecules acting by riboregulation or RNA basepairing on the target molecule and regulating physiological processes. In recent years several sRNAs have been functionally described, only a small number are characterised in pseudomonads. Hereby, the sRNA PhrS in *P. protegens* Pf-5 was studied for its effects on biofilm formation and motility by a targeted deletion mutagenesis approach. Our results show that PhrS in *P. protegens* Pf-5 putatively regulates respiration, colony morphological features, and is important for various phenotypic features associated with rhizosphere colonisation including biofilm formation, swimming and swarming motility.

Knowledge of the sRNA PhrS in Pf-5 is scarce. Work is needed to reveal the full PhrS regulatory pathway in Pf-5, e.g., its correlation with ANR and the impact of oxygen on its expression, the spectrum of regulated genes downstream of PhrS in the regulatory cascade, the interacting domain of PhrS with the Hfq protein, and the function of its translated peptide product. The in-depth characterisation of PhrS on its interference with cellular physiology and biocontrol property in *P. protegens* Pf-5 may suggest potential strategies for development of biological control effectiveness in Pf-5 and propel the investigation on its homologues in other bacteria.

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Chapter 4. Discussion & Conclusion

As the demand for food to satisfy the exploding population in the world is continuously growing, improvements in agriculture productivity to secure the global food supply are critical. However, the current usage of pesticide to manage plant pathogens and prevent crop loss in agriculture causes disastrous side effects for human health and the environment. To minimize these deleterious side effects, an alternative strategy of utilizing biological control organisms to manipulate plant pathogens is an attractive option. Some of the biocontrol bacteria are categorized in the *Pseudomonas* genus, the gram-negative rod-shaped gamma-proteobacteria ubiquitously spread in the environment. The rhizosphere inhabitant fluorescent pseudomonads include a broad range of strains having effective biocontrol properties to protect plants against harm caused by bacteria, fungi and nematodes. The biocontrol activities mediated by these bacterial strains include inducing the host's inherent systemic resistance against pathogens, producing anti-pathogen compounds that are antagonistic to pathogens, and resource exclusion through competition. The model biocontrol strain investigated here, *Pseudomonas protegens* Pf-5, evokes its suppression of the plant pathogens by secreting a range of anti-pathogen compounds. These compounds include antibiotics which inhibit the growth of pathogens, and siderophores which chelate the essential nutrient iron and hence suppress pathogen growth.

However, the capacity of biocontrol bacterial to mediate pathogen suppression in the environment is not always consistent with their capability to produce anti-pathogen agents. One reason is that the strains must also be successful in rhizosphere colonisation to achieve the effective protection on plants (Weller 1988). Previous studies on Pf-5 have described its production of anti-pathogen compounds and their encoding gene clusters in detail (Paulsen et al. 2005, Loper et al. 2007, Loper et al. 2008). In contrast, fewer studies have focused on the two key traits relevant to its rhizosphere colonisation: biofilm formation and flagellar motility. In the current study, the regulatory effects of a LuxR family regulator, PFL_3627, and a small RNA, PhrS, on biofilm development and flagellum-mediated motility of Pf-5 were investigated by gene knockouts. The whole genome microarray data identified the regulon of PFL_3627. The project aims to identify the regulatory effects of selected regulatory protein PFL_3627 and small RNA PhrS on biofilm formation and flagellum-mediated motility in Pf-5.

The production of AHL in Pf-5

The interaction of bacteria with their surroundings, including the perception of the neighbouring bacterial community and their eukaryote hosts, is of crucial importance to activate the cooperative production of public goods. The emergence of the concept sociomicrobiology guided microbiological research investigating the cooperative bacterial behaviour in groups resulting from communication within bacterial communities rather than actions of individual cells. Apart from direct cell-to-cell contact, the synthesis and exploitation of small diffusible signalling molecules has been identified as a widely used strategy in a number of bacterial genera for intercellular communication and information exchange. Quorum sensing (QS) is a well-studied example of bacterial communication and social interaction which occurs when communities reach the threshold of cell density that enables them to coordinate gene expression. In bacterial QS, the system of LuxI-R circuit utilizing the signalling molecule acyl-homoserine lactone (AHL) is a well studied paradigm. The LuxR-I system typically consists of a LuxI homolog to encode the AHL synthase for the signalling molecule production, and a LuxR homolog which is translated into the regulatory protein sensing AHL and controlling the expression of target genes (Ng and Bassler 2009, Miyashiro and Ruby 2012). In the Pf-5 genome, there have been several "LuxR solos" identified without the canonically paired LuxI homolog. The definitive conclusions about its production and utilisation of AHLs for signalling in QS cannot yet be drawn. To date, the biosensor strains in our study detected no AHL synthesis from Pf-5. It is consistent with a previous study using AHL biosensors screening on Pf-5 (Subramoni et al. 2011). In *P. fluorescens* strain F113 certain AHLs can be synthesized at a low level as by-products resulting from the activity of HdtS, an acyltransferase involved in phosphatidic acid biosynthesis (Laue et al. 2000). The sensitivity of biosensor strains were non-permissive for detection of AHL released from HdtS homolog by streaking (Laue et al. 2000). . In Pf-5 genome, PFL_0011 encodes an HdtS homolog and may potentially be able to synthesize AHLs. For conclusive identification of AHL synthesis in Pf-5, it would be worth conducting ethyl acetate extraction method on cell cultures (Makemson et al. 2006) followed by chromatographic analysis.

