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INTERKINGDOM SIGNALLING VIA A LUXR-FAMILY PROTEIN IN PLANT ASSOCIATED BACTERIA

DISSERTATION

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To my dad, the best man I have ever known and the person responsible for making me who I am today.

Gracias por todo

ABSTRACT

Many Gram-negative bacteria possess unpaired (i.e. lacking a cognate signal synthase) quorum sensing (QS) LuxR type proteins also called solos; these proteins are believed to extend the regulon of a complete 'resident' QS system or to eavesdrop on N-acyl homoserine lactone (AHLs) signals produced by other bacteria. It is now clear that cell-cell communication in bacteria also occurs with their eukaryotic hosts, a phenomenon called inter-kingdom signaling. Several plant-associated bacteria have a special type of LuxR solo that is able to bind and regulate gene expression in response to a yet unidentified plant molecule(s). The rice pathogen *Xanthomonas oryzae* pv. *oryzae* and the plant-beneficial soil bacteria *Pseudomonas fluorescens* possess the OryR and PsoR LuxR-type solos that respond to plant compound(s). Targets of regulation of both solos were found in this study through the use of genome-wide transcriptome and proteome analysis as well as phenotypical investigations. Interestingly, these proteins regulate different genes depending on the type of relationship the bacteria has with its plant host. In the rice pathogenic *X. oryzae*, OryR regulates a large set of genes including many loci involved in motility and in the beneficial *P. fluorescens* PsoR regulates the production of various bio-control agents. In addition, the commonalities and differences between these solo regulators and QS LuxR proteins were also investigated. In conclusion, OryR and PsoR belong to a new sub-family of LuxR regulators present only in plant-associated bacteria involving a novel inter-kingdom signaling circuit.

POVZETEK

Številne po Gramu negativne bakterije posedujejo proteine LuxR, ki so vključeni v medcelično komunikacijo (quorum sensing, QS). Zaradi odsotnosti vezave s sorodno signalno sintazo se imenujejo tudi proteini “solos”. Za te proteine velja prepričanje, da razširijo regulon celotnega rezidenčnega sistema QS, oziroma da se odzivajo na N-acetil-homoserin lakton (AHLs), ki ga izločajo druge bakterije. Vedno jasneje postaja, da pri bakterijah medcelična komunikacija poteka tudi z njihovimi evkariontskimi gostitelji, kar označujemo s terminom signaliziranje med kraljestvi (inter-kingdom signaling). Številne rastlinske bakterije imajo poseben tip proteinov LuxR solo, ki so se sposobni vezati in regulirati gensko ekspresijo kot odgovor na še neidentificirane rastlinske molekule. Riževa patogena bakterija *Xanthomonas oryzae* pv. *Oryzae* in rastlinam koristna talna bakterija *Pseudomonas fluorescens* imata proteine solos OryR in PsoR vrste LuxR, ki se odzivajo na rastlinske molekule. V naši študiji smo ugotavljali tarčne gene, ki jih omenjena protein solos regulirata, s transkriptomsko in proteomsko analizo ter s fenotipskimi raziskavami. Ugotovili smo, da ti proteini regulirajo različne gene v odvisnosti od vrste razmerja, ki ga imajo bakterije s svojim rastlinskim gostiteljem. Protein OryR pri riževem patogenu *X. oryzae* regulira velik nabor genov, vključno s številnimi lokusi, ki uravnavajo mobilnost. Pri koristni bakteriji *P. fluorescens* pa protein PsoR uravnava produkcijo različnih substance, vključenih v biokontrolo. V nadaljevanju smo ugotavljali skupne značilnosti in razlike med regulatornimi proteini solo in proteini QS LuxR. Zaključimo lahko, da proteini OryR in PsoR sodijo v novo poddružino regulatornih proteinov LuxR, ki se pojavljajo le pri rastlinskih bakterijah in predstavljajo novo signalno omrežje, ki povezuje različna kraljestva živih bitij.

PUBLICATIONS

This thesis is based on the following publications:

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KEY WORDS

Inter-kingdom

Plant-bacteria signaling

Xanthomonas oryzae

Pseudomonas fluorescens

LuxR family

Solo LuxRs

OryR

PsoR

Gene regulation

Flagellar regulation

Biocontrol

Rice

In planta expressed proteins

Secreted proteins

Protein U

ABBREVIATIONS

QS Quorum sensing

PAB Plant-associated bacteria

Xoo Xanthomonas oryzae pv. *oryzae*

EPS extracellular polysaccharide

F-T3SS Flagellar type III secretion system

AHL Acyl-homoserine lactone

AI-2 Autoinducer 2

Approx. Approximately

CFU Colony forming units

DNA Desoxyrribonucleic Acid

EDTA Ethylenediaminetetraacetic acid

EPS Exopolysaccharide

Gfp Green fluorescent protein

HTH Helix-Turn-Helix

HSL Homoserine Lactone

ISR Induced systemic resistance

KB King'S B medium

ORF Open Reading Frames

PCR Polymerase Chain Reaction

PGPR Plant growth promoting rhizobacteria

PGPB Plant growth promoting bacteria

AUTHOR CONTRIBUTION

I hereby declare that the research work described in this thesis is entirely my own work except sections 4.1.7 "Analysis of *in vivo Xoo* expressed proteins" and 4.4: "*In planta* proteomics as a method for studying pathogen expression", which were done in collaboration with my colleagues Dr. Giuliano Degrassi (Bacteriology Group, ICGEB; Trieste) and Dr. Michael P. Myers (Protein Networks Group, ICGEB; Trieste).

1 INTRODUCTION

1.1 *Xanthomonas oryzae* pv. *oryzae*

All known species of the genus *Xanthomonas*, a member of the gamma subdivision of the Proteobacteria, are plant-associated. They can be found living epiphytically or saprophytically without causing symptoms of disease but most are plant pathogens. They are known to cause disease in 124 monocotyledonous and 268 dicotyledonous plants (Chan and Goodwin, 1999). Although the genus as a whole has a very broad host range, pathogenic species and pathovars show a high degree of host plant specificity (Subramoni et al., 2006, Ryan et al., 2011). Among them, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is a pathogen of the staple crop plant rice (*Oryza sativa*) that causes bacterial blight of rice. *Xoo* is a motile bacterium with a single flagellum, which is induced under conditions of limited nutrition (Shen et al., 2001). *Xoo* cells are rod shaped and can vary in length from 0.7 to 2.0 µm and a width of 0.4 to 0.7 µm. Like most other Xanthomonads, *Xoo* produces the pigment xanthomonadin and high amounts of extracellular polysaccharide (EPS), which give its colonies a characteristic yellow and mucoid nature (Niño-Liu et al., 2006). *Xoo* was originally isolated in Japan in 1909, shortly after it was discovered to be the causal agent of rice disease and classified as *Bacillus oryzae*; the bacterium was then renamed *Pseudomonas oryza,* and later *Xanthomonas oyrzae* (Ishiyama, 1922). In 1978 it was reclassified as *X. campestris* pv. *oryzae* (Dye, 1978) until finally in 1990 its status as a species, *Xanthomonas oryzae* pv. *oryzae*, was recognized (Swings et al., 1990a). The diversity of races in Xoo is remarkable: more than 30 races of different virulence have been reported worldwide. Additionally, as in most xanthomonads, *Xoo* shows a high plasticity in its genome, has been shown to experience rapid evolution and has an unusual abundant and diverse set of insertion sequence (IS) elements (Ryan et al., 2011).

1.1.1 Rice & leaf blight

Rice is the staple food of almost half the world's population (Fairhurst and Dobermann, 2002) providing 20% of the world's calorie consumption (Kubo and

Purevdorj, 2004). This makes rice one of the most important crops, especially for developing countries. *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is the causal agent of bacterial leaf blight (BLB)(Swings et al., 1990b), a vascular disease generating lesions along the leaf, initially along the tip and edges. The lesions gradually expand and turn yellowish and eventually grayish-white. Bacterial leaf blight was originally characterized in the Fukuoka Prefacture in Japan in 1884 and is commonly found in South Easy Asia, although it has been reported in both tropical and temperate areas in Australia, Latin America and the Caribbean (Mew et al., 1993). Before the use of resistant varieties, bacterial leaf blight gave rise to crop loss of up to 50% (Mew et al., 1993) and up to 60-75% in tropical areas, depending on weather conditions, making this disease a major threat to human food safety (Niño-Liu et al., 2006).

1.1.2 Virulence

Xoo invades rice plants by swimming into the leaves through hydathodes or preexisting lesions and multiplies in the tissue connecting them. Once inside the plant, bacteria colonizes and clogs xylem vessels by producing large amounts of extracellular polysaccharides (EPS or xanthan), which clogs the vessels and ultimately leads to tissue necrosis and wilting (Shen and Ronald, 2002). In addition to the importance as a pathogen, the bacterium is known to be an ideal model for studying plant–pathogen interactions, race differentiation and evolution of plant pathogens. The rice–*Xoo* interaction has been studied at the molecular level and much work has been done over the past 20 years to identify the virulence factors important for rice infection and their corresponding genes with special reference to type III secretion system *hrc* (hypersensitive response conserved) and *hrp* (hypersensitive response and pathogenicity) genes (Zhu et al., 2000), and the *avr* genes, encoding Avr proteins. Other virulence factors include the EPS production *gum* operon (Dharmapuri and Sonti, 1999, Yoon and Cho, 2007), secretion *xps*F and *xps*D genes (Ray et al., 2000), type I secretion system *rax* genes (required for *avr Xa21* activity) (Shen et al., 2002) secreted proteins such as xylanase (Ray et al., 2000), cellulase (Sun et al., 2005), lipase/esterase (Rajeshwari et al., 2005) and chorismate mutase (Degrassi et al., 2010), motility with the flagellar gene operon *flh* and its regulator *flhF* (Shen et al., 2001), iron metabolism (Subramoni and Sonti, 2005a), the *xadA* gene which encodes Xanthomonas Adhesin like Protein A (Ray et

al., 2002, Das et al., 2009) and the virulence and pathogenicity regulating *rpf* genes (Jeong et al., 2008).

1.1.3 Motility

Flagellar regulation is multi-step and complex, in xanthomonads this regulation is a variation of the well-studied single polar flagellum system found in vibrios and pseudomonads (Dasgupta et al., 2003). Studies in *Xcc* have indicated a 3-step process (Lee et al., 2003, Hu et al., 2005, Yang et al., 2009). At the top of the cascade are the Class I genes, these include the master regulators RpoN2 (σ^{54}) and FleQ, along with the repressor FlgM. The master regulators coordinate the expression of Class II genes, which include the F-T3SS and basal body hook structure and regulators like FliA (σ^{70}), FleN and FlhF. Those in turn regulate the Class III genes, which include motor and chemotactic proteins, flagellin and the flagellar cap protein (Table 1-1, Figure 1-1).

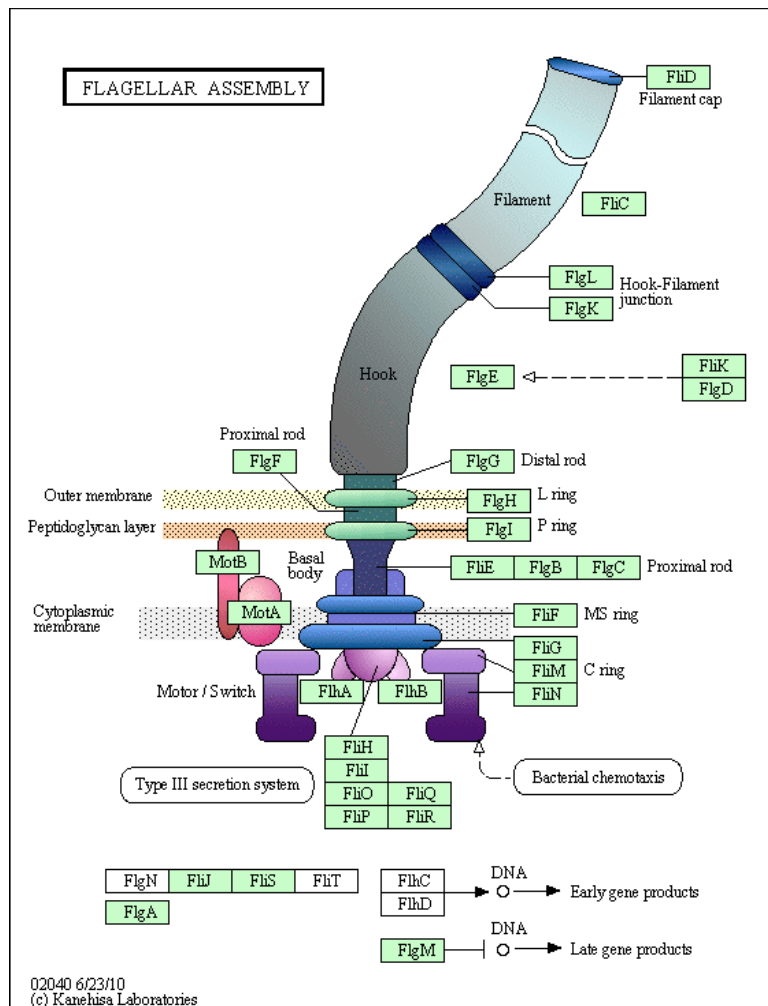

Figure 1-1. Flagellar assembly in *Xanthomonas oryzae*.
(image taken from: http://www.kegg.jp/kegg-bin/show_pathway?xoo02040)

Flagella have been shown to be important for virulence in plant and animal pathogens. In *Xoo*, the flagellum appears to be induced under limited nutrition conditions (Shen et al., 2001) and studies have demonstrated that *Xoo* cells move towards rice exudates of susceptible plants, while not towards resistant plants (Feng and Guo, 1975). This clearly indicates that chemotactic movement is essential for bacterial entrance into the plant (Shen and Ronald, 2002). Once inside the plant, the flagella seems to loose its importance for virulence, as has been shown in *Xcc*, since cells loose motility and flagella in the xylem fluid of cabbage leaves (Kamoun and Kado, 1990).

Table 1-1. Temporal classes of *Xoo* flagellar genes.
Ordered according to the type of promoter recognized by different sigma factors

Class I		Class II		Class III	
Genes	**Function**	**Genes**	**Function**	**Genes**	**Function**
fleQ *rponN2* *flgM*	Regulators	*fliEFGHIJK* *fliLMNOP* *fliQR* *flhB*	F-T3SS	*fliC* *fliD*	Flagellin Cap
		flhA	Regulator	**Other genes**	
		flgBCDEF *flgGHIJKL*	Basal body-hook	7 chemotactic	
		flhF *fleN* *fliA*	Regulators	2 motor proteins	

Modified from: (Yang et al., 2009)

1.2 *Pseudomonas fluorescens*

The fluorescent *Pseudomonas* are a diverse and highly adaptable bacterial group; members of this cluster include organisms as disparate as *P. aeruginosa, P. syringae, P. putida* and *P. fluorescens* (Couillerot et al., 2009). These bacteria owe their name to their ability to produce the fluorescent siderophore pyoverdine (PVD) or pseudobactin. The soil bacteria *P. fluorescens* has received much attention due to its potential as a biocontrol agent. Biocontrol strains grow aggressively on the surface of plant roots, forming microcolonies or biofilms in the spaces between epidermal cells and can inhibit the growth of plant pathogens through antagonism, direct competition and induction of plant response. *P. fluorescens* has been well characterized for its ability to produce a wide range of antimicrobial compounds such as siderophores, 2,4-diacetylphloroglunicol (DAPG), phenazines, hydrogen cyanide (HCN), chitinase and surfactants (Fenton et al., 1992, Keel et al., 1992, Vincent et al., 1991).

Iron is a scarce nutrient, especially in alkaline soils. Siderophores like PVD have a high affinity for iron, once they bind ferric iron, they can interact with specific member receptors and be taken up by the producing organism (or others with the same receptors), facilitating the acquisition of this vital element and at the same time, outcompeting possible plant pathogens with less efficient siderophores. This competition has been proposed as an important trait for biological control (Kloepper et al., 1980, Schroth and Hancock, 1982).

2,4-DAPG is a low molecular-weight polyketide that inhibits the growth of phytopathogens like fungi, helminths, nematodes and oomycetes. The *phl* locus is an 8-kb cluster involved in the biosynthesis, regulation, export and degradation of DAPG. The locus consists of 8 genes *phlHGFACBD* of which, *phlD* is the key biosynthetic gene (Vincent et al., 1991) (Haas and Keel, 2003).

Chitinase is an enzyme capable of degrading chitin, the polysaccharide that is a major component of fungal cell walls. Several bacterial species, including *Pseudomonas spp.,* have been shown to produce this enzyme in culture, and even higher levels when fungal cell wall is supplemented to the culture and has been suggested as a biocontrol agent by damaging fungal cell walls (Mitchell and Alexander, 1961, Neiendam Nielsen and Sørensen, 1999). Additionally, chitin binds $Cu^{2}+$ ions, which could allow biocontrol agents that produce it to influence copper availability in the rhizosphere (Haas and Defago, 2005).

1.3 Signaling

Cell to cell communication was, until not long ago, thought to be an ability unique to multicellular eukaryotic organisms. Microorganisms were believed to be solitary entities each independently struggling to find nutrients and appropriate living conditions. In reality, bacteria much rather live in communities and in this way they can combine efforts to achieve a common goal. These communities are exposed to ever-changing environmental factors, like temperature, pH, osmolarity, concentration of harmful substances and nutrient availability (Patankar and Gonzalez, 2009b, Whitehead et al., 2001). In order to counteract these adverse conditions, bacteria have evolved various systems to quickly sense and respond to these changes. Some examples include sigma factors, which allow bacteria to selectively activate transcription (Wösten, 1998). Likewise, the two-component signal transduction system is a surface exposed structure that permits the bacteria to channel exterior

signals to intracellular responses (Mascher et al., 2006). Furthermore, bacteria can also coordinate gene expression in response to cell-density resulting in population-wide responses through the use of small signal molecules; a phenomenon termed quorum sensing (QS)(Fuqua et al., 1994, Bassler, 1999, Whitehead et al., 2001).

1.3.1 Quorum Sensing

Various phenotypes like biofilm formation, virulence factor secretion, antibiotic production, sporulation, swarming, bioluminescence and competence require a finely coordinated community-wide expression in order to be effective. QS allows bacteria to do this in a few simple but fundamental steps starting with the synthesis of low molecular weight autoinducer molecules (Figure 1-2). These molecules most commonly diffuse freely out of and into the cell, and as the population of bacteria increases so does the concentration of these molecules. When a certain threshold level is reached, cognate receptors bind the molecule and activate coordinated gene expression in the entire population (Ng and Bassler, 2009).

1.3.2 Historical background of quorum sensing

QS was first discovered in the bioluminescent marine bacteria *Vibrio fischeri* (Nealson et al., 1970, Engebrecht et al., 1983, Wang et al., 1991) and this model has become a paradigm for quorum sensing in Gram-negative bacteria (Waters and Bassler, 2005). *V. fischeri* is a bacterium with two very distinct living styles, it can be found as a free-living organism in marine environments but it is also commonly found as a symbiont of various marine animals. Interestingly *V. fischeri* exhibits bioluminescence when in symbiotic mode. The best studied case is *Euprymna scolopes*, a nocturnal marine squid, that uses the light produced by the bacteria to match that of the moon and in doing so evades predators by preventing itself from being detected from below (Visick and McFall-Ngai, 2000). In exchange, the microorganism receives protection and nutrients from its host (Bassler, 1999, Miller and Bassler, 2001).