The LuxR solo PFL_3627 in Pf-5

The genome of the plant-associated bacteria *P. protegens* Pf-5 encodes an exceptional abundance of 11 hypothesized LuxR solo type proteins identified (Discussion in Chapter 2). In pseudomonads, the striking number of LuxR solos may include those that respond to plant

exudate compounds for plant associated bacteria (PAB) or canonical ones sensing endogenous or exogenously produced AHLs (Patel et al. 2014). More than one LuxR solo existing in the same bacterial strain with overlapping functions may have additive regulatory effects (Patel et al. 2014).

The biological significance of LuxR solos in prokaryotes is poorly understood at present, as only a small fraction of them have been investigated. The regulatory effects of LuxR solos of both canonical AHL signal recipients and plant compound recipients are diverse. Evidently modulation of the regulatory LuxR solos may result in impaired bacterial fitness, reduced biocontrol capability, modulated virulence activation, and changed motility (Gonzalez et al. 2013, Habyarimana et al. 2014, Patel et al. 2014, Qian et al. 2014).

The LuxR solo mediated cell signalling plays critical role in bacterial survival. The deletion of the LuxR solo gene PFL_3627 from Pf-5 resulted in defective bacterial growth (Figure 2.2 in Chapter 2), implicating a loss of competitive fitness. Similarly, in *P. putida* KT2440 inactivation of the LuxR solo PpoR impairs bacterial growth (Fernandez-Pinar et al. 2011). In the emerging phytopathogenic strain *Pseudomonas syringae* pv. actinidiae, the deletion of its LuxR solos severely impacted the bacterial fitness (Patel et al. 2014).

The regulatory effects imposed by LuxR solos encompassing various bacterial genera are often involved with bacterial motility and biofilm formation, putatively by controlling flagellum biosynthesis and extracellular matrix production. In the current study, the LuxR solo PFL_3627 from Pf-5 genome drastically stimulated bacterial swarming and modified the swimming motility *in vivo* (Figure 2.7 & 2.8 in Chapter 2), while the biofilm formation initiation at early stages were also fortified (Figure 2.4 in Chapter 2). The genome-wide microarray analysis comparing the mutant and wild-type strains demonstrated at molecular level that PFL_3627 influences the expression level of flagellar biosynthesis genes consistent with the more active motility and possibly advantageous initial surface attachment. The reciprocal regulatory effect on motility has been observedwith more than one LuxR family regulator. The LuxR solo SdiA in *E. coli* suppressed bacterial motility by down-regulating genes for flagellum assembly (Sharma et al. 2010). In contrast, the LuxR solo homologue PpoR in *P. putida* had a positive regulatory effect on its swarming motility (Fernandez-Pinar et al. 2011). Induction of swarming and swimming motility was also observed from the LuxR solo homologue OryR in rice pathogen *Xanthomonas oryzae* pv. oryzae (Gonzalez et al. 2013). In several cases, the flagellum and motility are recognized for their essential roles

during early stages of biofilm development (Pratt and Kolter 1998, Watnick and Kolter 1999, Wood et al. 2006). In addition to its effects on motility in the current study, the mutant on PFL_3627 showed increased biofilm formation at an early time point. It is noteworthy that mutation of other LuxR regulators has stimulative effects on biofilm formation, but the mechanism can differ. For instance, in *Vibrio cholerae* the LuxR type regulator VpsT mutation released the stress response regulator RpoS and promoted biofilm formation (Wang et al. 2014). Overall, our current work on Pf-5 provides clues of potential interplay between motility and adherence, evidenced by the fact that deletion of PFL_3627 facilitates initial surface attachment and early phase biofilm formation, possibly by modulating flagellum-mediated motility.

The array of signalling molecules recognised by PFL_3627 have not been yet determined. The first identified LuxR solo, PsoR in Pf-5, was suggested to bind plant-secreted compounds (Subramoni et al. 2011). Somewhat different from the consensus sequence of AHL-sensing LuxR proteins, this newly emerged subclass of LuxR regulators usually have substitutions in one or two of the six highly conserved amino acid residues in autoinducer binding domain, implicating structurally different ligand-binding than sensing AHLs (Gonzalez and Venturi 2013). In addition to that, plant compound binding LuxR solo genes generally have one or two *pip* genes in flanking regions (Gonzalez and Venturi 2013). However, as described in result from chapter 2, in contrast to the plant compound binding family, all six residues identical to AHL-binding LuxR regulatory proteins are conserved in the PFL_3627 protein investigated in our study, and PFL_3627 gene has no *pip* gene in adjacent region. In our experiments, nevertheless, the PFL_3627 protein did not respond to the four AHLs added here at biological effective concentration (Figure 2.11 to 2.13 in Chapter 2). A recent study conducted on the insect pathogen *Photorhabdus luminescens* revealed pyrones as a new class of molecular signal received by LuxR solos (Brachmann et al. 2013).