It was found that bioluminescence was only achieved when bacteria reached high cell density (10^{10} cells/ml). A pivotal experiment demonstrated that the bacteria could be stimulated to premature luminescence at low cell densities if spent supernatant from a high cell density culture was added (Nealson et al., 1970). Furthermore, this effect seemed species-specific given that supernatants from other bacterial cultures did not induce bioluminescence (Eberhard, 1972). This finding led

researchers to model that luminescence was induced by a density-dependant secreted molecule, which was termed autoinducer (Fuqua et al., 1996). This molecule, unveiled more than two decades ago (Eberhard et al., 1981b), is an acylated-homoserine lactone (AHL) that in *V. fisheri* triggers bioluminescence at high cell densities.

Moreover, a few years later it was found that AHL-based quorum sensing was not unique to *V. fischeri*, the discovery of QS systems in *Pseudomonas aeruginosa*, (Gambello and Iglewski, 1991a), *Erwinia carotovora* (Jones et al., 1993) and *Agrobacterium tumefaciens* (Piper et al., 1993, Fuqua and Winans, 1996b) led to a deep interest in this field, resulting in the identification of QS systems in more than 70 bacterial species in the following years (Williams et al., 2007). Some well-studied quorum sensing systems are listed in Table 1-2.

Table 1-2. Examples of AHL-based quorum sensing systems. Modified from (Lazdunski et al., 2004) and (Fuqua et al., 1996).

Organism	I/R genes	Cognate AHLs	Regulated phenotypes	References
Vibrio fischeri	*luxR/luxI* *ainS*	3O-C6-HSL C8-HSL	Bioluminescence Colonization factors	(Engebrecht et al., 1983, Engebrecht and Silverman, 1984, Eberhard et al., 1981a)
Pantoea stewartii	*esaR/esaI*	3O-C6-HSL	Exopolysaccharide production	(von Bodman et al., 1998)
Agrobacterium tumefaciens	*traR/traI* *trlR*	3O-C8-HSL	Virulence plasmid copy number and conjugal transfer	(Fuqua and Winans, 1994, Fuqua et al., 1995, Hwang et al., 1994)
Erwinia carotovora subsp. carotovora	*carR/carI*	3O-C6-HSL	Production of carbapenem and exoenzymes	(Bainton et al., 1992, Pirhonen et al., 1993)
Pseudomonas aeruginosa	*lasR/lasI* *rhlR/rhlI* *qscR*	3O-C12-HSL C4-HSL	Virulence, biofilm formation, others	(Gambello et al., 1993, Gambello and Iglewski, 1991b, Pearson et al., 1995, Chugani et al., 2001)
Rhizobium leguminosarum biovar viciae	*rhiR/rhiI* *raiR/raiI* *bisR, traR, traI, cinR, cinI*	C6-HSL C7-HSL C8-HSL C6-HSL C7-HSL C8-HSL 3OH-C8-HSL 3OH-C8-HSL 3OH-C14:1-HSL	Nodulation Unknown Plasmid transfer Growth inhibition	(Cubo et al., 1992, Gray et al., 1996, van Brussel et al., 1985)

1.3.3 Molecular basis of quorum sensing

1.3.3.1 AHL-based quorum sensing

Molecular analysis revealed that the *V. fischeri* luminescence gene cluster is composed of 8 *lux* genes: *luxA-E, luxG, luxI* and *luxR* (Engebrecht et al., 1983, Swartzman et al., 1990). *LuxA* and *B* encode for the components of the protein luciferase responsible for the light emitting reaction; *luxC-E* code for proteins responsible for synthesizing the necessary precursors utilized by the luciferase protein and *luxG* is a hypothetical flavin reductase. The cell-density-dependant bioluminescence in *V. fisheri* is the result of the action of two regulators present in the operon, the products of the *luxI* and *luxR* genes (Whitehead et al., 2001, Ng and Bassler, 2009). LuxI is the synthase responsible for AHL production and LuxR is a transcriptional regulator. At low population numbers, small amounts of AHLs are produced, which are quickly diluted in the outside environment. As population grows, so does the concentration of autoinducer in and around the cell; once a certain threshold is reached the transcriptional regulator binds the autoinducer (Fuqua et al., 2001). Only when the LuxR-type receptor protein binds the AHL signal is the protein correctly folded allowing dimerization and binding of target DNA sequences, the *luxI* gene is usually one of the targets creating a positive feedback loop (Hughes and Sperandio, 2008). This will in turn enhance or block the transcription of quorum sensing target genes, resulting in a population-wide activation of certain phenotypes (Figure 1-2). This form of gene regulation is referred to as quorum sensing (QS), a term first introduced by Fuqua *et al* (1994).

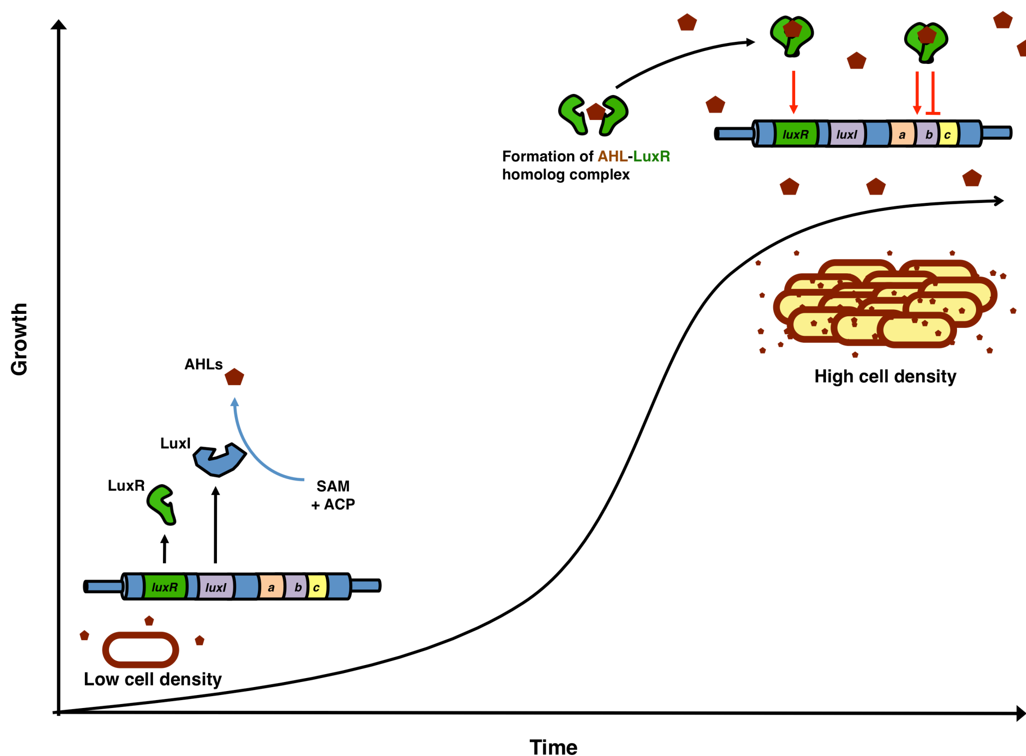

Figure 1-2. Typical Gram-negative bacteria quorum sensing system.
At low cell densities, basal levels of the transcriptional regulator LuxR and the LuxI sinthase are expressed; resulting in low levels of autoinducer (AHL). As the population increases, so does the concentration of autoinducer in the environment Once a threshold is reached, the receptor protein LuxR binds the autoinducer, usually dimerizing, which induces a conformational change that allows the LuxR-AHL complex to bind (or release) the promoter region of target genes. In most cases, the *luxI* gene is positively regulated, activating a positive feedback loop.

1.3.4 Autoinducers

1.3.4.1 Acyl homoserine lactones.

The most common signal molecules used by gram-negative bacteria are the acyl homoserine lactones (AHLs). These are composed of a conserved homoserine ring and a highly variable acyl chain (Figure 1-3a), and can usually cross the cell membrane (Hughes and Sperandio, 2008). AHLs are classified according to the length of their acyl chain into the categories of short, if their acyl moiety has 4 to 8 carbons or long when it has between 10 and 18. The acyl chain has two major variables, (i) in the acyl chain at position 3 it can be either saturated, unsaturated or have the presence or absence of oxo or hydroxyl groups and (ii) the acyl chain can vary in length having a minimum of 4 to a maximum of 18 carbons. These different AHLs most probably allows some specificity in mixed bacterial populations providing each species with its own “language”; alternatively if two species produce

the same AHLs this results in interspecies signaling (Fuqua et al., 2001, Whitehead et al., 2001).

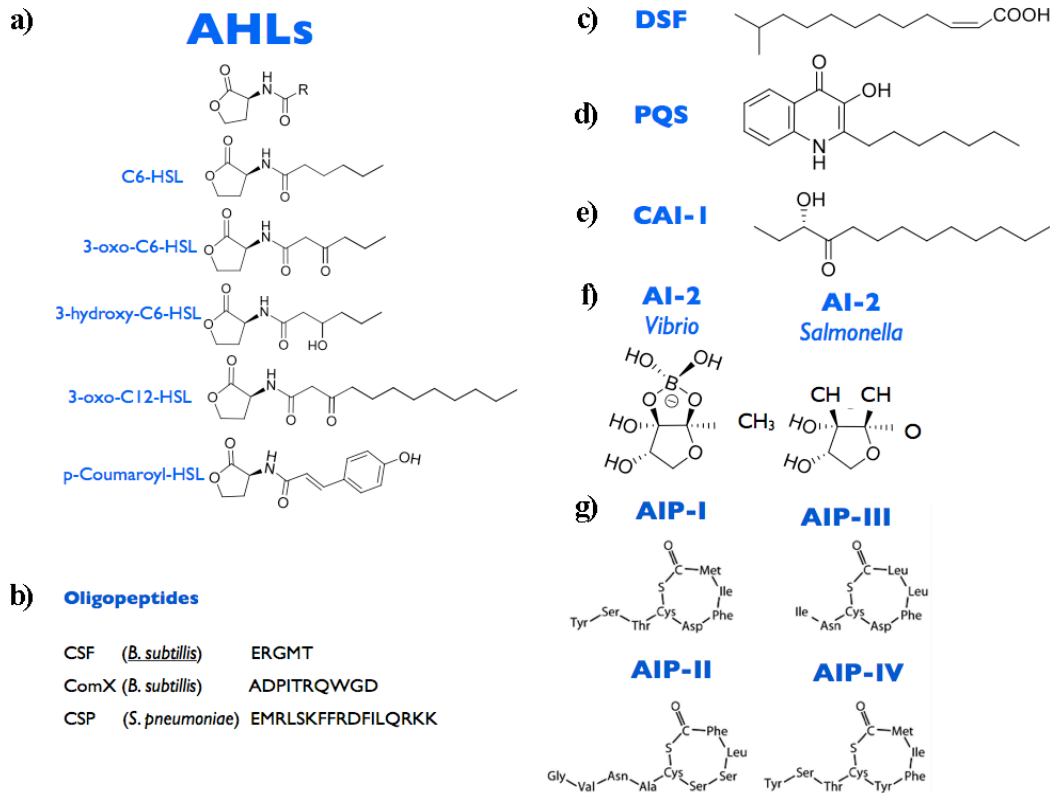

Figure 1-3. Structure of bacterial autoinducers.
(a) AHLs Homoserine lactones produced by several Gram-negative bacteria. (b) Amino acid sequences of three oligopeptides produced by Gram-positive bacteria (c) DSF produced by *Xanthomonas* spp. (d) Structure of the PQS autoinducer of *Pseudomonas aeruginosa*. (e) Structure of *V. cholerae* CAI-1 (f) Autoinducer 2 (AI-2), in the presence of boron, it exists as S-THMF borate, whereas in absence of boron it exists as R-THMF (g) Cyclic peptides from *Staphylococcus aureus*.

1.3.4.2 Autoinducer 2

AI-2 is a furanosyl borate diester (Figure 1-3f) first found in *Vibro harveyi* (Bassler et al., 1993). AI-2 is of special interest because it has been described as a global signal since it has been found in many gram-negative as well as gram-positive bacteria (Bassler, 1999). Interestingly, AI-2 signals from different bacteria can activate a *V. harveyi* indicator strain (Surette et al., 1999). AI-2 has been implicated in bioluminescence regulation in *V. harveyi* (Bassler et al., 1994) and type III secretion in *E. coli* (Sperandio et al., 1999).

1.3.4.3 Oligopeptides.

Oligopeptides are the autoinducers used by Gram-positive bacteria (Figure 1-3b). In this case the sensor is a two-component membrane-bound histidine kinase, which is

mediated by a phosphorylation cascade that regulates DNA binding of a response regulator (Bassler and Losick, 2006). Unlike AHLs, these peptides are not freely diffusible and must be transported across the cell membrane by special oligopeptide exporters (Waters and Bassler, 2005). A very well known example is the regulation of genetic competence in *Bacillus subtilis* that uses the ComX signal (a 6 amino acid peptide) (Okada et al., 2005).

1.3.4.4 Diffusible signal factor.

DSF is the autoinducer used by the xanthomonads (He and Zhang, 2008a) and was first discovered in *Xanthomonas campestris (Xcc)* when a screening for decreased virulence mutants revealed that a mutation in the *rpfABCDEFG* genes (regulation of pathogenicity factors), decreased the production exopolysaccharide and extracellular enzymes (Tang et al., 1996). The phenotype could then be restored with the co-inoculation of the wild type in close vicinity to the mutant indicating that *Xcc* could produce a diffusible signal factor (Barber et al., 1997). This system has also been reported in species including *X. oryzae, Xylella fastidiosa* and *Stenotrophomonas maltophilia.* A two-component system is implicated in DSF perception. DSF was recently identified as *cis*-11-methyl-2-dodecenoic acid (Figure 1-3c) and most important features are the unsaturated double bond at the α, β position and the *cis* configuration of the α, β double bond, the chain length and the methyl substitutions, without which, the response regulator has little or no affinity towards the autoinducer (Wang et al., 2004).

1.4 AHL autoinducer synthases

Two families of AHL synthases have been identified so far, belonging to the LuxI and LuxM families (Figure 1-2). The most common is the LuxI family responsible for AHL synthesis in many bacteria, including α, β and γ-protobacteria (Lerat and Moran, 2004). LuxI type proteins are usually between 194 and 226 amino acids long, have 10 highly conserved residues, most of them in the more conserved N-terminus (Fuqua et al., 2001). LuxI uses S-adenosyl-methionine (SAM) as a precursor to make the lactone ring and utilizes the existing acyl carrier proteins (ACP) from the lipid metabolism to obtain the acyl chains and joins them with an amide bond. LuxM proteins are a class of AHL synthases that use the same precursors as LuxI proteins

but have a different amino acid composition; members include AinS from *V. fisheri*, VanM from *Vibro anguillarum* and LuxM from *Vibro harveyi* (Bassler, 1999).

1.5 LuxRs

The LuxR family of regulatory proteins is large consisting of many subfamilies responding to various stimuli; one sub-family is the QS LuxR-type which responds to AHLs (Figure 1-2). These proteins are modular and about 250 amino acids long having two functional domains (Figure 1-4); an autoinducer-binding domain at the N-terminus (Shadel et al., 1990, Slock et al., 1990) and a DNA-binding helix-turn-helix (HTH) domain at the C-terminus (Choi and Greenberg, 1992, Choi and Greenberg, 1991, Fuqua et al., 1994). A short linker region bridges the two domains. LuxR proteins bind to DNA at a conserved site called a *lux* box, an inverted repeat recognition sequence that consists of a 20 bp palindrome. The *lux* box, which is usually located at -42.5 from the ATG (Devine et al., 1989, Stevens and Greenberg, 1997), is highly conserved although some variability exists that allow differential interaction of the regulator with its target genes (Whitehead et al., 2001, Camilli and Bassler Bonnie, 2006, Nasser and Reverchon, 2007). Existing evidence suggests that QS LuxR-type proteins can act as either a transcriptional repressor or an activator (Fuqua et al., 2001). In the latter case, binding of the autoinducer brings about a conformational change that allows the HTH domain to bind DNA upstream of its target genes; the LuxR/autoinducer complex then physically recruits RNA polymerase by interacting with its αCTD domain (Stevens et al., 1999) and activates transcription (Zhang et al., 2002). On the other hand, in some cases the LuxR-family protein acts as a repressor by binding to target gene promoters in the absence of signal molecule blocking access to RNA polymerase (Minogue et al., 2002). In this case, when signal molecule concentrations reaches quorum threshold and binds to the LuxR-type protein, conformational changes causes it to release the promoter and in turn, relieving repression (Minogue et al., 2002).

Structural analysis of TraR of *Agrobacterim tumefascens* (Zhang et al., 2002, Vannini et al., 2002) and SdiA of *Escherichia coli* (Yao et al., 2006a) have shown that the AHL-binding cavity of LuxR proteins are composed of five-stranded antiparallel β-sheets with three α-helixes on each side. Interestingly, the two domains can function independent of each other. When over expressed the N-terminal can bind autoiducers (Hanzelka and Greenberg, 1995) and the C-terminus

alone has been shown to activate transcription (Choi and Greenberg, 1991). Binding of the autoinducer is crucial for LuxR protein stability and correct folding. When over-expressed, in *E. coli* in the absence of its cognate autoinducer (3-oxo-C8-HSL), TraR is quickly proteolyzed (Zhu and Winans, 2001) or forms inclusion bodies (Zhu and Winans, 1999) but when 3-oxo-C8-HSL is added to the media prior to induction of over-expression, the TraR protein is folded and soluble. Similar results have been found in SdiA, although this protein can bind a wider range of autoinducers (Yao et al., 2006b). Additionally, most LuxR-type proteins dimerize when bound to their cognate autoinducer and can only bind DNA in this state (Nasser and Reverchon, 2007). Surprisingly, sequence alignments reveal that LuxR-type proteins show very low homologies in their primary structure (18-25%); nonetheless 95% of QS LuxR-type proteins share 9 highly conserved amino acid residues (Whitehead et al., 2001, Zhang et al., 2002). Six of these residues are hydrophobic or aromatic and form the cavity of the autoinducer-binding domain (Figure 1-4). These are (with respect to TraR): tryptophan 57 (W_{57}), tyrosine 61 (Y_{61}), asparagine 70 (D_{70}), proline 71 (P_{71}), tryptophan 85 (W_{85}) and Glycine 113 (G_{113}). The remaining three highly conserved residues, glutamine 178 (E_{178}), leucine 182 (L_{182}) and glycine 188 (G_{188}), are in the HTH domain (Fuqua and Winans, 1996a). Evolutionarily, there appear to be two distinct groups of LuxI/R proteins, the first family is found in proteobacteria from different divisions (α, β and γ) and the second is found only in γ-Proteobacteria indicating that it evolved in this group relatively recently, with a probable origin being around the divergence of the *Xanthomonads* and *Xylellas*, which lack LuxI/R homologs but before the divergence of pseudomonads which have them (Lerat and Moran, 2004). Interestingly, proteins from the first family are usually activators, which comprise most of the LuxR-type proteins described. The transcriptional regulators from the second group are mainly repressors and include EsaR from *Pantoea stewartii*, YpsR from *Yersinia pseudotuberculosis*, SpnR from *Serratioa marcescens*, and ExpR and VirR from *Erwinia* spp. (Nasser and Reverchon, 2007).

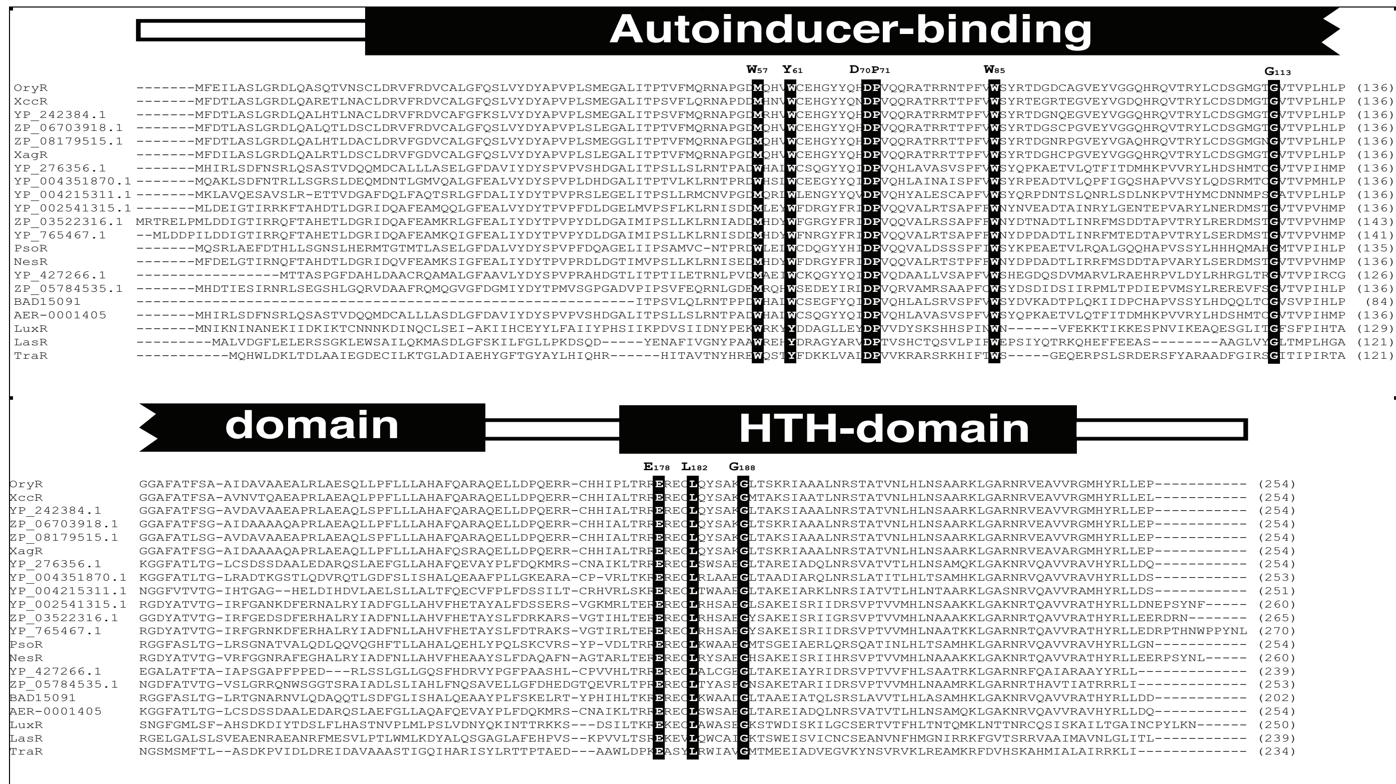

Figure 1-4. Alignment of LuxR-type proteins.

PAB Solo LuxRs: OryR *Xanthomonas oryzae* pv. *oryzae* KACC10331; XccR *Xanthomonas campestris* pv. *campestris* str. 8004; YP_242384.1 *Xanthomonas gardneri* ATCC 19865; ZP_06703918.1 *Xanthomonas fuscans* subsp. *aurantifolii* str. ICPB 11122; ZP_08179515.1 *Xanthomonas vesicatoria* ATCC 35937; NP_643297.1 *Xanthomonas axonopodis* pv. *citri* str. 306; YP_276356.1 *Pseudomonas syringae* pv. *phaseolicola* 1448A; YP_004351870.1 *Pseudomonas brassicacearum* subsp. *brassicacearum* NFM421; YP_004215311.1 *Rahnella* sp. Y9602; YP_002541315.1 *Agrobacterium radiobacter* K84; YP_003882209.1 *Dickeya dadantii* 3937; ZP_03522316.1 *Rhizobium etli* GR56; YP_765467.1 *Rhizobium leguminosarum* bv. *viciae* 3841; PsoR *Pseudomonas fluorescens* Pf-5; NesR *Sinorhizobium meliloti* 1021; YP_427266.1 *Rhodospirillum rubrum* ATCC 11170; ZP_05784535.1 *Citreicella* sp. SE45; BAD15091 *Pseudomonas azotoformans*; ZP_07006441 *Pseudomonas savastanoi* and QS LuxR: LuxR of *V. fischeri*; LasR of *P. aeruginosa* and TraR of *A. tumefaciens*. The 9 highly conserved amino acids are highlighted in black and their positions with respect to TraR indicated above.

1.6 LuxR Solos

Additionally to the archetypical LuxI/LuxR quorum sensing systems, many bacteria also have QS *luxR*-type genes, which are unpaired to a cognate *luxI* (Figure 1-5). In fact, an *in silico* analysis of 265 proteobacterial genomes showed that 68 had a *luxI/luxR* system, of these 45 contained more *luxRs* than *luxIs* and another set of 45 genomes contained only *luxRs* (Case et al., 2008). These unpaired proteins have been termed "orphans" (Fuqua, 2006) and more recently "solos" (Subramoni and Venturi, 2009). These solos are homologous to QS LuxR-type proteins having an autoinducer-binding domain in the N-terminus and a DNA-binding HTH domain in the C-terminus but unlike their counterparts they do not directly regulate the production of autoinducers via a positive auto-induction loop. These solo proteins can expand the regulatory repertoire of the typical LuxI/R QS systems by responding to endogenously produced autoinducers. Alternatively, they can also regulate target genes by "eaves-dropping" on exogenous signals produced by neighboring bacteria (Ahmer, 2004) or via interkingdom signaling when responding to eukaryotic signals (Zhang et al., 2007, Ferluga et al., 2007, Subramoni et al., 2011).

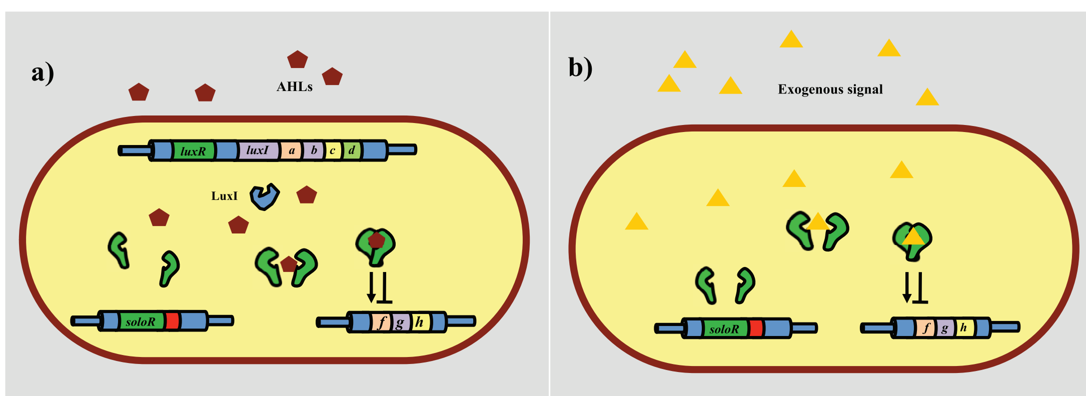

Figure 1-5. LuxR solos.
(a) LuxR solos found in bacteria with a classical quorum sensing system(s) and (b) LuxR solos found in bacteria lacking QS systems.

One of the best-characterized solo proteins is QscR of *Pseudomonas aeruginosa*. This opportunistic pathogen has a complex QS response composed of two hierarchically organized QS pairs where LasI/R regulates the second system (RhlI/R). QscR responds and binds to the endogenously produced 3OC12-HSL, synthesized by LasI, and has its own sizeable regulon, which overlaps with the ones of LasI/R and RhlI/R (Lequette et al., 2006). Another well-studied solos is SdiA,

which in *Salmonella enterica* regulates the *rck* (resistance to complement killing) and *srgE* (of unknown function) loci (Ahmer et al., 1998, Ahmer, 2004). SdiA is also present in *E. coli*, where it is involved in the regulation of genes related to cell division (Wang et al., 1991), antibiotic resistance, motility and chemotaxis genes (Rahmati et al., 2002, Dyszel et al., 2010, Hughes et al., 2010). Since neither *S. enterica* nor *E. coli* have AHL synthases in their genomes or produce AHLs, SdiA responds to exogenously produced AHLs (Ahmer et al., 1998, Ahmer, 2004, Michael et al., 2001, Yao et al., 2006a); various examples of LuxR solo proteins are listed in Table 1-3.

1.7 A sub-family of LuxR solos in plant-associated bacteria

Interestingly, there is a sub-group of LuxR-type solos in plant-associated bacteria (PAB) (Figure 1-6), whose members have substitutions in one or two of the highly conserved amino acids in the autoinducer binding domain, more precisely, W_{57} and Y_{61} that can be substituted by methionine (M) and tryptophan (W) respectively (Figure 1-4). This feature apparently gives these proteins the ability to bind and respond to plant signal molecules rather than AHLs allowing inter-kingdom signaling (Zhang et al., 2007, Ferluga et al., 2007, Ferluga and Venturi, 2009). This group includes OryR of *X. oryzae*, XccR of *X. campestris*, PsoR of *P. fluorescens*, and NesR in *S. meliloti* (Patankar and Gonzalez, 2009a). This novel sub-family of LuxR-solos found in PAB, although highly related to classical QS LuxR-type proteins, form a distinct cluster as can be seen in Figure 1-6. Moreover, these LuxR solos are usually in close proximity to the proline iminopeptidase (*pip*) gene (Figure 1-8), coding for a protein that cleaves the proline from the N-terminal of proteins (Sarid et al., 1959). The promoter region of the *pip* gene usually has a *lux* box-like element called the *pip* box. PIP is important for virulence (Zhang et al., 2007) but its biological function is not well understood.

1.7.1 XccR

Xanthomonas campestris (*Xcc*) is an economically important pathogen of a wide variety of plants, including many of agricultural interest. Like all xanthomonads, it has a special cell-density dependant communication system; unlike the classical AHL mediated LuxI/LuxR scheme, the xanthads employ a diffusible signal factor (DSF) and a two-component system RpfC/RpfG (Fouhy et al., 2006).

Table 1-3. List of functionally characterized LuxR solos. Modified from (Subramoni and Venturi, 2009)

LuxR 'solos'	Organism	AHL QS systems	Binding molecule/s	Functions Regulated	Reference
AvhR	*Agrobacterium vitis*	AvsI/AvsR	Not yet determined	Ability to cause necrosis on grapes and HR on tobacco plants	(Hao et al., 2005)
AviR	*Agrobacterium vitis*	AvsI/AvsR	Not yet determined	Ability to cause necrosis on grapes and HR on tobacco plants	(Zheng et al., 2003)
BisR	*Rhizobium leguminosarum pv. viciae*	CinI/CinR RhiI/RhiR RaiI/RaiR TraI/TraR	3OH-C14:1-HSL	Symbiotic plasmid pRL1J1 transfer and growth inhibition in the presence of 3OH-C14:1-HSL	(Danino et al., 2003, Wilkinson et al., 2002)
BlxR	*Brucella melitensis*	None	Not yet determined	Regulation of virulence factors like type IV secretion system and flagella	(Rambow-Larsen et al., 2008)
CarR	*Serratia marcescens*	SmaI/SmaR	Ligand independent	Carbapenem antibiotic production	(Cox et al., 1998)
ExpR	*Sinorhizobium meliloti*	SinI/SinR Mel	C14-HSL 3-oxo-C14-HSL C16:1-HSL 3-oxo-C16-HSL C18-HSL	Production of symbiotically active EPSII, succinoglycan production, motility, chemotaxis, nitrogen fixation, metal transport etc.	(Bartels et al., 2007, Hoang et al., 2004, McIntosh et al., 2008, Rambow-Larsen et al., 2008, Patankar and Gonzalez, 2009a)
TraR	*Agrobacterium tumefaciens*	TraR/TraI		Inhibition of conjugation of Ti plasmid	(Fuqua and Winans, 1994, Oger et al., 1998)
QscR	*Pseudomonas aeruginosa*	LasI/lasR RhlI/RhlR	3-oxo-C12-HSL 3-oxo-C10-HSL	Timing of expression of AHL production and virulence factors	(Chugani et al., 2001, Lequette et al., 2006)
SdiA	*Escherichia coli* *Salmonella enteric serovar typhimurium*	None	3-oxo-C8-HSL 3-oxo-C6-HSL 3-oxo-C4-HSL 3-oxo-C10-HSL 3-oxo-C12-HSL C6-HSL C8-HSL	Functions involved in adhesion and resistance to complement killing	(Ahmer et al., 1998, Ahmer, 2004, Michael et al., 2001, Yao et al., 2006a)
VjbR	*Brucella melitensis*	None	C12-HSL	Regulation of virulence factors like type IV secretion system and flagella	(Delrue et al., 2005)
XccR	*Xanthomonas campestris pv. campestris*	None	Plant Signal molecule	Proline imino peptidase (*pip*) gene expression; Virulence on Cabbage	(Zhang et al., 2007)
OryR	*Xanthomonas oryzae pv. oryzae*	None	Rice Signal molecule	Proline imino peptidase (*pip*) gene expression; Virulence on rice	(Ferluga et al., 2007, Ferluga and Venturi, 2009)
NesR	*Sinorhizobium meliloti*	SinI/SinR Mel	C14-HSL 3-oxo-C14-HSL C16:1-HSL 3-oxo-C16-HSL C18-HSL	Nutritional and environmental stress response, plant nodulation	(Patankar and Gonzalez, 2009a)
PsoR	*Pseudomonas fluorescens Pf-5/ CHA0*	None	Plant signal molecule	Anti-microbial agents	(Subramoni et al., 2011)

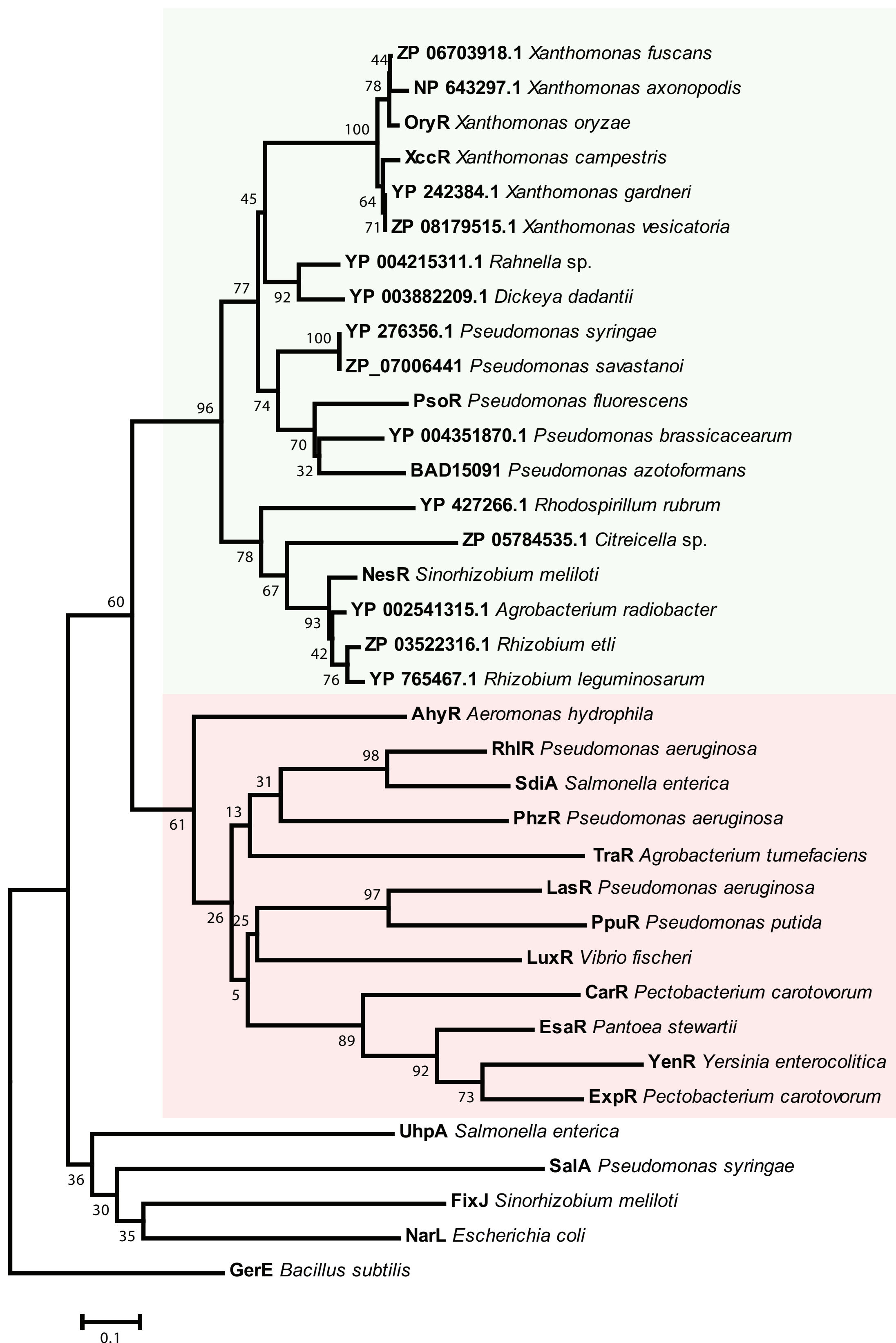

Figure 1-6. Phylogenetic tree of transcriptional regulators.
PAB LuxRs are highlighted in green, and QS LuxRs are in red.

Nonetheless, many species of xanthomonads contain, in their genomes, a gene coding for a LuxR solo protein. XccR from *X. campestris* was the first LuxR solo reported to respond to a plant compound (Zhang et al., 2007). This solo, a 254 amino acids protein homologous to QS LuxRs, could not be activated by AHLs, possibly also due to the difference in the first two residues that are highly conserved in LuxR proteins (W_{57} and Y_{61}). Additionally, a luxbox-like 20 bp palindromic sequence was detected in the promoter region of the *pip* gene adjacent to *xccR*; it displays very high identity overlapping with *lux* boxes found in QS systems (Fuqua et al., 1996). Interestingly, instead of an autoinducer synthase, in *X. campestris* this neighboring gene codes for a proline iminopeptidase (PIP), a protein that cleaves proline residues from the N-terminus of peptides (Sarid et al., 1959). Both *pip* and *xccR* are important for pathogenicity since knockout mutants showed an almost 10 fold reduction in virulence when tested on cabbage. Furthermore, over-expression of XccR activates the PIP promoter from its *pip* box plays as confirmed by both promoter fusion and direct binding to the promoter region, which was confirmed by gel-mobility shift assays. But perhaps the most fascinating result was that this activation could be observed *in planta* without the need of XccR over-expression; studies concluded that a plant compound(s) induces this regulation via XccR. Induction of the PIP promoter fusion increased 8 fold *in planta*. Furthermore, this induction was XccR and *pip* box dependant, since disruption of either resulted baseline activities. Interestingly, *pip* promoter activities were highest in a *pip* mutant, suggesting autoregulation.