The small regulatory RNA PhrS

Unlike protein regulators, the small RNA molecules have emerged lately as regulatory molecules in prokaryotes. While the majority of sRNAs have been investigated in *E. coli*, their functional details in pseudomonads remain scarce. In our study, the small RNA PhrS was potentially linkedto oxygen utilisation and colony morphology; its deletion impaired biofilm formation and inactivated swimming and swarming motilities. Nevertheless, its regulatory pathway at a molecular level remains to be addressed.

The sRNA PhrS in *P. aeruginosa* has been previously demonstrated to positively regulate the production of *Pseudomonas* quinolone signal (PQS) at translational level and subsequently stimulate the biosynthesis of the virulence factor pyocyanin (PYO), which is a redox-active compound (Sonnleitner et al. 2011). It appears that in *P. aeruginosa* the redox-active compounds including PYO activate the transcription factor SoxR (Dietrich et al. 2008) and putatively affect the colony size and structure. Similarly *phrS* inactivation in Pf-5 resulted in the modulation of colony morphotype and reduction of colony size. PQS and PYO have not been identified in Pf-5, however it is not impossible for PhrS to regulate some unidentified redox-active compound to control the expression of SoxR and subsequently microcolony formation. Based on limited amount of available data on PhrS in Pf-5, in reference to its homolog in *P. aeruginosa*, the expression of *phrS* would be particularly favoured under low oxygen concentration condition as it may activate alternative electron acceptor other than oxygen. The biofilm formation by Pf-5 was also impaired upon *phrS* deletion. PhrS from *P. aeruginosa* is controlled by the Hfq regulatory protein and oxygen-responsive regulator ANR (Sonnleitner et al. 2011). The regulation of PhrS by Hfq and ANR in Pf-5 remains to be studied. However, as biofilm formation by bacteria is a cooperative behaviour for environmental adaption and resistance to cytotoxicity, PhrS in Pf-5 may be an indispensable molecular player for health, sustainability and survival of bacterial population under various conditions.

The mutation of *phrS* (PFL_6286) in Pf-5 generated a significant defect in its biofilm formation over the 24 hr period (Figure 3.4 in Chapter 3) and inactivated its swimming and swarming motility (Figure 3.5 in Chapter 3). The impairment on biofilm by regulatory sRNA was reported previously on the RyhB mutant in *E. coli* (Mey et al. 2005). Apart from controlling biofilm formation, sRNAs extend their regulatory roles to bacterial motility. Similar to the essential role of PhrS in motility of Pf-5 according to current study, the sRNAs MicA and McaS are two positive regulators identified for motility in *E. coli* K12 strain MG1655 (De Lay and Gottesman 2012).

Concluding remarks

The study conducted here investigated the rhizosphere colonisation correlated traits: biofilm formation and motility regulated by the LuxR type regulator PFL_3627 and small RNA PhrS in the biocontrol strain *P. protegens* Pf-5. These fine-tuned regulatory pathways in response to fluctuating environmental conditions control the two phenotypes in Pf-5 to achieve

efficient colonisation and ecological adaptation. Successful root colonisation by Pf-5 contributes significantly to its biocontrol capabilities and is essential for its suppressive activities on plant disease *in situ*. Despite these, limited progress has been made to elucidate its genetic interplay regulating these phenotypes. In our study, the investigation of the regulatory effects by LuxR family regulator PFL_3627 and sRNA PhrS in Pf-5 on biofilm formation and motility implicates potential influence of these regulatory systems on rhizosphere colonisation and efficiency of biological control. The removal of PFL_3627 from Pf-5 generated a mutant which speculatively is strengthened in its ability to colonise rhizosphere surface. Although its transcriptomic analysis did not conclusively suggest whether it produced different amounts of the majority of secondary metabolites which antagonize phytopathogens, the available results that it may move more actively and adhere to various surfaces and niches more stably show the possibility for the mutant to provide increased *in situ* protection on plants. Meanwhile, the present data indicated the sRNA PhrS has an upregulating effect on both motility and biofilm formation. Based on that the deletion of *phrS* inactivated motility and impaired substantially the biofilm stabilized on surface, it is predicted that the mutant would lose partly, if not severely, its ability of rhizosphere colonization in the field.

The LuxR family regulator PFL_3627 acts as a suppressor for motility and early phase biofilm formation. As the LuxR solo, it putatively receives AHL or some unidentified signalling molecule involved in QS. The disclosure of its mediated QS cascade and regulated transcriptome would imply beneficial strategy to target its QS mechanism, modulate its root colonisation capability and thereby improve its biocontrol efficiency. Nevertheless, the information source of LuxR solos in plant-associated bacteria is in scarcity. The regulatory functions of LuxR solos are diverse encompassing virulence factor expression, antibiotic biosynthesis, motility, bioluminescence, bacterial proliferation and biofilm formation, and their biological significance includes amplification of regulon by paired LuxR or response to exogenous stimuli. The wide spread soil pseudomonads are good candidates to gain insight into the LuxR solos of diverse functions and mechanisms.