1.7.2 OryR

The solo OryR is a close homolog to XccR and is found in *X. oryzae* pv. *oryzae* (*Xoo*), a Gram negative bacteria that causes leaf blight disease on the rice plant (*Oryza sativa*). *Xoo* is a vascular pathogen that enters plant leaves through the thylakoids and once inside divides and produces a high amount of exopolysaccharides that clogs the xylem system. Like *Xcc, Xoo* does not produce AHLs (Ferluga et al., 2007) but genome sequencing (Lee et al., 2005) has shown that it has a LuxR solo at the locus XOO1268; the predicted protein is 254 amino acids long. OryR has a conserved autoinducer-binding domain in their N-terminus, and a helix-turn-helix domain at the C-terminus. Like XccR, OryR in its AHL-binding domain has two substitutions in the key positions W_{57} and Y_{61} that are switched for a methionine (M_{57}) and a tryptophan (W_{61}) respectively (Figure 1-4). A common

feature of many QS LuxR proteins is their ability to become soluble only in the presence of their cognate autoinducer (Fuqua et al., 2001). Interestingly, OryR was found to be insoluble when over-expressed in the presence of a wide range of AHLs but is readily soluble as if rice plant macerate is added to the growth media, indicating that it binds a plant-produced molecule and that it is probably induced and expressed *in planta* (Ferluga et al., 2007).

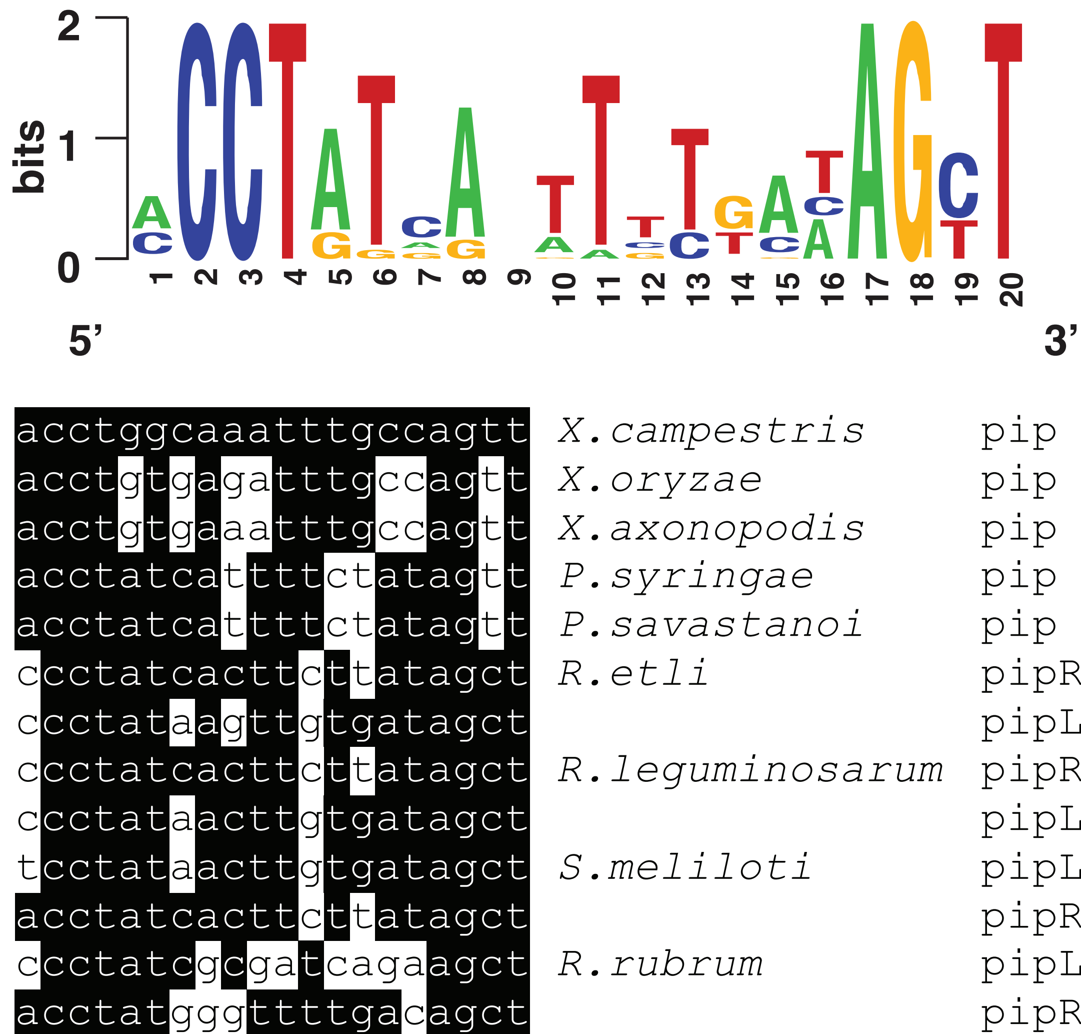

Figure 1-7. PAB *pip* boxes.
Consensus sequence of various *pip* boxes found upstream of the *pip* gene(s) and examples found in plant-associated bacteria. Some organisms have more than one pip gene. L denotes a *pip* gene upstream of the solo and R downstream. The consensus sequence was made using the Weblogo tool (http://weblogo.berkeley.edu/**).**

Additionally, *oryR* is genetically linked to the *pip* gene, and as is characteristic of this family of transcriptional regulators, it regulates *pip* expression via a highly conserved *pip* box found in its promoter region (Figure 1-8). Furthermore, a *pip* transcriptional fusion revealed that this regulation is rice-molecule dependant, since activation is only observed when rice macerate is added to the media. Interestingly,

this induction is enhanced when *Xoo*-infected rice is used for making the plant extract, indicating that molecule production by the plant might be induced by infection (Ferluga and Venturi, 2009).

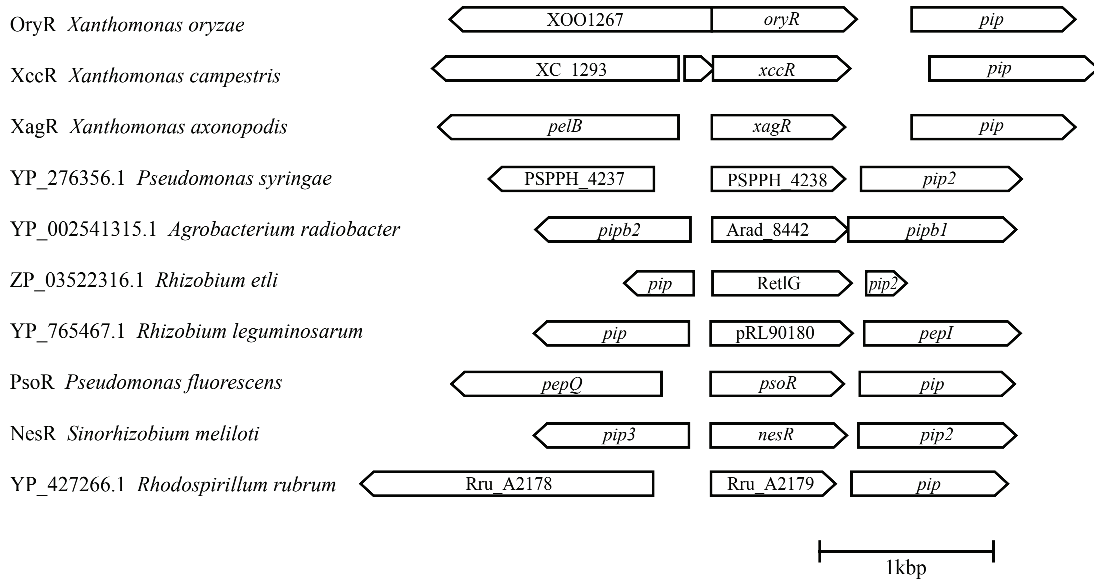

Figure 1-8. Genomic arrangement of LuxR solos in plant-associated bacteria.

1.7.3 PsoR

Fluorescent *Pseudomonas* are ubiquitously found in the rhizosphere of plants. These bacteria have been widely studied as potential bio-control agents, thanks to their ability to produce a wide range of anti-microbial compounds like 2, 4-diacetylphloroglucionol (DAPG), pyoluteorin, hydrogen cyanide (HCN) and the siderophores pyochelin and pyoverdines (Haas and Keel, 2003, Haas and Defago, 2005, Loper et al., 2007). Genome sequencing of two plant-growth-promoting strains *P. fluorescens* Pf5 and CHA0 revealed the presence of a LuxR solo, which has been termed PsoR (Subramoni et al., 2011). The predicted protein is 252 amino acids long and although highly similar to XccR and OryR, PsoR displays some unique features, perhaps due to the mutalistic relationship this bacteria has with plants. First, on a sequence level, PsoR conserves the tryptophan at position 57 (W_{57}) of QS-LuxRs but shares the substitution of tyrosine at position 61 for a trypthan (W_{61}) of other PAB-solos. This single substitution could indicate binding of a related but different plant signal molecule (PSM). Significantly, PsoR-PSM interaction appears to be plant specific as PsoR-solubility studies in presence of plant macerate demonstrated interaction with PSM in the presence of rice and wheat macerates but not in

cucumber (nor any AHL tested). Secondly, even though *psoR* is a member of the PAB solos and so it is closely linked to *pip*, the latter does not contain a *pip* box in its promoter region and is not regulated by PsoR, as confirmed by promoter-fusion assays (Subramoni et al., 2011), possibly reflecting the non-pathogenic nature of *P. fluorescens*.

The biological role of PsoR is also in accordance to its niche, recently Subramoni *et al* (2011) found through genome-wide transcriptome analysis that the over-expression of PsoR produced an up regulation of various anti-microbial related genes like DAPG, chitinase and some iron acquisition genes. Expression levels of the GFP based transcriptional reporter fusion of the *phlA* gene (in the DAPG operon) showed a five-fold increase in the PsoR over-expressing strain compared to the wild type; meanwhile levels in the *psoR* knockout mutant decreased by 3 fold. Additionally, flow cytometry observations revealed that the *psoR* over-expression did not increase *phlA* reporter activity in the cucumber rhizosphere. Similar results were observed in biocontrol experiments. The ability of *P. fluorescens* Pf-5 wild type, *psoR* knockout mutant and over-expressing strains to protect wheat or cucumber plants against the pathogen *Pythium ultimum* was compared in a natural soil microcosm. Again here, PsoR was important in protecting wheat but had no effect in cucumber. These results suggest that binding of PSM by PAB-LuxR could be plant specific and different proteins could bind different types of molecules, which are most likely closely related in their structure and composition.

1.7.4 NesR

Unlike the examples of the PAB solos mentioned above, NesR of *S. meliloti*, belongs to the group of solos found in bacteria that have, in their genomes, a "classical" QS system. *S. meliloti* can be found living in the soil or in symbiotic association with the alfalfa plant (*Medicago sativa*) and its QS SinI/SinR system is responsible for the production of various AHLs (Marketon and Gonzalez, 2002, Marketon et al., 2002) and, in combination with to the AHL-responsive solo ExpR, regulates various phenotypes like motility, chemotaxis and the production of EPS II and succinoglycan (Marketon and Gonzalez, 2002, Marketon et al., 2002, Teplitski et al., 2003).

Additionally, *S. meliloti* also has a PAB-LuxR called NesR. Like XccR and OryR, NesR has two substitutions at the key positions 57 and 61 (Figure 1-4) and interestingly, two *pip* genes flank it upstream and downstream, both of which have

pip box-like elements (Figure 1-8). Genome-wide transcriptome analysis revealed that this solo affects the active methyl cycle, finding significant negative fold-differences expression of genes coding for methionine synthases *bmt*, *metH, metK* the hydrolase *ahcY* and the reductase *metF*. Complementation by plasmid borne over-expression resulted in restoration of wild type levels or up-regulation of all these genes. Interestingly, expression of these genes remained unchanged in a *sinI* mutant showing that, as is the case with the other PAB-LuxR, NesR regulation is AHL-independent (Patankar and Gonzalez, 2009a). Furthermore, the *S. meliloti* NesR knockout mutant showed decreased ability to grow in high salt concentrations, the presence of the detergent DOC, the ability to grow in media with glycine betaine as sole carbon source and it is less competitive for nodulation with respect to the wild type (Patankar and Gonzalez, 2009a).

1.7.5 XerR, the negative regulator of *xccR*

A new player in the expression and regulation of PAB LuxR solos has been recently found in *X. campestris.* XerR is a member of the two-component signal transduction system response regulator NtrC family that negatively regulates the PAB solo XccR (Wang et al., 2011). XerR acts as a repressor by directly binding to the promoter region of the *xccR* gene, as evidenced through electrophoretic mobility assays, preventing RNA polymerase from binding to the transcriptional sites. This repression is relieved *in planta* and the binding of XerR to the *xccR* promoter was substantially inhibited by the presence of plant extract in a dose-dependent manner. Interestingly, the same plant extract improves the binding of XccR to the *pip* promoter indicating a tight plant-signal molecule dependant regulatory circuit between XerR and XccR (Wang et al., 2011).

2 Aims and Objectives

The microbial world is hugely diverse and complex. The myriad of co-existing species possess signaling mechanisms to provide them information of their whereabouts, of their surrounding neighbors and the environmental conditions they find themselves in. The scope of this thesis involves exploring the role of inter-kingdom signaling between the rice plant (*Oryzia sativa*) and two bacterial species associated to it, the pathogen *Xanthomonas oryzae* and the beneficial plant-growth-promoting *Pseudomonas fluorescens*. Both of these bacteria possess a special LuxR-type transcriptional regulator (OryR and PsoR respectively), which is uniquely found in plant-associated bacteria, which responds to an unidentified plant compound. Interestingly, these proteins, although very similar, regulate different phenotypes in these microorganisms, reflecting on the different relationship each has with the host plant. Below are the thesis objectives:

A. Identify the role of inter-kingdom signaling between the plant and the bacterial species.
B. Identify the targets of regulation of OryR and PsoR in response to rice signal molecule
C. Investigate if the two bacterial species share similar signaling networks/pathways/mechanisms
D. Evaluate the degree of conservation and exchangeability between QS-LuxRs and PAB-LuxRs
E. Develop an *in vivo* proteomics approach in order to identify *Xoo* proteins that are expressed *in planta*.

3 MATERIALS AND METHODS

3.1 Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 3-1. *X. oryzae* pv. *oryzae* strain were grown at 30°C and 150 rpm agitation in either PYS or PS medium. *E. coli* strains DH5α, M15 and S17-1λ-pir were grown in LB at 37°C and 150 rpm agitation. *P. fluorescens* strains were grown in LB or KB media at 30°C and 150 rpm (See Appendix 7-1 for media composition). Media containing macerated rice material was prepared as follows: 20-25 g of the areal part of the plant (leaves and upper part of the stem) was macerated in the presence of liquid nitrogen, added to 400 ml PYS (*X. oryzae*) or LB (*P. fluorescens* and *E. coli*) medium and autoclaved. This medium was filtered and used for all experiments. Antibiotics, when required, were used at the following concentrations: ampicillin, 75 μg/ml (*X. oryzae*) 100 μg/ml (*P. fluorescens* and E. *coli*); kanamycin, 50 mg/ml (*X. oryzae* and *P. fluorescens*) or 100 mg/ml (*E. coli*); gentamicin, 10 mg/ml; tetracycline, 10 μg/ml (*X. oryzae* and E. *coli*) or 125 μg/ml (*P. fluorescens*) and rifampicin 100 μg/ml (See Appendix 7-1.6 for a detailed list of antibiotics).

Table 3-1. Strains used in this study

Strain	Characteristic/Sequence	Reference
X. oryzae pv. *oryzae* strain XKK.12	Wild type strain	(Ferluga et al., 2007)
X. oryzae pv. *oryzae* XKK.12ORYR	*oryR*::Km insertion mutant; Km^{r}	(Ferluga et al., 2007)
E. coli strain DH5α	F'/endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1 (lacZYA-argF)U169 deoR [80dlac(lacZ)M15recA1]	(Sambrook et al., 1989)
E. coli strain S17-1λ-pir	RP4-2Tc::Mu-Kn::Tn7 pro hsdR recA	(Simon et al., 1983)
E. coli strain M15	Derivative of *E. coli* K-12, containing pREP4 plasmid ensuring the production of high levels of lac repressor protein; Km^{r}	Qiagen, Hilden, Germany
P. fluorescens CHA0	Wild type; biocontrol agent; DAPG^{+}, PLT^{+}, PRN^{+}, HCN^{+}; Amp^{r}, Cm^{r}	(Schnider-Keel et al., 2000)
P. fluorescens CHA0PSOR	*psoR*::Km of P. fluorescens CHA0; Amp^{r}, Cm^{r,} Km^{r}	(Subramoni et al., 2011)

Rif^{r}, rifampicin resistance; Km^{r}, kanamycin resistance; Cm^{r}, chloramphenicol resistance; Amp^{r}, ampicillin resistance.

3.2 Reagents and chemicals

All chemicals used for the culture media preparation were purchased from Difco (Franklin Lakes, NJ, USA) and Sigma (St. Louis, MO, USA). Molecular Biology reagents were acquired from New England Biolabs (Ipswich, MA, USA), Promega (Madison, WI, USA) and Ambion (Austin, TX, USA).

3.3 Recombinant DNA techniques

Recombinant DNA techniques, including digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments and ligations with T4 DNA ligase were performed as described (Sambrook et al., 1989). The composition and preparation of solutions used are provided in Appendix 7-1.

3.3.1 Genomic DNA extraction

All DNA manipulations and transformation of *E. coli*, were performed as described previously (Sambrook et al., 1989). Genomic DNA from *X. oryzae* and *P. fluorescens* was isolated by Sarkosyl-pronase lysis (Better et al., 1983).

3.3.2 DNA agarose electrophoresis

DNA samples were analyzed routinely on agarose gels (0.8-2.0% w/v) in TAE 1x (40mM Trisma base, 1mM EDTA pH 8.0, 0.1142% glacial acetic acid). To visualize DNA, SYBR Green I nucleic acid gel stain (Invitrogen, Carlsbad, CA, USA) was added 1:10000 into the gel solution just prior to pouring the gel. Electrophoresis was performed in Biorad (Hercules, CA, USA) minisub$_{TM}$ cell chamber, at 5 V/cm, and TAE 1x was used as running buffer (Sambrook et al., 1989).

3.3.3 Cloning of PCR products

Cloning and expression vectors used are listed in Table 3-2. For cloning purposes, DNAfragments were PCR amplified from approx. 50 ng of chromosomal DNA. For general PCR reactions, 0.6 U of GoTaq Flexi (Promega, Madison WI, USA) were used, and the reaction mix was composed by 200 µM dNTPs, 1.5 mM $MgCl_2$, 0.1µM of each primer. Restriction sites were added to the primers in the 5' ends when required. PCR reactions were performed in an Applied Biosystems PCR GeneAmp 2400 thermocycler. The DNA template was initially denatured at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C, annealing at 55-65 °C for 30 seconds and extension at 72 °C for 1-3 minutes, according to the length of the fragment to be amplified. A last extension cycle at 72°C during 5 minutes was used to ensure completion of strands. For introduction of point mutations 50 ng of template DNA were amplified using 1 U of high fidelity Vent$_R$ DNA Polymerase (New England Biolabs, Ipswich, MA, USA) and a reaction mixture that contained 2.5 mM dNTPs, 1.5 mM $MgCl_2$ and 125 ng of each primer. The DNA template was initially

denatured at 95°C for 30 seconds, followed by 16 cycles of denaturation at 95°C, annealing at 55 °C for 1 minute and extension at 68 °C for 4 minutes.

In all cases, fragments were separated by electrophoresis and extracted from the gel by using the EuroGOLD gel extraction kit (Euroclone, Italy). Blunt ended fragments were cloned into pMOS-Blue® (GE Health Care) and protruding A- fragments were cloned into pGEM-T easy® (Promega, Madison WI, USA), according to the instructions of the manufacturer. Subsequently, each fragment was excised with restriction enzymes, ligated into the final vector, and sequenced (Macrogen, Korea) to verify the identity and orientation of the insert.