Apart from regulation imposed by LuxR family proteins, the sRNA has been a new emerging class of parameter controlling the genetic interplay and physiology in bacteria. As the majority of sRNAs has been investigated in *E. coli*, the larger genomes of pseudomonads are predicted to contain more abundant sRNAs, however few of them have been characterised.

Our investigation reporting the negative effects of PhrS on biofilm formation and flagellum-mediated motility in Pf-5 will add to the unravelling of the roles of sRNAs in pseudomonads.

Overall, this thesis launched the investigation on a selected LuxR family protein and a sRNA, two classes of regulators in plant-associated pseudomonad strain Pf-5 that may have effect on its biocontrol properties. However, a crucial next step would be to directly assay the pathogen suppressive effects of these knockout mutants on plants in a greenhouse setting and in test plots in the environment. Previous study indicates that in *P. fluorescens* strain F113 the mutation on regulatory genes impaired motility and biofilm formation on abiotic surface but did not affect rhizosphere colonisation (Barahona et al. 2010). In addition, to fully address the regulatory pathway at molecular level, future work is expected to continue with the discovery of the messenger molecule received by PFL_3627, bifunctional role of PhrS and its regulon. Overall, it is conceivable that the disclosure of these information will provide clues of improving biocontrol efficiency in utilizing the organism Pf-5 and shed light on the complicated network of molecular regulation in bacteria.

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Appendix: putative sRNAs predicted in PF-5

*The gene labels after each sequences indicates their intergenic locations or antisense to open reading frames; the names after gene labels indicate their sRNA families and unannotated ones belong to unknown families.

TAGCCATATTCAGCTCCTGTTTAGCTGCTTGTGGTTAGCGGGTCTTGGGTGTTAGA
GCACTCAAGGCCCGCGTCTTT

PFL_0166-67 FinP

ATAAAACAGCCCCCGGCAGAGCGATCTGCCGGGGGCTGTTGGCTTTGCCGCCTG
GAGCGGGGCGGCAGGCG

PFL_0257-58 6C

GTCGCTGCCAAATCAGCGGCCGGGTGTAGGAACCTGATCATAAGAAGCACCCAG
CGCTTTGGGTGTTGTTTGC

PFL_0354-55 isrK-Hfq-binding

GCGCCTCCCTAAAAAACGCCCGGCACCTGCCGGGCGTTTTCATTGGCGGACACTT
TTTGCAGGAGCCAGCTTGCTGGCGAAAGGGCCCTCAAGACCTGCGCCTGCCTGG
CGGACCATTTCGCCGGCAAGCCTGTTCCTACAAAGGGGGGAGTTTGGCGAGTTTC

PFL_0455-56

ATCAGCAGGTCATGCTGGCTACTGCCCAGATCCTGCAATTGGCTGTT

PFL_0487 snoZ256

GGCATCAGCTGGCGGCAAGATTATCCGCGCACTGCAATAGGTGCAACCGGGTGC
AACCCATTTTTAGCTGGAAAAAGAAGCAGCTTGTCAGGAATAAAATTCCTGCAT
GGAGTGGCTTGCCTCCTACTGGTGCCTTT

PFL_0495-96 Deinococcus_Y_RNA

AATCCTGCCCTGATTCCCCGGGGCGCTGAAGAACGGCCCGCTGCGTCACCGCAG
CGGGCCGTTTTTTTTGCGT

PFL_0496-97 ctRNA_pGA1

CGGTTCTTGTCGGGGTGCCTTGCTATGAGGCTGAGATCGGATAATCCGGATCCCG
TTGAACCTGATCAGGTTAGCGCCTGCGTAGGGAACAAGATTTCTCGTCAC

PFL_0537-38 TPP

CGGGACGGTGAATCACTTCTTCCAGAGGGTTGCCCGCAGCGGATGAGGCTGCGG
GCGGGGATCTTACACCAAGCGTTATAAAGTAACATTATAACTGTGCGCGACTGA
AGCGCGCGGAGTGTACAGAGCCCTCTGGAACGCGCATCTCGGCTGAT

PFL_0646-47

GGCGAAATCCCTTCGTTGGTTGATGCCTGATTATAACTATGCGCGGCGTAATGAC
CGCACGGCAAGGGCGTGTGCCCTTGCCGGGGTCAACGACAGCC
PFL_0664-65

AGGACTTCCCTGTTGTGGGGCCGGATCATGAGAGTTCGAAAAAGGGTTGGCATG
ATACCCGCTCTCGATGACCGGATAAAGGCTGAATTGGATAAAATCTGAACAGTT
CTTCTGTAACGTCCTGGGAGCAATCCAGGCGAAACAGTCATAAAACTGTCGTTAA
TCCAGAGTCGTCCGTGAACTTGTGCAGCCGAT

PFL_0668-69

AGAAGCTGCCGGGGGTTGCGCATTCTGGAGGAGCCGGCTTGCCGGCGAAAGCGC
TCTTGCGTCCCACGCCCATCCCCCCGGAAGCTATACGCCTTCAGGAGACCCCG

PFL_0681-82

AGCCGTTCGCCGGCCAGCCGGCTCCTACAGGTTCGTGTAGGAGCCGGCTGGCCG
GCGAAGGCCG

PFL_0718-19

GGTTGCCGGCTCCTCTGAAAGTGGGTTCAGGTTAGCCAGCTCAGTGATGCCCTGT
CTGCTGATCCAGGGTCGCCGCAAGGGGCGAGGTGCGCAGTCTAGCGAAAGATTT
GCCGGGGGACAAAACTTGCATTTTGCCCCCCGTTCCAGAGCCTTCGCTGGCCTGT
GCAAGGGCTGCCCGGGGCAGCCGCGGCC

PFL_0724-25

GAGTAGGTCATCGTCAAGATTAAATTCCGAAACCCCAACTGCGCATGCAGTTGG
GGTTTTGTTTTTGCGC

PFL_0739-40 ctRNA_pGA1

GCTCCCCCATTCCCCGCCTATCCCCCGTAGGAGCCGGCTTGCCGGCGAAGGGGCC
CCCGGGTTTTGCGCAACTCTTGAAGTTGCATTCGCTGGCAAGCCAGCTTCTACGG
TTATCC

PFL_0770-71

TTATCGTCGTGAAACGATCGAAGGCGACCCTAGGGTCGCCTTCGTGTTTTGTGCC
TATATAAAAGAAGCGC
PFL_0823-24 ROSE

TAAGGCCGCTCCCAAACTAGATGTCTGGTTATTTATACAGTGTTTTGTTTCGAGCT
GACAATGCTTTCCCTTAGCTCGATTTGCTTGGTCGTTCTGGCCTTTTAGTGCGCTC
GGTAATGTCCACATACAGGATTTATCGGGTCGGAACGACTTTTGCTGTGGCTGCG
GACTTGAACCTTGAAAATGCGACCCCATATAGCTGGGCAGAAGAGTGAGCCCGG
CTCACAGACGATATTGAAAGGAGAATTTC
PFL_0825-26

GATGCATCTGATGCAGGGTTGAAGCGCAATGCTTGGACCCTGCTTCAAACCGCCA
AGCGATGACCTGTCGCATTCCTCTGCCTTTAGGGCTCATTAGCGGTGGACCGAAA

AAGCCTTTTTCTGTAAGCTACAGCTTTAGTGTGTCCACTAAAAGCGCGCAGATAA
TTCGACGAAGAAGCGGGGTGACGTGTCTATACGTCATTCCGCTTTTTTGCAACCT
GCGATCGCCCTTTCAGGCTCTATTTACGGGAGGTCTTC
PFL_0829-30

TATAGATACAGGGATTTGTCCCTGGCCTGTTGAAACTGTTCTTGAACAGTTTCCTG
ATATAAGGATTGCCCACATGGCACTCAGCGTTGAAGAAAAAGCTCAGATCGTAA
CCGACTACCAGCAAGCTGTT
PFL_0846-47

TTATCGTCGTGAAACGATCGAAGGCGACCCTAGGGTCGCCTTCGTGTTTTGTGCC
TATATAAAAGAAGCGC

PFL_0859-60 ROSE

GTACCTCAATCAGCCGGTTAGGCGTTCCCGCCATCCTTGGGGTCAAGGACGGCGG
GCGTTTCTTAT
PFL_0877-78

CCCACATCTCTATATAGAAGCCCCCCGTCAGAGTCCTGCTATGCGGGGTTCGCAG
GCGCGGGGCTTTTTTGTATGATGTCCGACCCGCGCGCACAGGGCGCCGATTGAGG
TTGAGC
PFL_0925-26

TTCACATGCGTGGCCTTGAGCCGCGCCCATCTATCCGCGTCATAGCCAGAATGCT
CAGGTGCCCACGCGGATGGCTTGGTAGTCTTGCGGCGCCTGAGCT
PFL_0928-29

GAAGGGCTCGCGTCGCGGGTAGTGGGAAAACTATACCAGCGAGGGAAGGCCCTG
TGCCTGCAGGCAACGGGCTTGCCGGCGAATGCGATCTTGCGGGGTTTTTTCGTTG
GCAAGCCAGCTTCTACGCTAGGCGCAAATATTGCGCGAGGTGTGGTAGGAGCCG
GCTTGCCGGCGAA
PFL_0950-51

TCCTGCATCGTCACGCTAGCCTTTGACGATCATGGCCCGCCGGCCAAGCCGGCGG
GCCATTAATAGAGAGCCGCGCAGTTTGCGGCTGTATTCCACGGCCGGCCTAGAGT
CGGCCGTTTTATGGGGAGTTCAGA
Pfl1102-1103

TTCTAATTCTCTCCTCAGGCGGCGGCAGCGGAGCCCCTACATGACGGGGTCGCC
AGGTTTGGGAGCGATAGTCTGCGCAACCAGGCTAATAGTCCGCAGCATCCCAAA
CCGCGCGAGATTCTAACAGCTTGAAGGCGTTCGCGTTAGATGCG