New England Biolabs (Ipswich, MA, USA) restriction enzymes were used to cut genomic and plasmid DNA. Generally 0.5-1 μg of DNA were digested by using 10 U of enzyme in a final volume of 25 μL, with the addition of the buffer suggested by the provider. Reactions were incubated from 2 h to 10 h, and when required heat inactivation at 65°C was performed to stop the reaction. For ligation reactions, a 1:2.5 ratio of vector to insert was used, and 1.5 U of T4 DNA ligase (Promega, Madison, WI, USA) were added in a final volume of 20 μL. Ligation reactions were incubated for 12 h at room temperature.

3.3.4 Plasmids

Plasmid DNA isolation from *E. coli* was performed by using EuroGold columns (EuroClone, Italy) according to the manufacturer’s instructions. Briefly, overnight cultures were submitted to alkaline lysis and neutralization. After removal of the cellular debris the lysates were loaded in a silica column and eluted with previously heated (75 ºC) sterile water.

Table 3-2. Plasmids used in this study.

Plasmids	Relevant characteristics	Reference
pMOS*Blue*	Cloning vector, Amp^r	GE-Health Care, Little Chalfont, UK
pGEM-T easy	Cloning vector, Amp^r	Promega, Madison, WI, USA
pMP220	Promoter probe vector, IncP, LacZ, Tc^r	(Spaink et al., 1987)
pQE30	Expression vector, ColE1 replication origin, T5 promoter, His epitope. Amp^r .	Qiagen, Hilden, Germany
pBBRMCS-5	Broad-host-range vector Gm^r	(Kovach et al., 1995)
pBBRPsoR	pBBR mcs-5 with 1162-bp EcoRI-HindIII fragment containing *psoR*, Gm^r	(Subramoni et al., 2011)
pUFR047	Wide host range vector, IncW, Gm^r, Amp^r	(De Feyter et al., 1993)
pQF50	Broad-host-range vector, pRO1600 replicon, Amp^r,	(Farinha and Kropinski, 1990)
pSS122	Promoter probe vector, IncW, Amp^r Gm^r	(Ferluga and Venturi, 2009)
pSS122PIP	*pip* promoter cloned with *HindIII-PstI* in pSS122	(Ferluga and Venturi, 2009)
pXoopipprom	*Xanthomonas oryzae* pv. *oryzae* proline imino peptidase (Xoopip) promoter cloned in pMP220; Tc^r	(Subramoni et al., 2011)
pLasI220	*lasI* promoter of *P. aeruginosa* cloned in pMP220; Tc^r	(Rampioni et al., 2007)
pPpuI220	*ppuI* promoter of *Pseudomonas putida* cloned in pMP220; Tc^r	(Bertani and Venturi, 2004)
pGluI220	*gluI* promoter of *Burkorderia glumae* cloned in pMP220; Tc^r	(Suárez-Moreno et al., 2010)
pCvI220	*cvI* promoter of *Chromobacterium violaceum* cloned in pMP220; Tc^r	(Suárez-Moreno et al., 2010)
pQEBRAR	*braRXEN* of *Burkorderia brasiliensis* cloned into pQE30 expression vector; Amp^r	(Suárez-Moreno et al., 2010)
pQEXENR2	*xenR2* of *Burkorderia xenovorans* cloned into pQE30 expression vector; Amp^r	(Suárez-Moreno et al., 2010)
pQEXENR	*xenR* of *B. xenovorans* cloned into pQE30 expression vector; Amp^r	(Suárez-Moreno et al., 2010)
pQEUNAR	*braRUNA* of *B. unamae* cloned into pQE30 expression vector; Amp^r	(Suárez-Moreno et al., 2010)

Gm^r, gentamicin resistance; Tc^r, tetracycline resistance; Amp^r, ampicillin resistance

3.3.5 Bacterial transformation and conjugation

E. coli competent cells were made by using the protocol described in Appendix 7-6 (Hanahan et al., 1991). Transformation was performed by mixing 5 µL of plasmid DNA with 100 µL of competent cells. The mixture was incubated in ice for 30 minutes, and then heat shocked at 42°C for 90 seconds. The heat shock was stopped by incubation on ice for two minutes, after which 1 mL of pre-warmed LB was added. Cells were then grown for 1 hour in agitation at 37 °C, and transformants were selected on LB agar plates with the proper antibiotics.

X. oryzae competent cells were prepared using the protocol described in Appendix 7-7. Plasmids were introduced into *Xoo* by electroporation following the protocol described in Appendix 7-8, when possible or by biparental conjugation using the *E. coli* S17-1 λ pir strain as donor (Simon et al., 1983), as described in Appendix 7-9.

Table 3-3. Plasmids constructs generated in this study

Plasmid	Description	Primer	Sequence (5'-3')	Restr. Site	Insert size (bp)
pUFROryR	OryR with promoter region cloned pURR047. Amp^r Gm^r	OryRF OryRHindIIIRv	TAGGATCCAGACGCCGCCGAAGATC GCAAGCTTTTATGGCTCCAGCAACC	BamHI HindIII	1111
pBBROryR1	OryR cloned in pBBRMCS-5 under lac promoter. Gm^r	OryRxpBBR_fw OryRHindIIIRv	TACTCGAGGGGCAACCACCGGTACC GCAAGCTTTTATGGCTCCAGCAACC	XhoI HindIII	793
pBBROryR2	OryR cloned in pBBRMCS-5 (TcR) under lac promoter. Tc^r	OryRpBBRTcFw OryRpBBRTcRv	ATCTCGAGCCTGAGCATGCCTGCGT TAGGATCCTGACCTGCGCCTGCCT	XhoI HindIII	793
pSS123	FlhF promoter region cloned in pSS122. Amp^r Gm^r	flhFKpnIFw flhFXbaIRv	GGGGTACCAAGCGTCACGCCATCAGTACC GCTCTAGATTTCATGCGAATTGCCTCGAGG	KpnI XbaI	473
pSSOryRpnt	OryR with point mutations cloned in pSS122PIP. Amp^r Gm^r	OryRpntF OryRpntR	GAAGCTTAGACGCCGCCGAAGATC GAAGCTTTTATGGCTCCAGCAACC	HindIII HindIII	1111
pBBRPsoRpnt	PsoR with point mutation cloned in pBBRMCS-5. Gm^r	PsoRPntF PsoRPntR	TGAATTCCCCTTCGCTGTTAAGGATCAAC TGGATCCTCAGTTACCCAGCAGGCGA	EcoRI BamHI	1159
pQEOryRHTH	HTH region of OryR cloned in pQE-30. Amp^r	OryRHTHF OryRHTHR	TGGATCCCAGGAACGCCGCTGCCA TAAGCTTTTATGGCTCCAGCAACCG	BamHI HindIII	219
pQEPsoRHTH	HTH region of PsoR cloned in pQE-30. Amp^r	PsoRHTHF PsoRHTHR	TGGATCCCGCAGTTATCCGGTTGATC TAAGCTTTCAGTTACCCAGCAGGCG	BamHI HindIII	210
pMP220PflChit	Chitinase promoter of *P. fluorescens* cloned in pMP220. Tc^r	PFL2090promFP PFL2090PromRP	GGGGTACCGCGGTTGCAGACAGCTAT GCTCTAGAGCCAGGTTCCCAGCAGCC	KpnI XbaI	500
pUFRProtU	*Xoo* Protein U with promoter region cloned in pUFR047. Amp^r Gm^r	pruFw pruRv	GGATCCGCAGACACCACCACC AAGCTTTCAATAGACGATCGT	BamHI HindIII	471
pQEPSSR	*pssR* of *Pseudomonas savastanoi* cloned in pQE30 expression vector; Amp^r	BamPagRFw HindPagRRev	TCGGATCCAAGGATACATACTACAAC TCAAGCTTTCAGTTGTCCAGCAGCCG	BamHI HindIII	706
pQEPAGR	*pagR* of *Pantoea agglomerans* cloned in pQE30 expression vector; Amp^r	BamPssRFw HindPssRRev	TCGGATCCAAGATAAACGGGGCCCCT TCAAGCTTTCAGACCATGCCCATGTT	BamHI HindIII	715
pMOSOmpP1	Fragment of XOO4582 cloned in pMOS. Amp^r	ompP1F ompP1R	TCTACCGCTTCCATTCTCAG CGGGTTCACGTCATAGGAG	- -	519

pMOSPilJ	Fragment of XOO1749 cloned in pMOS. Amp^r	pilJF	CTGTTCGATGCGTTCTCTTC	-	592
		pilJR	TCTGATCCATGCCCTGAATC	-	
pMOSXOO1805	Fragment of XOO1805 cloned in pMOS. Amp^r	XOO1805F	AAGGCGTTGAGGTTCAGAG	-	743
		XOO1805R	GTCGTACTTCCAGTTATCGG	-	
pMOSXOO3526	Fragment of XOO3526 cloned in pMOS. Amp^r	XOO3526F	TCACCAACAGCGTCAAGAAC	-	490
		XOO3526R	TTGTCGGAGGCAAAGTCAG	-	
pMOSPeptid	Fragment of XOO0439 cloned in pMOS. Amp^r	peptidF	GCAACGATGTGATCTTGCAG	-	584
		peptidR	GAGTTTGGCCACCACGTTAT	-	
pMOSCystPep	Fragment of XOO1487 cloned in pMOS. Amp^r	cystpepF	ACCGAAAGCATTAGCAAACC	-	523
		cystpepR	TCGGCGTATTGATGTCGTAA	-	
pMOSProt	Fragment of XOO0680 cloned in pMOS. Amp^r	proteaseF	ATCCCGTTGTTGGTCAGTTC	-	522
		proteaseR	TCTGACTACTGCCGAGCAAA	-	
pMOSProtU	Fragment of XOO1982 cloned in pMOS. Amp^r	protU	GTACGGCTGAGCGTATTGGT	-	336
		protU	CACCACCTTCAACGTCAAGA	-	
pMOSMucD	Fragment of XOO1851 cloned in pMOS. Amp^r	mucDF	GAGGCAACCTGATGAACCAT	-	521
		mucDR	GGTGAGCTTGACGGTGACTT	-	
pMOSXOO0007	Fragment of XOO0007 cloned in pMOS. Amp^r	XOO0007F	GCCACGCTGGACAAGTATCT	-	510
		XOO0007R	CTGATAATCGGGCTTGAGGA	-	

Gm^r, gentamicin resistance; Tc^r, tetracycline resistance; Amp^r, ampicillin resistance. Restriction sites introduced into plasmids are underlined

Briefly, overnight cultures from donor and acceptor were harvested and washed twice, and optical density was determined. Volumes equivalent to 5 $x10^8$ cells from donor were mixed with the corresponding volume of 8.5 $x10^8$ cells from the acceptor. The mixture of cells was then centrifuged and resuspended in 100 µL of media; cells were spotted on top of a 0.45 µM nitrocellulose filter, previously placed on a PSA agar plate. The conjugation mixture was incubated overnight at 30 °C. Cells were then resuspended in 1 mL PY broth, serially diluted and plated on PSA agar, with proper antibiotics. Conjugations were counter-selected in PSA Media with the appropriate antibiotics and incubated for 2-3 days at 30ºC. Mixtures of donor cells plus acceptor were used as negative control.

3.4 Generation of knock out mutants

For construction of genomic insertion mutants, an internal gene fragment of approximately 600 bp was amplified by PCR using the primers listed in Table 3-3. Primers were designed based on the sequences of *Xoo* KACC10331 genome (Lee et al., 2005) using the Primer3 Tool (Rozen and Skaletsky, 2000). PCR amplification products were cloned into the vector pMOSBlue using the pMOSBlue Blunt Ended Cloning kit (GE Healthcare, Milano, Italy) or pGEM T-Easy (Promega) following the supplier's instructions and later verified by DNA sequencing. Constructed plasmids were then electroporated into *Xoo* XKK.12 as described in Appendix 7-8. After electroporation, selection was performed on PSA medium containing ampicillin 75 mg/ml.

3.5 Mutant verification

The fidelity of the recombination event was confirmed by PCR amplification of a region of the genome where integration occurred; for this, a primer was designed in the mutated gene and one from the integrated vector (either one of the universal primers U19 or T7 found upstream or downstream of the multiple cloning site of the pMOS*Blue* plasmid used to generate insertion mutants), see Table 3-3. The use of U19 or T7 depended on the orientation of the inserted gene fragment into the blunt end vector (See Table 3-4 for primers). The amplified fragment was then verified by DNA sequencing (Macrogen, Korea). Examples of sequencing and verification strategy are listed in Appendix 7-4.

Table 3-4. Primers used for mutant verification.

Locus	Gene	Primer name	Sequence	UP
	hflK	hflkVER	GCAACAAGGGCGGAGACGG	U19
Xoo4582	ompP1	ompP1VER	CCCAGAGTGAAGCCCACTT	T7
Xoo1749	pilJ	pilJVER	CCGATTTCCTGCGAGGATTC	T7
Xoo1805	Hypothetical protein	XOO1805VER	GTCTACGGTGCTGACGCCA	U19
Xoo3526	Hypothetical Protein	XOO3526VER	CGTGCTGCGTGACCCGAAGA	U19
Xoo0439	peptidase	XOO0439VER	TTGCTTTCAGTAAACGTGAT	T7
Xoo0680	Protease	XOO0680VER	AGCCGAAGTTGGAATAGA	U19
Xoo1851	Periplasmic protease	XOO1851VER	ATTTCACGTGACAGCAAC	T7
Xoo0007	Hypothetical protein	XOO0007VER	AGCCGATATCGATAGGATAC	T7
Xoo1487	Cysteine protease	XOO1487VER	CTGTTTTCCACGATCAGTC	T7

UP: Universal primer

3.6 Mutant complementation

3.6.1 OryR

Complementation of *Xoo oryR* mutants was performed as follows: (1) a 793-bp fragment containing the *oryR* gene of *Xoo* XKK.12 under the *lac* promoter in the high copy plasmid pBBR-MCS5 and (2) an 1111 bp fragment containing the *oryR* gene with its native promoter region in the low copy plasmids pUFR047. The primers used to amplify from genomic DNA are listed in Table 3-3. These fragments were then cloned into pGEM-T easy vector and subsequently cloned into pBBRMCS-5 Gm^r (XhoI-HindIII) or Tc^r (XhoI-SacII)(Kovach et al., 1995) yielding pBBRORYR1 and pBBRORYR2 respectively. The fragment including *oryR* and its promoter region were cloned in pUFR047 (BamHI-HindIII) to yield pUFROryR (Table 3-3). All constructs were verified by sequencing (Macrogen, Korea).

3.6.2 Protein U

In order to complement the Protein U knock-out genomic mutant, the plasmid pUFRPrU was constructed as follows: the gene *prU*, including its own promoter region, was PCR amplified using genomic DNA as template using oligonucleotides pruFw and pruRv as DNA primers. The 471 bp product obtained was cloned in pGEM-T Easy Vector (Promega, Madison, WI) as a BamHI-HindIII fragment, after verification by DNA sequencing was cloned as a BamHI-XbaI fragment in the corresponding site in the pUFR047 vector, generating pUFRPrU (Table 3-3).

3.7 Elucidation of the *X. oryzae* OryR regulon through genome-wide transcription analysis

In order to find targets of regulation of OryR, a microarray analysis were performed under the following conditions: i) wild type Xoo XKK.12 grown in PY media supplemented with rice macerate and ii) Xoo XKK.12 OryR- mutant grown in PY media supplemented with macerated rice. Two biological replicates of RNA samples were collected for each of the two conditions as described below.

3.7.1 Total RNA isolation

RNA isolations were carried out from cultures of *X. oryzae* strains carrying pSS122PIP grown in PYS media and PYS supplemented with rice. The cultures were incubated (30°C, 180 rpm) until they reached an OD at 600 nm of 2.0. RNA isolation was carried out from 2 x 10^9 cells using the Ribopure^{TM}-bacteria RNA isolation kit (Ambion Inc., Austin, TX, USA), following manufacturer's instructions. Isolated RNA was treated with DNase at 37°C for 1 h and purified following directions given by the manufacturer. The purity of RNA was assessed by a PCR on total RNA (250 ng) with GoTaq polymerase (Promega, Madison, WI, USA) using primers specific for *X. oryzae* KACC. RNA quality and concentration was assessed by nanodrop (Thermo Scientific, Wilmington, DE, USA).

3.7.2 Microarray experiment and analysis

A custom microarray for *X. oryzae* (Roche NimbleGen OID22515) was designed and manufactured by Roche-Nimblegen (Madison, WI, USA) in a 385 K (385,000 probe platform) chip based on the genome sequence of *Xanthomonas oryzae* KACC10331 (NC_006834). The array detects a total of 3931 open reading frames; 10 probes were designed per gene (when possible) and placed on the chip 9 times. This adds up to a total of 353790 specific probes. The empty spaces were filled with random (negative) probes. Labeling, hybridization and scanning, were performed by Roche NimbleGen, Inc. (Madison, WI). Briefly, slides were hybridized with Cy-3-labeled samples and scanned with a NimbleGen MS200 microarray scanner. Data analysis was performed using raw fluorescence intensity values with microarray suite version 5.0. Gene functions were annotated from the National Center for Biotechnology Information (NCBI) database. Data analysis was carried out using the ArrayStar software (DNASTAR, Inc., Madison, WI).

3.7.3 Semi-quantitative RT-PCR and analysis

For validation of microarray data, the most positively and negatively regulated genes were chosen for semi-quantitative RT-PCR analysis as follows: Reverse transcription was performed in a 20-µl reaction mixture containing 2.5 µg of total RNA, 200 ng of random primers/mg of RNA (Promega, Madison, WI) and 30 U of AMV reverse transcriptase following the manufacturer's instructions. Conditions used for RT were 65°C for 3 min, 25°C for 10 min, 42°C for 90 min, and 70°C for 10 min. The Xoo16s_Fw and Xoo16s_Rv primers were used to measure the transcription of 16S rRNA. Second strand synthesis was performed using Go Taq Flexi polymerase (Promega, Madison, WI) with 1 ml of undiluted (any test gene) or 1:100 diluted (16S rRNA) cDNA reaction as the template. The number of PCR cycles for each gene was standardized so that the product amplification is in the linear range. 10 µml of the PCR reaction was analyzed by agarose gel electrophoresis. The intensity of the bands were measured and normalized to that of 16S rRNA using the ImageJ software (Abramoff et al., 2004) to obtain the fold difference. Each gene was validated twice by RT-PCR analysis of RNA samples from three independent isolations.

Table 3-5. Primers used for SQ-RT-PCR.

Gene locus	Primer name	Sequence	Amplicon size (bp)	Reference
16s	Xoo16s_Fw Xoo16s_Rv	TAGCTCAGGTGGTTAGAGCGC CAACGCGAACATACGACTCAA	240	(Lee et al., 2008)
Xoo3196	PilY1_Fw PilY1_Rv	AGTCGGCATTTCCATACGTC GCGGGTTGGTTACTTTACGA	296	This study
Xoo0329	Polyvinyl_FW Polyvinyl_Rv	CAAACCAATTTGCCGGTATC GCCAACCACACCTTCAGTTT	326	This study
Xoo0031	Xoo0031_Fw Xoo0031_Rv	CTGCTTCCTGGATTTTCTCG TTTCTCACTGCCTGATGCAC	255	This study
Xoo2580	FlgL_Fw FlgL_Rv	TGAACTCAGACACGCTGACC CGACTGCATCTGCTGAAAAA	293	This study
Xoo2619	FlhF_Fw FlhF_Rv	CGACTACAAGCTGGTGCTGA ATCTTCAAGGCGAAGAACGA	343	This study
Xoo1701	Xoo1701_Fw Xoo1701_Rv	AGTTATTGCTGACGCCTGCT ATCCCTTGGGGAACAATTTC	265	This study
Xoo1580	RhsD_Fw RhsD_Rv	GCAGGTATGGGCAGAGTGAT TTGGCTATTGTGAGCGACTG	258	This study
Xoo0281	Cellulase_Fw Cellulase_Rv	TCGATCGACACCATTCGTAA TACCAAATTCGCCGAGAAAC	298	This study

3.8 Determination of promoter activity

3.8.1 β-glucuronidase-based reporter gene fusion assay

β-glucuronidase activities were determined, as described in Appendix 7-2, for *X. oryzae* XKK.12 wild type and *oryR*-mutant, carrying the pSS122 or pSS123 plasmids. Activity was measured at various optical densities (OD_{600}) in PYS media

and PYS supplemented with rice (cultures were started at an OD_{600} of 0.2). All experiments were performed in triplicate, and the mean values are given.