PFL_1108-09

TACTGCAATACGGGATCGGCTGACACGGTGCGATCCCGTAGGAGCCGGCTTGCC
GGCGATCTCCAGGCCGAAGGCCTGGGGGCAATGAACCTGTGATGGA
ATTTCAAAAGAC

pfl1137-1138

AAAAAAACGCCGCCGACCTGAAGAGGTCGGCGGCGTTTTGTCTTACCTGCCTATA
TAGGCAGG
PFL1143-44

GGTGCGAGAGGGCTGCCGGCCAGCTGCGCGGCAGCCCCGATCAAGGCAATTGCG
AGGTGTCGTTCTGGTTCATCTGGCGACACTTTGTTGTCGGGTTGCG
ACTTGTTCTACCCGGCCAATGGGGCACAAAAGGGCCGGGTTCCTCCGACTTTCGA
CTGCTTGTGTGCGCTGTGTCGGCTGTGTATCTTGATCAGCCTTTG
GCCGTTTGGGCGGCCACACCCCCATTTTTGAGATTGGCCCCACGAGGCGAATCCG
AGCGTTTCTAAATAAAAAATTGAGGAGCACATCG
PFL_1147-1148

CGTTTTGCTTGAAGCGGTCAAGGAATTGCCCCCTTTGGAGATGCTGGAGATGAAA
GGTCACGGATTATGCCACGACCGCGCCGATTCGGCGTCGGCTGCT
GCGGGGCTTTGTCGCATTGTGTTTCCACGCCCGGGCGCCGTTGCGACTTTTGTGC
AGCGGCGTAAACGCAGCTTAAGCGGCTAATATGGCACTTCTGCGG
TGAATTCCTGCCGCCG
PFL_1171-1172

TACCATTTCCATATCAAGTGTGCACAGCCGCTAGACTGCCTCCTTGATTTGCTAG
AGGAGCGTGCGTTAGTCGCGCGCTCCCACTCTTTACTACAGGCTTCCCCCCGAA
PFL_1186-87

CGGTGAGGTGCCATCATTGCGCCTCATCGTCACTTGAATTTTTAATCTCGACCTCA
ACGCGTCCGTCCCTTAAGGTAGGGCGCGAATAGACAACCGGCCG
GCCCAAGCTCCCTTGGACGTCGAGCCCAGACGGACGCCT
PFL_1191-92

TCCGTTACCAGCCTGTTCCGCTCACGTAGAGGGAACAGGCTCCAGCGTTGCCAGG
GATAAAGGCGCCGCTTGAGCCTGAGGTCGAACTCACCAAAGGACTATAACA
PFL_1208-09

GGGCTGCCGTCTGAAGGTCCTGCGGACCTTTTCGCAGCGTGCGGCAGCGGCTACA
GGTTTTTTGGCAGGCTGGGTGATTCATTTCCTGCGCGCCTCAGGAGGGCTTG

PFL_1227-28

GATGGCTCTCTTATTAGTTAGCCAGCTAAGATGCCGCAAAGCTTCACGCTTGACC
ACCCTGGCTTGGCTGGGCATGCTTGGCGCCGATTAGGGCGCCGGCGCTGTTTGAA
CCACTGTTTTGCTCAGGCTTTTCGCGGCGTTCGTGCTCTTTGGGTGCGGTATTCTG
GCGTGCCTACGTGTTGCTTTGGTGAGGGCTGAATGCATCAGTCCTGCCATGAAAA
ACAGGATCATTTGAAAA

PFL_1237-38

GTCTAACCTCGGTATTGGGTGTCGAGCCACTTGGGGGGCAAGTGCGACGGTTGG
GCGCTGGCTGCGCCTCTGATCAAAAAGTGCGCAATTCTAGCGATGGTCGAAGCG
CTTGACCAGCTTTATGCGGGGGAAATACGACGAGCGCTGGACTATGCTTCCCCCC
GTTATGCCTGGTGGTTTTCATACGACGTTAGTTAAGGCCTTAGTAGTAATAAAAA
CCTGCAAGTTGCAGGGCAAACTGCACAAGTTTAGAGAATAGCGCTTGTATAACC
TTGCGCTTTCTATATAAGAAGCCCCGGTCTTGGTAGTAGGAGTCATTG

PFL_1247-48

CCCGGGACGCATGGGCGTCTTTTCAACGAGGGCGCCGACAAAAGTGACGGGCAC
AAAAAAGCCCCGCTGTTGAGGGCGGGGCTTTTTTACTGGATCAGTACAAG

PFL_1259-60

TCGGGTACTCCGTTCTGCGCGGGATGAATTAGCGGGGTATGATAGCGGCCGATTC
GAACCACTGAAAATTTATCCAGCGTTTTGTGCGCCCGAAAAACC

PFL_1278-79

AGTGCTCCACTCTTACTGTTGTAATTTTTATAGTTCCGGCGGCCTTGGTCGCAGAA
CCGAATCAGATATTTCGTGAATACCCGTCGGAAAATGCCCCTGGCAACTGTACCG
GTACAGTGTAGAGCGCCGGTTGTTCGCTTGAAAAGCTGGCTGCTGGGGGCAAAA
ACGCGGGGTGTCGCTTAATTGAAAGAAAAAGACAGATTTGCGGCGTGGCTTGCG
CCCGGTTCGGCGTCAGTCCCTGGCGCTTCTGAGCTTCTCGATCGGTGTCGCGCTT
GCTTCTGGGTTTTTCTGCCAGAGCCCCGACGTTATGATTGCGCCGATTTCATTTCT
GGACAGTCCC

PFL_1300-01

CTGGaagCGATCCGGgtgCTGGtGGCGgGCTGatagtccggttgtcagcactgaacaaaaGGCCCGCac
tgtGCGGGCCTTTTTTT

PFL_1306-1307 tryptophan operon leader

GCTGCCTGACTCCCGCTTTGAAAAAGCCGACTATCTTAACGGAACGACGCTTCCA
GTCCAAAGAACTCTGATCAATCCCCTGGATTAAAAGGTAGCGTACGGCGTGCATT
CGTCGGGTGGCGGGGCACTATCGGCTGACGACTGGGTCACAGTTTTGCGACCGA
TGGCTCAAGTCGCCCATGCCGCAGCCGACAGCCTGCCGACAGGAGACCAATAAT

PFL_1307-08

CCCACCCAGCTGGGGACTATGGCGTGCTACCTGTGTAGGAGCCGGCTTGCCGGC
GAAGAGGCCCGTGAGCCTTGCACCGCCCTTGGGGACGCCTTCGCTGGCAAGCCA
GCTCCTACAGCGGTGCGATACCGGTGTTGTTCTTGTAGGAGCCGGCTCGCCGGCG
AACAGCTCCTTGAGCCTTGCACCGCCCTCGGGCGAGGATTTTTTACCGTCGGGTG
AATAACTTCGTGCCCCGGCGGGGCTTGCTCCCTAAAATACCCGCCATTCCCTGAT
CGACACCGCGCATCGCCCGAGGCTCCCGCCCCC

PFL_1355-56

ACCCCTTCGCCAGCCAGCCGGCTCGTGCAGGCGGCTCACCGCCTCCGTAGGAGCT
GGCTGGCCAGCGAACCGCACACCTTTGAAGGAGAAAGCCCC

PFL_1364-65

TCCGCCGCGATCAATGTAGGAGCTGGCTTGCCAGCGAAAGCGCCCTTGCCGGCG
ATGCCGGACTCAAGGGGCTGTTCGCCGGCAAGCGGGCTCCTACAGAGCGTTTTTT
TTTGACTGACAAGGAGTTTCC

PFL_1389-90

ATGGTTTCAAGCGGATTCACGGCAGGTTGCCAAGAGTCTGCCGGGGCGAATGGC
CCGGTGCATCCGTTTGCGAACCGCAGAGCGATCGTGGGCTCTGTACGAAAACTA
CCTGCGCTTCGATCAGACTGCGTTAAAAACAGGCTCGGAATGCTCATTTAGGTTC
CTAAACTCCGCTTCCTCGCCTGTTTTTGCCTTGCCTGATCTTTGCTCGGCGAGTTT
TCGTACA

PFL_1420-21

CTGCGGCTTCTGTAGCCGCTGTCGCAGGCTGCGAGCGGCCCGAAGGGGCGTTAA
AAAACTTTGAGGGCGCCGCACCTCC

PFL_1425-26

CCTGGAGTACTCCGTTATTGTTCTTATTTTGACGAAAACGCTTCGAGGCGTTTTGC
GGGTCAGCGGCCTTCACGGCCAGGGCAGTCGAGACTGTCCGTGGCGGGACTCTA
AAGGGCCAACCTTTCGCTAACCTTTCAGTGCACTTTCGTGGCTTTCGGGCTTCAC
AGCGGCGGTTGCGGCGGTACACTCCGCGCCAAAGAGCGGCGTCGATCCCCCCTG
AAACGAGGTAAACAGCC

PFL_1440-41

GCCTTTGTTGGTTGTGTAAGAGGTCAGTGATGCCCGCTCCACAATTGAGAAGATG
AAATGTTTGGTCAGCAATTTCATTTGCAAAATTCCCAGAGTTTTTCTATAGGTCAG
AACCCCCTCGGTTCTGACCTTATTTGTCGTTCCTGGAACAAAGATGCCGCCCCTG
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CGT

PFL_1450-51

CAGCGTTGGCCGTGCCCTGGAACAGGGTGAATGGCGGGTTGAGGACATCAACCG
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PFL-1459-60

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CGGCT

PFL_1497-98

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CC

PFL_1498-99

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ATCAAGGTCAGCGGCAGTCAGCGGAGGGTAAAGCTTGGGTAAAGAAGGTATTTC
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PFL_1592-93

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PFL_1600-01

GCTTGTATCCGGGCCGTTACTTGCCGGTAGCGGCATAAGACCGCCGTTTTTCCGT
CTTCTGCCAACTCTGTTTTTTCCTCTGTAATCAGGCAAAGCCTTGCCCCGTCTGGG
GATCACCACTGCGTGAAAAAGTTGGTGCGCTTATTGCTTATGGCTTCGCAGTACA
GCGGTGGGCGGCAAACGTCCGGCAGACAGAGGAAAGACT