3.8.2 β-galactosidase activity

β-galactosidase activities were determined essentially as described by Miller (Miller, 1972) with the modifications of (Stachel et al., 1985). The protocol used for the β-galactosidase activity is described in Appendix 7-3.

3.8.3 Reporter fusions

3.8.3.1 Xoo flhF promoter

Detailed examination of various promoter regions within the flagellar operon of *X. oryzae* revealed a *lux* box-like element upstream of the *flhF* gene. In order to elucidate if the *flhF* gene was under the transcriptional regulation of OryR, a transcriptional fusion construct for the *X. oryzae flhF* promoter in pSS122 (Ferluga and Venturi, 2009) was made by amplifying a 473-bp fragment containing the *flhF* promoter region from *X. oryzae* XKK.12 genomic DNA using primers flhFKpnIFw and flhFXbaIRv. This fragment was cloned into pGEM yielding pGEMflhFprom; this plasmid was verified by sequencing (Macrogen, Korea) and the fragment was subsequently cloned into pSS122 (KpnI/XbaI), yielding pSS123 (Table 3-3). The construct was introduced in *Xoo* wild type and *Xoo oryR*- knockout mutant by bi-parental conjugation and *β*-glucuronidase activities were measured at different optical densities in rich media and rich media supplemented with rice.

3.8.3.2 Point mutations of PAB LuxR-solos

With the aim of restoring the AHL-binding capabilities of OryR and PsoR point mutations that re-established the highly conserved amino acid residues typical of QS-LuxRs (Figure 1-4) were introduced into both proteins using the Megaprimer method (Sambrook et al., 1989). For OryR, a 1111-bp fragment containing the *oryR* gene and its promoter region was amplified from genomic DNA using the primers OryRpntF and OryRpntR and subsequently cloned into pMOS*Blue* yielding pMOSOryR. This construct was then subjected to PCR amplification in order to introduce the point mutations using the megaprimers OryRptMutF (5’-AAT GCC AGG CGA TTG GCA GCA TGT CTA CTG CGA GCA CGG GTA C-3’) and OryRpntMutR (5’-GTA CCC GTG CTC GCA GTA GAC ATG CTG CCA ATC GCC TGG CGC ATT-3’) as described in Materials and Methods section 3.3.3. The same method was used for

PsoR, an 1159 bp fragment containing *psoR* and its promoter region was amplified using the primers listed in Table 3-3 and cloned into pGEM. Point mutations were introduced into this construct using the primers PsoRpntF (CCA GGG ACT GGC TGG AGC TCT ACT GCG ATC AGG GTT ACT ACC) and PsoRpntR (GGT AGT AAC CCT GAT CGC AGT AGA GCT CCA GCC AGT CCC TGG). PCR products were then treated with the restriction enzyme DpnI at 37 ºC for 3 hrs to remove template DNA and sequenced to verify the inclusion of the desired substitutions (Macrogen, Korea). OryR was then cloned into pSS122PIP (HindIII/HindIII) and PsoR into pBBRMSC-5 (EcoRI/BamHI). Mutant strains *X. oryzae oryR-* and *P. fluorescens psoR*$^{-}$ (pXoopipprom) were transformed with these plasmids and grown in the presence of a wide range of AHLs AT 1 µM concentration (Table 3-6). Activation of target *X. oryzae pip* promoter was measured by *β*-glucuronidase (*X. oryzae*) or *β*-galactosidase (*P. fluorescens*).

Table 3-6. Composition of AHL cocktail mixes tested in point mutations experiments.

Unsubstituted (C)	Hydroxyl-AHLs (OH)	Oxo-AHLs (Ox)
C4, C6, C7, C8, C10, C12, C14, C16, C18	OHC6, OHC8, OHC10, OHC12, OHC14, OHC16, OHC18	OC6, OC8, OC10, OC12, OC14, OC16, OC18

3.8.3.3 Promoter switching of QS and PAB LuxRs

To evaluate the cross-regulation of promoters by QS-LuxR and PAB-LuxR solos the following experiments were designed:

3.8.3.3.1 QS-LuxR with PAB target promoters

To evaluate the capability of quorum sensing LuxR type proteins to activate PAB target promoters in the presence of AHLs: *E. coli* M15 strains with different QS-LuxRs cloned in the expression vector pQE30 (Table 3-2) were tested for their ability to activate the target *X. oryzae pip* promoter using the reporter plasmid pXoopipprom (Table 3-2) in the presence of their cognate AHL (See Table 3-7 for details). For pQEPagR a 706 fragment containing the *pagR* gene coding for a luxR homologue of *Pantoea agglomerans* was amplified from genomic DNA using the primers BamPagRFw and HindPagRRev and cloned in the expression vector pQE30 cut BamHI-HindIII to give pQEpagR (Table 3-3). The same procedure was followed for PssR, a luxR homologue of *Pseudomonas savastanoi*, using the primers

BamPssRFw and HindPssRRev; the 715 bp fragment was cloned in the expression vector pQE30 cut BamHI-HindIII to give pQEPSSR (Table 3-3).

3.8.3.3.2 PAB-LuxR solos with QS *luxI*-gene promoters

To evaluate the capability of PAB-LuxR solos to activate QS *luxI* gene promoters in the presence of rice-macerate: *P. fluorescens* CHA0 strains with either pBBRPsoR or pBBROryR were tested for their ability to activate different QS-*luxI* gene promoters cloned in pMP220 and promoter activities were registered (See Table 3-7 for details).

Table 3-7. QS-LuxRs and I promoters used in the promoter switching experiments

QS-R protein	Organism	AHLs tested
PssR	*Pseudomonas savastanoi*	OC6, OC8
PagR	*Pantoea agglomerans*	C4, C6
XenR	*Burkordelia xenovorans*	OC14,OC12
UnaR	*Burkordelia unamae*	OC14,OC12
BraR	*Burkordelia brasiliensis*	OC14,OC12
XenR2	*Burkordelia xenovorans*	OHC8, OHC6, OC8
QS-I promoter		
PmeI	*Pseudomonas mediterranea*	-
BgluI	*Burkorderia glumae*	-
Cvi	*Chromobacterium violaceum*	-
PpuI	*Pseudomonas arouginosa* PUPA	-
LasI	*Pseudomonas arouginosa*	-

3.8.3.4 Helix-turn-helix domain expression

To determine if the HTH domain of OryR and PsoR are sufficient to activate target promoters (pXoopipprom) the DNA-binding Helix-turn helix domains of both proteins were cloned into the expression vector pQE30 as follows: For OryR a 219 bp fragment including the HTH domain of OryR was amplified from genomic DNA using the primers OryRHTHF and OryRHTHR and cloned into pGEM. The fragment was then cloned into pQE30 (BamHI/HindIII) and sequenced (Table 3-3). For PsoR a 210 bp fragment was amplified from genomic DNA with the plasmids PsoRHTHF and PsoRHTHR following the same procedure (See Table 3-3 for details). Promoter activities were measured by β-galactosidase assay.

3.9 Phenotypical analyses

3.9.1 Bacterial motility assays

Xoo. Fresh colonies from PSA agar plates were stabbed into motility plates (per liter: peptone 0.3 g, yeast extract 0.3 g, agar 3 g for swimming or 4 g for swarming) and

motility plates supplemented with rice macerate (5%). Plates were incubated at 30 ^{o}C and diameter of movement was measured after 7 days (Shen et al., 2001).

3.9.2 EPS production and quantification

To quantify the EPS production, single colonies were streaked and grown in PSA agar and allowed to grow for 3 to 5 days. Cells were harvested by adding 2 mL of phosphate buffer (pH 7.2) to the plates and gently scraping with a sterile spreader. 0.5% (v/v) formaldehyde was added to the cell suspension and dilutions of bacterial cells were plated out on PSA agar for estimation of cell numbers. EPS was stripped off from bacteria by gentle stirring and bacterial cells were removed by centrifuging at 16000 g. Four volumes of chilled acetone were added to the supernatant and the EPS was left to precipitate at 4°C for 12 hours. The precipitate was recovered by centrifuging at 3000 g and washed at least twice with acetone (Dharmapuri and Sonti, 1999). The pellet was allowed to dry at room temperature and dissolved in 1 ml of sterile water. EPS was estimated by the boiling phenol method (Dubois et al., 1956). 0.4 mL of the EPS sample was mixed with 0.2 mL of 5% (w/v) phenol followed by 1 mL of concentrated sulphuric acid. The mixture was incubated for 10 minutes at room temperature and then at 30°C for 20 minutes. Absorbance was measured at 490 nm against a reagent blank. A calibration curve was constructed using glucose concentration from 10µg to 100µg/mL.

3.9.3 Siderophore production

To evaluate the siderophore production of *P. fluorescens* wild type, PsoR knockout mutant and PsoR over-expressing strains were grown in iron-limiting conditions. Bacterial growth (optical density of 600 nm) was followed in KB media and KB media supplemented the iron chelator 2,2′-dipyridyl (Sigma) at the following concentrations: 1.5, 2.0 and 2.5 mM. All strains were also grown KB media supplemented with 100 mM iron sulfate as control. This assay gives an indication of the different abilities that the strains under study have towards the response to iron-limiting conditions.

3.10 *In planta Xoo* protein samples for proteomic analysis

3.10.1 Rice inoculation

Infection of rice plants with Xoo XKK.12 wilt type and *oryR*$^{-}$ knockout mutant and xylem sap collection was performed as follows: *Xoo* strains were grown in PY liquid

medium at 28°C for two days before being used for inoculation of rice plants. The bacterial concentration was adjusted to 1 x 10^9 CFU/ml with sterile demineralized water prior to inoculation of rice plants. Inoculation was carried out on the Italian rice cultivar Baldo, which showed high sensitivity to *Xoo* XKK.12 infection (Ferluga et al., 2007). Rice was grown and infections performed as previously described (Degrassi et al., 2010). Three to four leaves per plant were inoculated using the clipping method (Kauffman et al., 1973) and water was used as negative control. Inoculated plants were kept for 24 hours in humid chambers (>92% relative humidity) at 30±4 °C, and were then transferred to greenhouse conditions (26-28°C, 65% relative humidity with a photoperiod of 16 hours of light and 8 hours of darkness) for disease development for an additional 13 days. Lesion lengths were measured and infected leaves were used for sampling of xylem sap as follows: infected plants were placed in a humidity chamber, the dried blighted part of the infected leaves were cut 2 cm from the edge of infection. Drops oozing out from the xylem were constantly collected using a 200 µL pipette during the subsequent 4 hours, placed in sterile Eppendorf tubes and centrifuged at 16000 x g at 4°C for 30 minutes to remove bacterial cells. These xylem sap samples (approximately 500 µl for each sample) were used for proteomic analysis via mass spectroscopy.

3.10.2 Mass spectroscopy analysis of xylem sap samples

SDS-PAGE to xylem samples was used as follows: to remove the sugars and other non-protein compounds found in xylem sap; the collected xylem sap was diluted to 1 x Laemmli sample buffer and boiled for 5 minutes and then run onto 4-10% Nupage gel (Invitrogen, Carlsbad, CA, USA). The samples were allowed to run 1 cm into the gel and then the gel was stained with colloidal comassie blue (Pierce). The stained area of the gel was cut into 5 bands and processed for in gel digestion with trypsin using standard procedures. The resulting peptides were cleaned up using STAGE-tips 25. LC-MALDI of the digests was performed using an Applied Biosystems 4800 mass spectrometer coupled with an Ultimate 3000 HPLC via a PROBOT target spotter (Dionex). The peptides were separated using an in house packed column (20cm x 75 um) containing Jupiter proteo resin (Phenomenex, Torrance, CA, USA). The gradient ranged from 5-50% Acetonitrile in 0.1% TFA in 90 minutes. The gradient was developed at 300 nl/min and subsequently mixed with a 3 mg/ml solution of CHCA flowing at 1 µl/minute and spots were acquired every

10 seconds using a PROBOT plate spotter. The top 10 precursor ions from each spot were subjected to MS/MS analysis with 1500 laser shots and a laser intensity of 2600.

MGF files were created using TS2Mascot.exe (MatrixScience, Boston, MA, USA) with the mass range parameter set to a minimum of 60Da and a maximum mass equal to the mass of the precursor. Only monoisotopic peaks with a signal to noise ratio > 10 were included in the final MGF.

The MGF files were searched using the X!tandem search engine (version 2009.04.01.1). Searches were conducted using a precursor mass error of 100 ppm and a fragment mass error of 0.4 Da, complete carbamidomethylation of cysteines, partial oxidation of methionine, and partial deamidation of asparagine and glutamine. All data files were searched against *Xoo* strains: KACC10331 (NC_006834), MAFF_311018 (NC_007705) and PXO99A (NC_010717). The reverse database sequences were concatenated to the forward sequences in order to estimate the false discovery rate. Only proteins with an FDR < 5% were included in the analysis.

3.10.3 Over-expression, purification and antibodies against Protein U.

The gene encoding Protein U was amplified by PCR using genomic DNA as template using the following oligonuceotide primers: pruFw 5' - GGA TCC GCA GAC ACC ACC ACC – 3' and pruRv 5' - AAG CTT TCA ATA GAC GAT CGT – 3'. Primers were designed according to the sequence from the *Xoo* KACC10331 annotated genome (Lee et al., 2005). The resulting PCR product of 471 bp was cloned in the pGEM-T Easy Vector as a BamHI-HindIII fragment, DNA sequenced and then excised as a BamHI-XbaI fragment, and cloned in the corresponding sites of the 6xHis-tagged vector pQE30 (Qiagen, Hilden, D) yielding pQEprU. Expression and purification of 6xHis-tagged Protein U was carried out in *E. coli* M15 (pREP)(pQEprU), according to the instructions of the supplier (Qiagen, Hilden, D). Polyclonal antibody against 6xHis-tagged Protein U was generated by injecting purified proteins into mice. Protein U production in *Xoo* culture supernatants was evaluated by Western blot analysis (Renart et al., 1979) using the 6xHis-tagged antibody. Supernatants from *Xoo* cultures grown in M9CA and PY media with and without the addition of macerated rice were taken at an OD_{600} of 2.0, the total protein precipitated by trichloroacetic acid (TCA), protein concentration measured (BioRad protein assay kit) and approximately 50 μg of protein from each *Xoo* culture loaded

onto an SDS PAGE and blotted onto a PVDF membrane. After incubation with the polyclonal antibody and the secondary horseradish peroxidase-labeled antibody, Protein U was detected with 3,3-diaminobenzidine tetrahydrochloride tablets (Sigma Chemical Co.), according to the instruction of the supplier and as previously described (Degrassi et al., 2010).

3.11 Promoter trapping of PsoR regulated promoters of *P. fluorescens*

The experimental strategy used to identify PsoR–regulated genes was to insert *P. fluorescens* genomic DNA fragments digested with two different restriction enzymes (AluI or Sau3AI*)* within the multiple cloning site of vector pQF50 (SmaI or BamHI). DNA ligations between pQF50 and the genomic restriction DNA fragments were transformed in *E. coli* DH5 (pBBRPsoR) and plated on selective medium containing 100 μg of ampicillin (Sigma-Aldrich)/ml, 20 μg of X-Gal (Sigma-Aldrich)/ml. Blue colonies, indicating promoter activation, were selected. Positive blue colonies were isolated and the pQF50 recovered; this plasmid was then cloned into *E. coli* DH5 (pBBR, empty vector) in order to differentiate between constitutive promoters and PsoR-regulated promoters. The latter were sequenced and *ß*-galactosidase assay was performed to measure promoter activity in the presence PsoR and with an empty pBBR plasmid (See Figure 3-1).

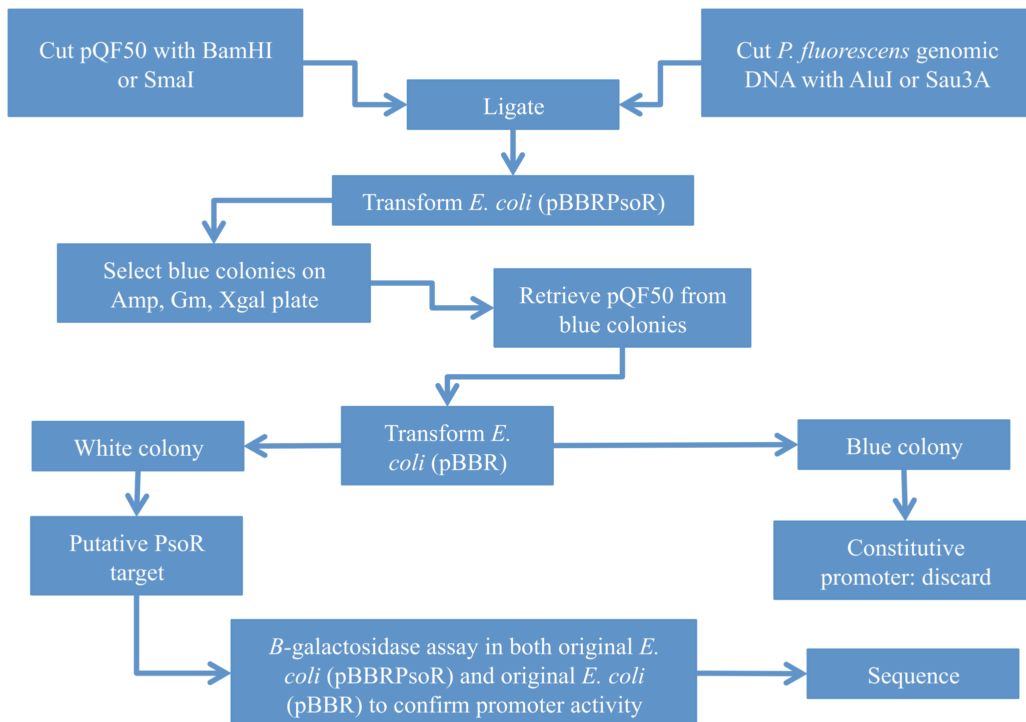

Figure 3-1. Promoter trapping strategy.

3.12 Statistical analysis

All experiments were performed at least three times and means plus standard deviations (SD) are given. In all instances the software Prism 5 for Mac OS X (GraphPad Software, San Diego California) was used for statistical analysis and calculations; including standard deviation (SD), *P*-values, *t*-tests and ANOVA. A *P*-value of < 0.05 was considered significant

4 RESULTS AND DISCUSSION

4.1 *Xanthomonas oryzae* pv. *oryzae* OryR targets

4.1.1 Determination of the OryR regulon by genome-scale transcriptome analysis

It was previously established that the solo OryR responds to an unknown low molecular weight rice molecule, triggering the transcription of the adjacent *pip* gene (Ferluga and Venturi, 2009). It was now of interest to determine if and which other gene(s) were transcriptionally regulated by the OryR/plant molecule complex. In order to establish the growth stage to purify RNA for transcriptome analysis, I used a *Xoo* (pPIP122) reporter strain in which the OryR target pip promoter is fused to a promoterless β-glucuronidase gene in plasmid pPIP122 (Ferluga and Venturi, 2009). Maximum levels of β-glucuronidase activity were observed when *Xoo* was grown in media with the addition of rice macerate (PY + Rice, see Materials and Methods Section 3.1) to an OD_{600} of 2.0 (Figure 4-1). The *Xoo oryR* mutant harboring the *pip* transcriptional fusion (pPIP122) on the other hand remained inactive at all times. Therefore, I concluded that the OryR/plant compound complex maximally activated the *pip* target gene when the bacteria are grown to an OD_{600} of 2.0, in the presence of macerated rice. I used these growth conditions to determine the other OryR gene targets. As can be observed in Figure 4-1, there is a slight variation in growth in the *Xoo* wild type strains between rich PYS media (white boxes) and PYS+rice (black boxes). This phenomenon has been observed in all *Xoo* strains so far tested, probably due to the complex nature of rice macerate, which most likely contains compounds harmful to bacteria. In fact, if larger amounts of macerated rice are added to the media, growth is inhibited altogether.