PFL_1612-13

TGTCCTCGACCCGGGTCGCGGATGCACGCATTACCTATTCGGGCACCGGCTCGTT
TGCCGATGCCAACCAGCCTG

PFL_1615 GRIK4_3p_UTR

ATTACGAGATCCTGCTGGTGGCTGCCGGCAGTCGCGACCCGGACATGGCTGACT
G

PFL_1620 GRIK4_3p_UTR

TGCACATTCCTTTTGTTCGAGAGTGAAGGCAATTTGCCGCCGACTATAGCCGGGG
CGCTATATCTCTTTAAAGATTAAAAAGTACTTTGC

PFL_1694-1695

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TCACCATGGACGAAATCATCTGGCCGATCACGGTCTTGGGCACGAT
ATCGCCATAGCCCAC

PFL_1695 K_chan_RES

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GTTC

PFL_1703-04

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CCCGCAGGTTG

PFL_1708-09 Entero_5_CRE

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PFL_1729-30 isrK

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PFL_1730 msr

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PFL_1786-87 sar

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CCCGCAGGTTG
PFL_1707-1709

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TCACGCACGGTTGTGCGTCAAATCCGTTCTTTGAGTAATTTTAAACGGCTGTTTTA
GCGGTTACTCTTGGCGGGCGTCATTCAACACTCTGCTTGTCGCAAGGAGCTTTTC
CTC

PFL_1825-26

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PFL_1828-29

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PFL_1836-37

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AGCAATGGCTGGCGATGGTGATCTAGAGCGGTCGCGGCTTGTTGGCAAGGCGTG
CAGTAAAAAGCCCGGCGCGAAGGCCGGGCTGGTTCAGGTAGCAAATCCGCGGGT
T

PFL_1839-40

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TTTTTGTGGGGT

PFL_1849-50 suhB

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TATAGCCCGCTTCGGCGC

PFL_1849-50 SNORA76

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TCGCTAGAGTTCGGCAGTCCTTTTGCTCGCTCAATTTAAATAATCAGAACCAATG
GGAGTGTCTT

PFL_1854-55

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PFL_1859-60

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AAACATCTGCGGTCTGGGCGCGTATCTATATAAGGCAGCACCGACACCTTCGATC
GTGTTCTTTCTGTTACAGGAGTTTCTCG

PFL_1870-71

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PFL_1874-75

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PFL_1875-76

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PFL_1894-95 SRP-bact

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PFL_1929-30

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PFL_1936-1937

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PFL_2042 rtT

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TGTTTTTCTGGC
PFL_2055-56 suhB

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GGCGCTGCGGTGGCGGTGAGGC
PFL_2069-70

AAAATCCGCTCTGCTGGGCGGTCTAGAAGGGGCTCACCGAAAGGTGAGCCCCTT
TTTTGTTTCTGG
PFL_2084-85 suhB

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PFL_2095-96

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PFL_2121-2122 suhB

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PFL_2192-93 isrK

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TTGGCAATCCT
PFL_2240-41 Cobalamin

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PFL_2287-88

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PFL_2339-40

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PFL_2240-41

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PFL_2240-41 P1

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TTAATGCGAATC
PFL_2362-63 snoZ223

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PFL_2475-76

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PFL_2478-79 bantam

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GCAAGCCGGCTCCTATGGGTTTGAAATTGGCTTCAAT

PFL_2478-79 Parecho-CRE

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CGAACGGCTCCGAAGGAGATGCGGGGATATTGAAGGCGCTAGAGGTTCGGCTGG
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GCAGGCTCAGCAGCGGCTACAGGGGTGCAGGAAGGCGTTCTGAGCCGGCGC

PFL_2580-81

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PFL_2641-42 yybP-ykoY

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PFL_2685-86

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GTTGAT

PFL_2701-02 isrK

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PFL_2927-28

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PFL_2970-71 cobalamin

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PFL_3216-17

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PFL_3259-60

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CCACTGCATGGGCGTCGACGACGCCGGTGGGAAGGCGCAGCAATCGCCGGTCCG
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PFL_3655-56 Cobalamin

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GCGAAGTGTCAGATCCACTGTGCCAAGGCATGGGAAGGCGACACTTGCTGGCAC
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PFL_3655-56 Cobalamin

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PFL_3846-47

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CTGAAGAAAC

PFL_3983-84

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PFL_4013-14

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PFL_4077-78

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PFL_4131-32

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PFL_4234-35

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PFL_4407-08 mini-ykkC

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PFL_4419-20

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PFL_4431-32

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AGAACGGGA

PFL_4507-08 FMN

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PFL_4753 RtT

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PFL_4779-80

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PFL_4842-43

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PFL_4888-89

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PFL_4969-70

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PFL_4978-79 isrK_Hfq-binding

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PFL_5084-85

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TGTGTT

PFL_5350-51

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ATGC

PFL_5589-PFL_6310

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PFL_5743-44

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PFL_5751-52

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PFL_5753-54

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TTAGGC

PFL_5780-81

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PFL_5797-98 SAH-riboswitch

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GCCTGCGCCGATGACGGGA
PFL_5834-35

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PFL_5919-20

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PFL_5921-22

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PFL_6116-17

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PFL_6253 SgrS