RNA was purified from three biological replicates of wild type *Xoo* XKK.12 (pPIP122) and *oryR* mutant *Xoo* XKK.12ORYR (pPIP122) cultures grown in PY rice medium at OD_{600} of 2.0; prior to RNA purification β-glucuronidase activity was established in order to confirm activity of OryR. Transcriptome analysis revealed that OryR is a global regulator, influencing the transcription levels of 305 genes by two-fold or more (Figure 4-2), representing 7.5 % of the protein coding genes in *Xoo*.

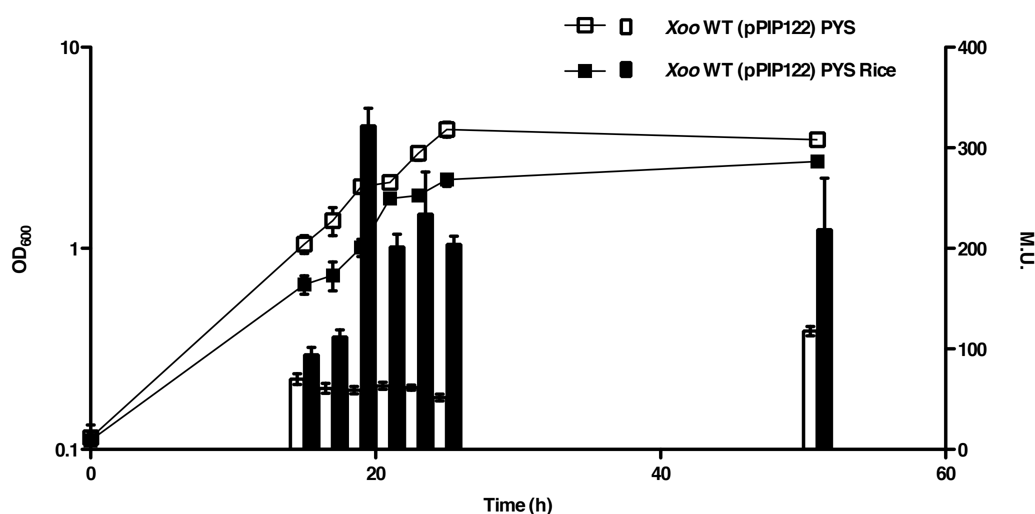

Figure 4-1. Determination of pip-promoter activation by OryR.
***Xoo* was grown in rich PY media (white) or PY media + rice macerate (black). ß-glucoronidase levels (bars) and optical density (boxes) were measured at different time points. Maximum pip induction was found at an optical density of 2.0. M.U., Miller units, OD600 optical density at 600 nm. Values given are the means of three biological replicates; error bars denote standard deviation (SD).**

OryR positively regulated 191 of these genes (Table 4-1), while 114 were down regulated (Table 4-2). It is worth mentioning that these set of genes include both direct and indirect regulation. Eight genes were chosen for validation with semi-quantitative RT-PCR, which gave results comparable to the transcriptomic data (Table 4-3).

4.1.2 Positive regulation by OryR

It was established that 191 genes (the cut off of 2-fold increase in expression was chosen), were positively regulated by OryR (Figure 4-2a, Table 4-1). The largest percentage of positively regulated genes correspond to hypothetical proteins (28%); 31% of *Xoo* KACC10331 ORFs are hypothetical proteins, which is a consistent number for most of the sequenced prokaryotic genomes (Karaoz et al., 2004). A closer analysis of these hypothetical proteins with the STRING tool (Snel et al., 2000) showed that most of these proteins are conserved among bacteria; some of them even have homologs in groups as distant as eukaryotes and archae. Interestingly, four of them, hrpE (XOO0076), XOO0105, XOO2584 and XOO4877 are shared only with its close relative *Xcc* and another four, XOO4700, XOO4725, XOO4755 and XOO4791 are unique to *Xoo*. Cell process and metabolism genes are the second most represented set of genes (25%).

A significant finding was the over-representation of movement-related genes among the positively regulated list; there are 30 flagellar genes (Table 4-1), accounting for 14% of the up-regulated genes above the 2-fold cut-off value (P < 0.01). Considering that in *Xoo* most flagellar genes are found in a single cluster of about 40 ORFs (i.e. from XOO2565 to XOO2621; Lee et al. 2007), this represents a major genetic locus of OryR regulation (Figure 4-3). The OryR/plant molecule positively regulated genes include: flagellum organization and biogenesis genes (GO:0043064): *flgE, flgF, flgG, flgH, flgI, flgJ, flgK, fliC*; motor activity genes (GO:0003774) *flgB, flgC, flgE, flgF, flgG, flgH, flgJ, flgK fliF, fliG, fliJ, fliK, fliM, fliN*; as well as important regulators like *flhF* and *fliA*. The two flagellar genes *motC* (XOO2830) and *motB* (XOO2831), which are found in a chemotaxis cluster downstream of the main flagellar cluster are also regulated by OryR.

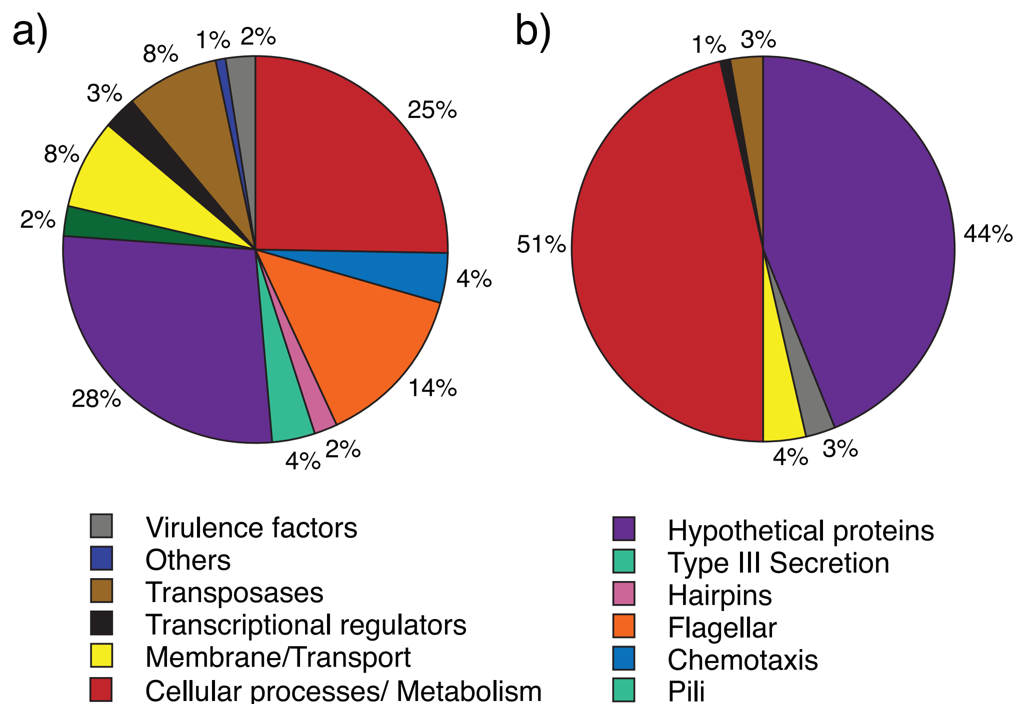

Figure 4-2. OryR transcriptome analysis.
Functional classification of differentially expressed genes found in a transcriptome analysis. a) positively regulated genes. b) negatively regulated genes. Only genes with a fold difference greater than 2 are included.

Additionally, there are 9 chemotaxis-related genes, 6 of which are found in close genomic proximity to the main flagellar cluster and two are found in the above mentioned chemotaxis cluster (XOO2836 and XOO28480) and one in a different chemotaxis cluster further upstream (XOO1468) which is also regulated by OryR. Furthermore, there are 5 pili-related genes, including *pilY1* (XOO3196) and *pilE1*

(XOO3195), which are the two most strongly induced genes. Overall, 22% of all the genes positively regulated by over 2 fold by OryR are motility-related genes, providing a clear indication of the role of this transcriptional regulator.

Table 4-1. OryR positively-regulated genes.
Classification by predicted function of genes positively regulated over 2-fold (Classification was performed with the ArrayStar Software package).

Predicted function	No.	Predicted function	No.
Cellular processes/ Metabolism	**42**	**Hypothetical proteins**	**61**
Chemotaxis		**Pili**	
chemotaxis protein	6	type IV pilin	1
chemotaxis protein methyltransferase	1	pili assembly chaperone	1
chemotaxis related protein	2	PilY1	1
	9		**3**
		Membrane/Transport	
Flagellar		outer membrane protein W	1
flagellar basal body L-ring protein	1	outer membrane hemin receptor	1
flagellar basal body P-ring protein	1	polar amino acid transporter	1
flagellar basal body rod modification protein	1	polyphosphate-selective porin O	1
flagellar basal body rod protein FlgB	1	preprotein translocase subunit SecE	1
flagellar basal body rod protein FlgC	1	putative inner membrane protein translocase component YidC	1
flagellar basal body rod protein FlgF	1	SsrA-binding protein	1
flagellar basal body rod protein FlgG	1	succinate dehydrogenase membrane anchor subunit	1
flagellar biosynthesis protein FlhA	1	sugar transporter	1
flagellar biosynthesis protein FliP	1	TonB-dependent receptor	5
flagellar biosynthesis regulator FlhF	1		**14**
flagellar biosynthesis switch protein	1	**Transcriptional regulators**	
flagellar FliJ protein	1	RNA polymerase sigma factor FliA	1
flagellar hook protein FlgE	1	transcriptional regulator	2
flagellar hook-associated protein FlgK	1	AraC/XylS family transcriptional regulator	1
flagellar hook-associated protein FlgL	1		**4**
flagellar motor protein	1	**Transposases**	
flagellar motor protein MotD	1	IS1404 transposase	2
flagellar motor switch protein FliM	1	IS1479 transposase	2
flagellar MS-ring protein	1	ISxac1 transposase	3
flagellar protein	9	Transposase	8
flagellar rod assembly protein FlgJ	1		**15**
flagellin	1	**Others**	
	30	bacteriophage endolysin protein	1
Hairpins		VirK	1
Hpa1	1		**2**
HpaA	1	**Virulence factors**	
HpaB	1	virulence associated protein	1
	3	virulence regulator	1
Type III Secretion		Cellulase	2
putative type III effector HolPsyAE	1		**4**
HrpD5	1		
HrpD6	1		
HrpG	1		
	4		
Total positively regulated			**191**

Finally, there were 15 up-regulated transposases (8%), this could be due to the high amount of IS elements found so far in sequenced *X. oryzae* genomes, which make up to 10% of total genes, a feature that has been suggested to give the *Xoo* genome a

great plasticity and rapid adaptability (Salzberg et al., 2008) allowing for its great diversity of genomes and pathovars (Leach et al., 1995).

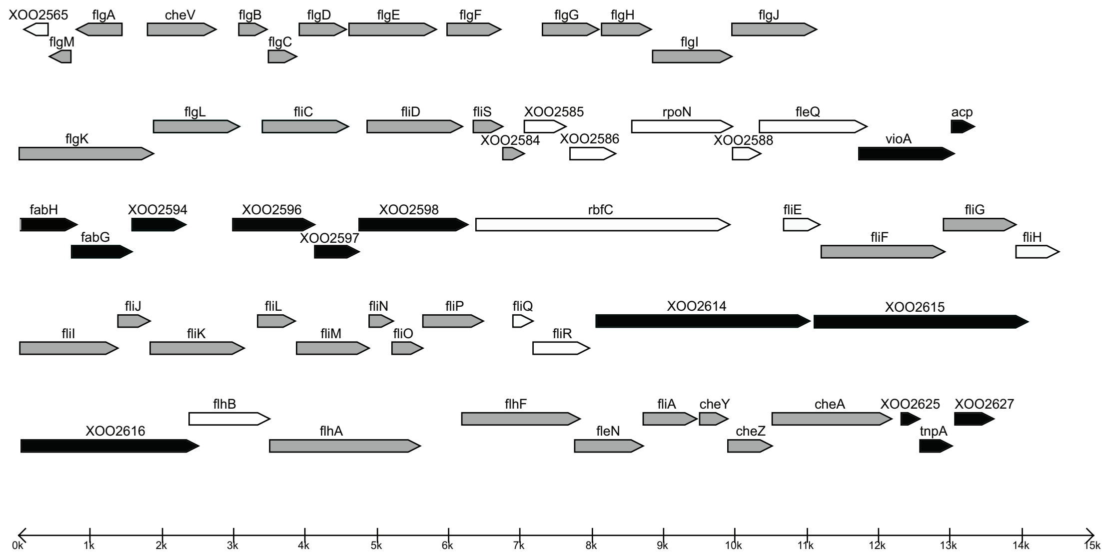

Figure 4-3. *X. oryzae* flagellar cluster.
Grey boxes represent flagellar genes positively regulated by OryR by at least over 2 fold. White genes represent flagellar genes positively regulated less than 2 fold and black boxes represent non-flagellar genes. Image was made using the Microbes on line tool (Dehal et al., 2009).

Transcriptomic studies have revealed the highest number of genes regulated by OryR corresponding to hypothetical proteins; this is not surprising due to the high number of proteins of unknown function found in bacterial genomes that can range between 30 and 40% of the total genome (Karaoz et al., 2004). Of these, 11 are unique to *Xoo* and could be interesting for future research. Importantly however, the positive regulation of a very large set of motility-related genes caught my attention and was further investigated. These include flagellar, chemotactic and pili genes (Table 4-1, Figure 4-3). Flagellar regulation is multi-step and complex; in xanthomonads this regulation is a 3-step variation of the well-studied single polar flagellum. A 4-step regulation system is on the other hand found in vibrios and pseudomonads (Dasgupta et al., 2003). The 3-step regulation system in xanthomonads has been mainly studied in *Xcc* (Lee et al., 2003, Hu et al., 2005, Yang et al., 2009); class I genes include the master regulators RpoN2 (σ^{54}) FleQ, and the repressor FlgM coordinate the expression of class II genes, which include the F-T3SS and basal body hook structure and regulators like FliA (σ^{70}), FleN and FlhF. These then in turn regulate the class III genes, which include motor and chemotactic proteins, flagellin and the flagellar cap protein (see Table 1-1). Interestingly, the OryR-positively regulated genes identified

here do not include the class I master regulators RpoN2 and FleQ, which are known to be expressed constitutively (Claret and Hughes, 2000). Nonetheless, OryR-positively regulated genes included T3SS, basal body-hook, motor proteins, flagellar cap and flagellin (*fliC*) itself, representing a very significant percentage of the OryR regulon.

4.1.3 Negative regulation by OryR

114 genes were down-regulated over the selected 2-fold threshold (Figure 4-2b, Table 4-2). The highest percentage (51%) corresponds to various cellular processes and metabolism genes. Again, here hypothetical proteins are highly represented, with 44% of the total genes down regulated above 2-fold. Of these XOO0696, XOO0697 and XOO4439 are shared only with *Xcc* and 7 hypothetical proteins are unique to *Xoo*; XOO1454, XOO1701, XOO1880, XOO4713, XOO4739, XOO4758 and XOO4884. Interestingly, there are some previously studied virulence factors found in the negatively regulated genes. These include two cellulases (XOO0281 and XOO4019) and a peptidase (XOO1259).

Table 4-2. OryR negatively-regulated genes. Classification by predicted function of genes positively regulated over 2-fold (Classification was performed with the ArrayStar Software package).

Predicted function	No.
Hypothetical protein	**49**
Virulence factors	
protease	1
cellulase	2
	3
Membrane/Transport	
RhsD protein	1
RhsD protein precursor	1
outer membrane protein	1
TonB-dependent receptor	1
	4
Cellular processes/ Metabolism	**54**
Transposases	
putative transposase	1
transposase	1
IS1479 transposase	1
	3
Total negatively-regulated	**114**

Table 4-3. Validation by SQ RT-PCR of various differentially expressed genes.

Gene ID	Gene name	Fold difference	
		Microarray	SQ RT-PCR*
Xoo3196	PilY1	32.56	13.6 ± 3.73
Xoo0329	Polyvinylalcohol dehydrogenase	25.83	40.7 ± 8.60
Xoo0031	Hypothetical protein	10.18	2.26 ± 0.03
Xoo2580	Flagellar hook-associated protein flgL	5.70	2.46 ± 0.01
Xoo2619	FlhF	2.67	3.67 ± 0.06
Xoo1701	Hypothetical protein	-34.75	-40.5 ± 2.54
Xoo1580	RhsD protein	-5.02	-6.84 ± 0.02
Xoo0281	Cellulase	-4.29	-1.78 ± 0.30

* ± denotes standard deviation (SD).

4.1.4 OryR is important for swimming and swarming in a rice-dependant manner

Following the result from the transcriptome analysis (see above), it was of interest to determine the possible role of OryR in motility of *Xoo*; the ability of *Xoo* wild type and *oryR* knockout mutant to swim and swarm was therefore assayed. Swim plates indicated that both the wild type and the *oryR* knock out mutant were motile in swimming media and displayed the typical circular dissemination pattern from the point of inoculation (Figure 4-4 d and e, Table 4-4). Both strains showed a slow spread through the plates reaching a diameter of about 20 mm in 4 days. Interestingly, when macerated rice was added to swimming media, there was a dramatic increase in swimming by the wild-type strain, reaching in 4 days a diameter of about 35 mm, i.e. almost a 40% increase compared to media lacking rice macerate (Figure 4-4 a and b). The importance of OryR in swimming was observed when macerated rice is included in the media as *oryR* mutants swam significantly less ($P < 0.05$) (Figure 4-4b, Table 2).

Swarming motility assays show a similar trend. In this case however, neither the wild type nor the mutant showed swarming motility on swarming media after 14 days (Figure 4-5d, e and f, Table 4-4). Swarming was however observed when media was supplemented with rice macerate (Figure 4-5a) being significantly reduced ($P < 0.05$) in the *oryR*-knockout mutant (Figure 4-5b). This clearly indicated that under the conditions I tested swarming was dependent on the presence of compound(s) present in rice macerate and that OryR played a role in this response. I complemented the *oryR* mutant by introducing the *oryR* gene in a high copy plasmid (pBBROryR) and a low copy plasmid (pUFROryR) and performed the swimming and swarming experiments under the same conditions. The presence of the *oryR* gene *in trans* in a plasmid did not restore the swimming and swarming phenotype to wild-type levels with either plasmid (Figure 4-4c and f, Figure 4-5c and f, Table 4-4); in fact it further

decreased the ability of the mutant to swim and swarm (pUFROryR complementation not shown). This most likely indicated that over-expression of *oryR* in a multicopy plasmid had adverse effects on the regulation of gene(s) necessary for swimming and swarming.

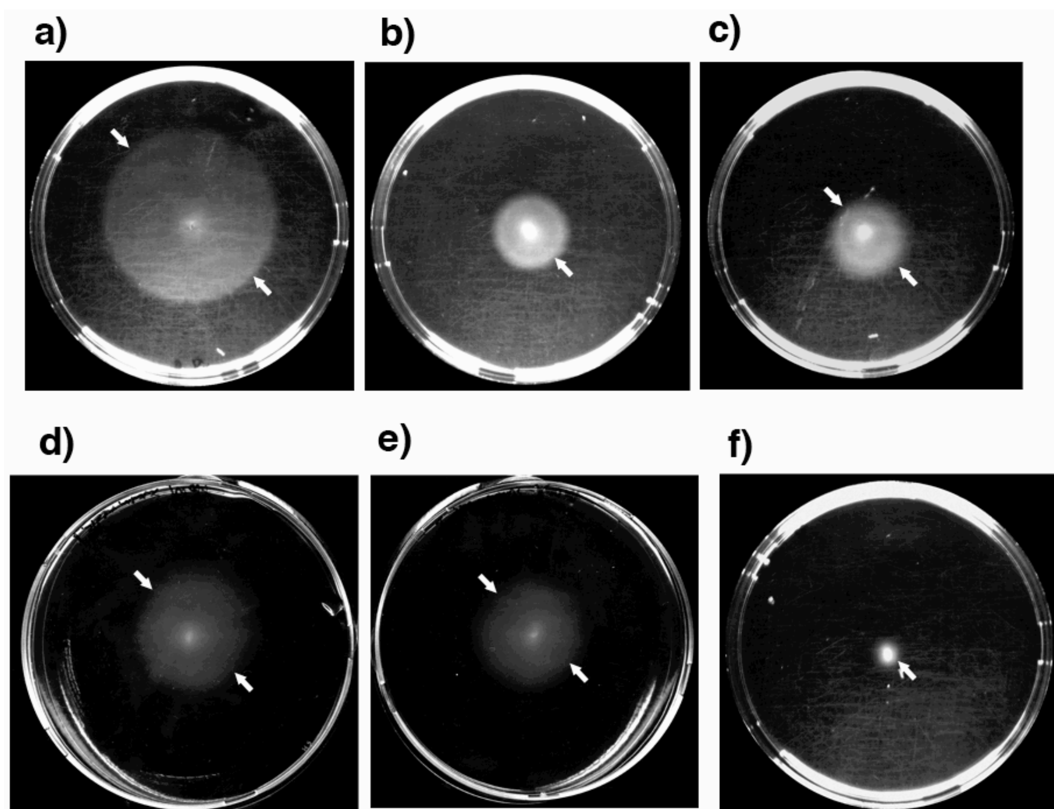

Figure 4-4 *Xoo* swimming assay.
Swimming motility in 0.3% agar with the addition of rice macerate (a,b and c) or without (d, e and f). A significant increase in swimming is observed for the wild type when macerated rice is added to the media (a); this increase is not observed in the oryR mutant (b); complementation in high copy plasmid pBBROryR does not fully recover the phenotype (c). No difference is observed between wild type (d) and oryR mutant (e) in regular 0.3% agar swimming media while swimming motility is greatly reduces by over-expression of OryR (f) complemented strain. White arrows indicate the edge of the colony.

In summary, these experiments have indicated that both swimming and swarming respond to rice macerate in *Xoo* under the conditions I tested and that OryR played a role in inducing both of these types of motility in the presence of rice macerate.

It is believed that OryR acts predominantly in the presence of a rice-signal molecule(s) thus its role needs to be studied in media supplemented with rice macerate or directly *in planta*. Furthermore, a recent study in *Xcc* has shown the presence of a repressor, termed XerR, that inhibits XccR in culture media but this repression is relieved *in planta* (Wang et al., 2011). This coincides with what I

observed in analyzing swimming and swarming phenotypes in solid growth media (Figure 4-4 and 3-5); i.e. *Xoo* requires rice macerate in the media in order to swim and swarm.

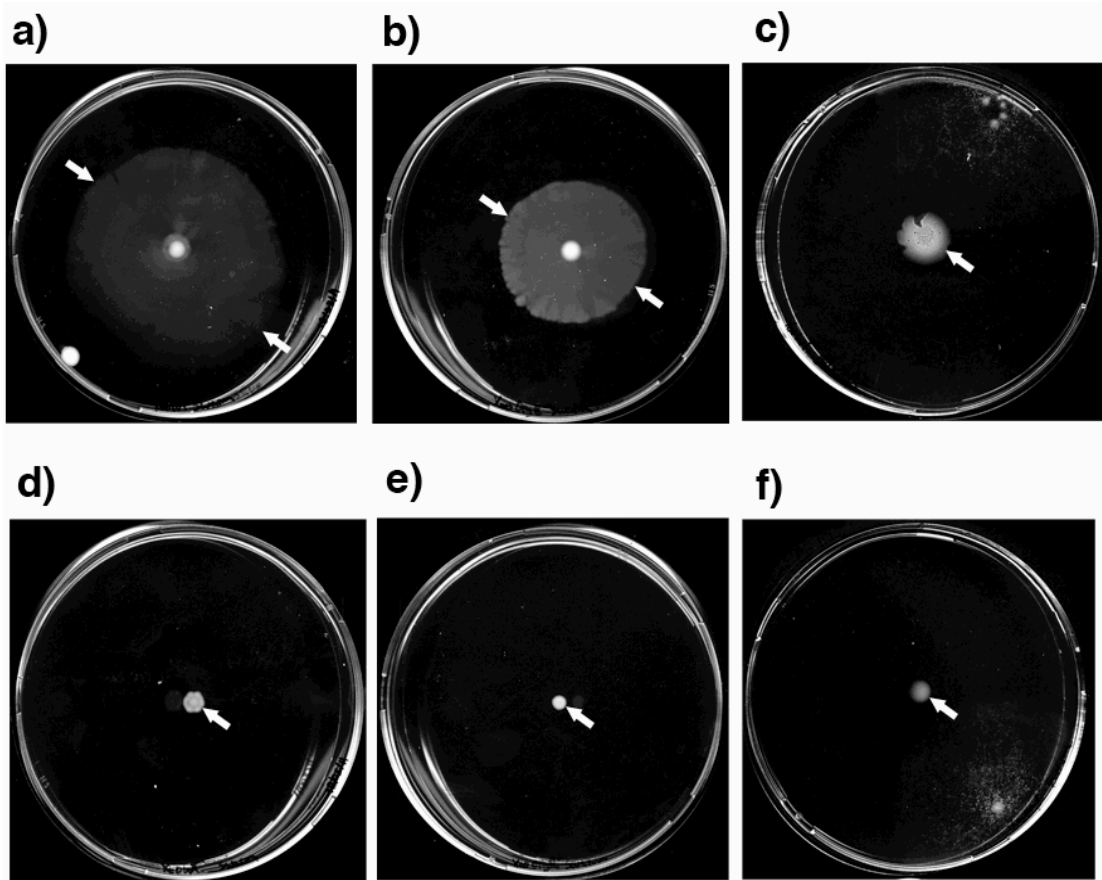

Figure 4-5 *Xoo* swarming assay.
Swarming motility in 0.4% agar with the addition of rice macerate (a,b and c) or without (d, e and f). Swarming is greatly enhanced in *Xoo* wild type when rice is added to the media (a); the effect is less evident in the *oryR* knockout mutant (b); over-expression of *oryR* in pBBR doesn't recover the phenotype. No swarming motility is observed for in any strain in normal swarming media d) wild type, e) *oryR*⁻ or f) complemented strain. White arrows indicate the edge of the colony.

The regulation of flagella by OryR in the presence of rice macerate most likely plays an important role in the observation that swimming and swarming are affected in the *oryR* mutant. Since flagella are known to be important for virulence (Josenhans and Suerbaum, 2002), this could also explain previous observations of a decreased virulence in the *oryR* mutant (Ferluga et al., 2007).

Table 4-4. *Xoo* swimming and swarming motilities.
Wild type and *oryR*⁻ knockout mutant strains in regular motility media vs. media supplemented with rice macerate.

Strain	Media	*Xoo* WT	*Xoo oryR-*	*Xoo oryR-* (pBBROryR)
Swimming (mm)	SM	22.0 ± 0.7	17.8 ± 2.0	8.7 ± 1.2
	SM + Rice	34.6 ± 5.6*	27.0 ± 6.3*	12.7 ± 0.6
Swarming (mm)	SM	6.3 ± 0.3	3.7 ± 0.3	5.1 ± 0.2
	SM + Rice	65.7 ± 4.0†	31.3 ± 9.6†	13.3 ± 1.5

* and † denote a significant difference ($P < 0.05$) between values with the same symbol, ± denotes standard deviation (SD).

4.1.5 Exopolysaccharide quantification

Decreases in motility can be due to reduced exopolysaccharide production and since xanthomonads are known to produce large amounts of EPS (Subramoni et al., 2006); their levels were measured in both the wild type and OryR knockout mutant, no significant difference was observed (Table 4-5). This is in accordance to micro-array data that did not show a significant fold difference in expression of the EPS *gum* genes.

Table 4-5. *Xoo* exopolysaccharide production.

Strain	EPS (μg/10^8 cells)
Xoo wild type	7.928 ± 0.68
Xoo oryR::Km	7.518 ± 0.79
Xoo oryR::Km (pBBROryR)	8.178 ± 3.64

SD: Standard deviation

4.1.6 OryR activates the *flhF* flagellar regulator

A closer look at the flagellar genetic region of *Xoo* revealed a lux-box-like element in the gene promoter of the FlhF flagellar regulator (XOO2619); this was highly homologous to the lux-box like sequence present in the OryR-regulated *pip* promoter (Figure 4-6a). The FhlF regulator has been known to be important for motility as knock out mutants show considerable decreased motility in *Xoo* (Shen et al., 2001). This regulator has been reported to have an RpoN/FleQ-dependant promoter and to be under the regulation of these master regulators in *Xcc* (Hu et al., 2005, Yang et al., 2009). The presence of a lux-box-like element in the promoter region of *Xoo* and the regulation of this gene as detected in the micro-array experiment led us to believe that OryR plays a role in *flhF* regulation. In order to test if OryR regulates this flagellar regulator, its promoter region was cloned in the pSS122 reporter plasmid (Ferluga and Venturi, 2009) and introduced into XKK12 wild type and the derivative *oyrR* knockout mutant. ß-glucuronidase activity was monitored at different optical densities in both PY and PY with rice leaf macerate added. This experiment

indicated that this regulator has very low activity in rich PY media for both strains but when the media was supplemented with rice macerate, activity increases to a maximum of 3 fold (Figure 4-6b) at an OD of 2.0. More importantly, this increase in gene promoter activity is OryR dependant since it remains at baseline levels in the OryR knockout mutant.

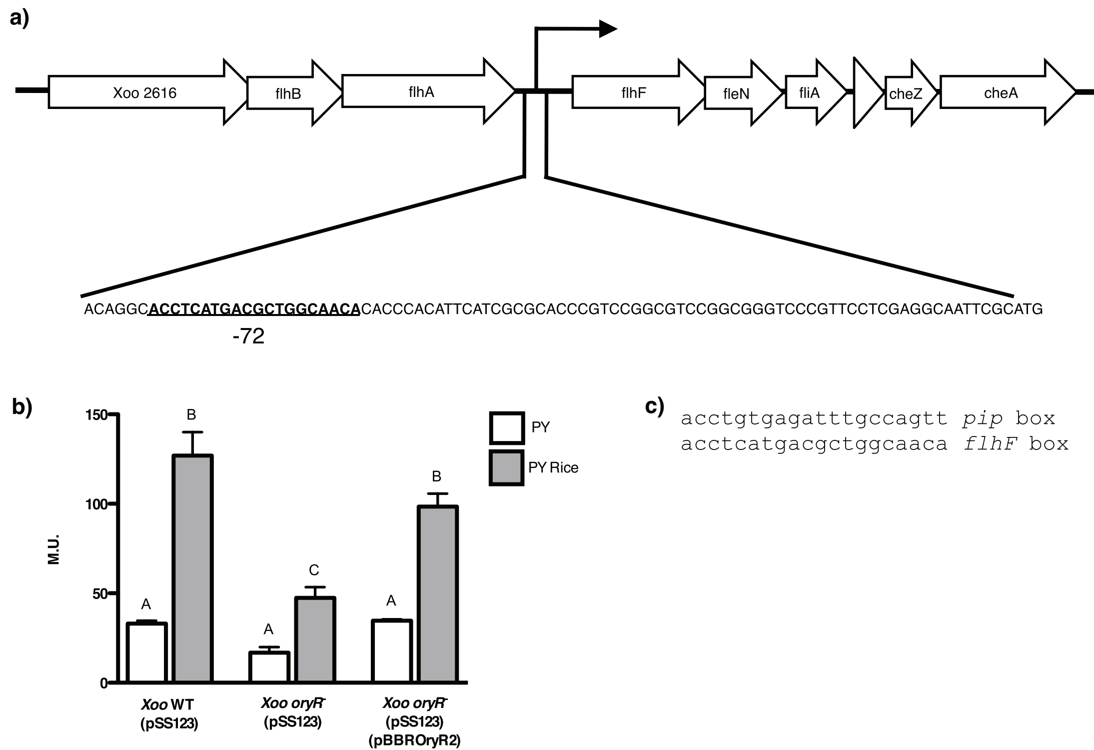

Figure 4-6. *Xoo* FlhF flagellar regulator
a) Promoter region of the *flhF* gene with a putative *lux* box-like element (bold) centered around position -72 from the transcriptional start site. b) ß-glucuronidase assay of the *X. oryzae* flhF promoter. *Xoo* wild type, *oryR* insertion mutant and *oryR* complemented mutant; grown in PYS and PYS+rice. M.U. Miller units. Means with different letters are significantly different ($P < 0.05$; *t*-test). c) Alignment between *Xoo pip* and *flhF* boxes.

The lux-box like element was found at position -72 upstream of the *flhF* flagellar regulator. It was found that this promoter had very little activity in rich media, but showed a sharp increase in activity in media supplemented with rice plant macerate. Importantly, this increase was shown to be OryR-dependent (Figure 4-6b). In *Xoo,* FlhF was identified in a yeast two-hybrid screen of a genomic library and was found to be important but not absolutely required for motility (Shen et al., 2001); these data are in accordance with these results.

4.1.7 Analysis of *in vivo Xoo* expressed proteins

Xoo initially enters and spreads along the rice leaf through the vascular system. In order to gain insight into the *in planta* expressed movement-related secreted proteins,

infected vascular xylem sap was collected as described in the Materials and Methods section 3.10. These samples were then submitted to proteomics analysis and this revealed a set of *Xoo* proteins that were expressed during the infection. This methodology therefore allows the identification of proteins expressed by *Xoo in planta* and also permits the quantification of their relative abundance. The PAI (the protein abundance index) is a parameter developed to obtain approximate quantitative information using the number of peptides per protein normalized by the theoretical number of peptides (Ishihama et al., 2005); the higher the PAI number the more abundant the protein in the sample. This approach was used to further verify/validate micro-array data obtained in this study with a particular attention to secreted and flagellar-related systems. *Xoo* wild type and *oryR*- mutant infected xylem saps were collected from 14 day infected rice; the bacteria were removed by centrifugation and the remaining proteins and subjected to proteomics analysis. In the wild-type infected vascular sap samples, identified proteins included various well-studied virulence factors such as 1,4-beta-cellobiosidase (YP_202674), cysteine protease (YP_200126) (Furutani et al., 2004a), chorismate mutase (YP_199373) (Degrassi et al., 2010), a lipase/esterase (YP_202009) (Rajeshwari et al., 2005), a cellulase (YP_198920) (Furutani et al., 2004a, Sun et al., 2005), among others (See Appendix 7-11 for the full lists of proteins). Interestingly, flagellin (YP_201220) was the second most abundant protein in the list of proteins found in the wild type sample with a PAI of 2970.3 (Unique/Mr* 10000). Flagellin on the other hand was found at position 50, with a 4 fold decrease in relative abundance in xylem sap from *oryR* mutant infected plants. As most of the other proteins in the list exhibited similar abundances between wild type and OryR mutant this is most likely the result of the regulation of flagellin by OryR, rather than differential growth rates between the wild type and *oryR* mutant strains.

4.2 *Pseudomonas fluorescens* PsoR targets

The plant beneficial soil bacterium *Pseudomonas fluorescens* has, in its genome, a gene coding for QS LuxR type proteins (PFL_5298). This protein termed PsoR is part of the family of transcriptional regulators found in plant-associated bacteria, which respond to plant compounds (Figure 1-6). The *psoR* gene is flanked by *pepQ* (PFL_5299; coding for creatinase) upstream and by *pip* (PFL_5297; coding for proline imino peptidase) downstream. Both *psoR* and the *pip* gene are transcribed

from the complementary strand of genomic DNA. The role of PsoR in the regulation of the biocontrol agents 2,4-DAPG and chitinase, and its solubility in the presence of wheat and rice macerates, have been described by Subramoni et al (2011), who also found several targets through the use of genome-wide transcriptome analysis. Interestingly, PsoR regulation seems to be plant-specific in *P. fluorescens.* In the following section more validation of additional findings of the microarray experiment were performed, with respect to iron acquisitionas well as looking for additional targets of positive regulation through promoter trapping in the heterologous *E. coli* system.

4.2.1 Iron acquisition

Microarray analysis between *P. fluorescens* wild type and a PsoR over-expressing strain indicated a negative regulation of iron-acquisition related genes (Table 4-6) by PsoR in (Subramoni et al., 2011).

To corroborate if iron acquisition is PsoR-dependant in *P. fluorescens*; wild type, *psoR* mutant and PsoR overexpressing strains were grown in KB media in presence of the iron chelator 2,2′-dipyridyl;, results indicate that PsoR appears to be a repressor of iron acquisition genes. The *psoR* mutant strain showed an improved growth capacity in various concentrations of 2,2′-dipyridyl with respect to the wild type. Additionally, the PsoR over-expressing strain displayed a greatly reduced capacity to grow in iron-limiting conditions of KB media, which was further increased by the addition of the chelator (Figure 4-7a). These effects were not due to growth defects or antibiotic-related growth decreases as all three strains grew uniformly when KB was supplemented with 100 mM $FeSO_4$ (Figure 4-7b).

Table 4-6. Differential expression of iron-transport related genes in *P. fluorescens*.

Locus	Function	Fold diff.	R value
PFL5169	Iron-regulated outer membrane virulence protein	-1.52	3.06
PFL5964	Iron ABC transporter, periplasmic iron-binding protein	-1.42	2.69
PFL0225	Ferric siderophore transporter, periplasmic energy transduction protein TonB	-2.15	3.95

Pseudomonads are known for producing siderophores with very high affinity for iron (Haas and Defago, 2005), this feature gives them advantage in the competition to colonize the rhizosphere as Fe^{3+} is considered a limiting factor due to its reduced availability at neutral pH in the presence of oxygen (Loper and Buyer, 1991). This has been proposed as one of the ways in which this group of bacteria reduces or

prevents the growth of potential plant pathogens in this niche (Haas and Defago, 2005, Haas and Keel, 2003). Additionally, iron is important for attachment and colonization of host organisms in *P. fluorescens* (O'Toole and Kolter, 1998, Ratledge and Dover, 2000), an important trait for biocontrol strains, as *P. fluorescens* is most effective as an antagonist to plant-pathogens when attached to plant roots (Haas and Defago, 2005). Given the importance that iron has on plant-bacterial interactions it is highly likely that its acquisition is tightly regulated and the solo PsoR could play an important role, indicating through the increasing concentration of PSM near the plant the correct time to repress siderophore production.

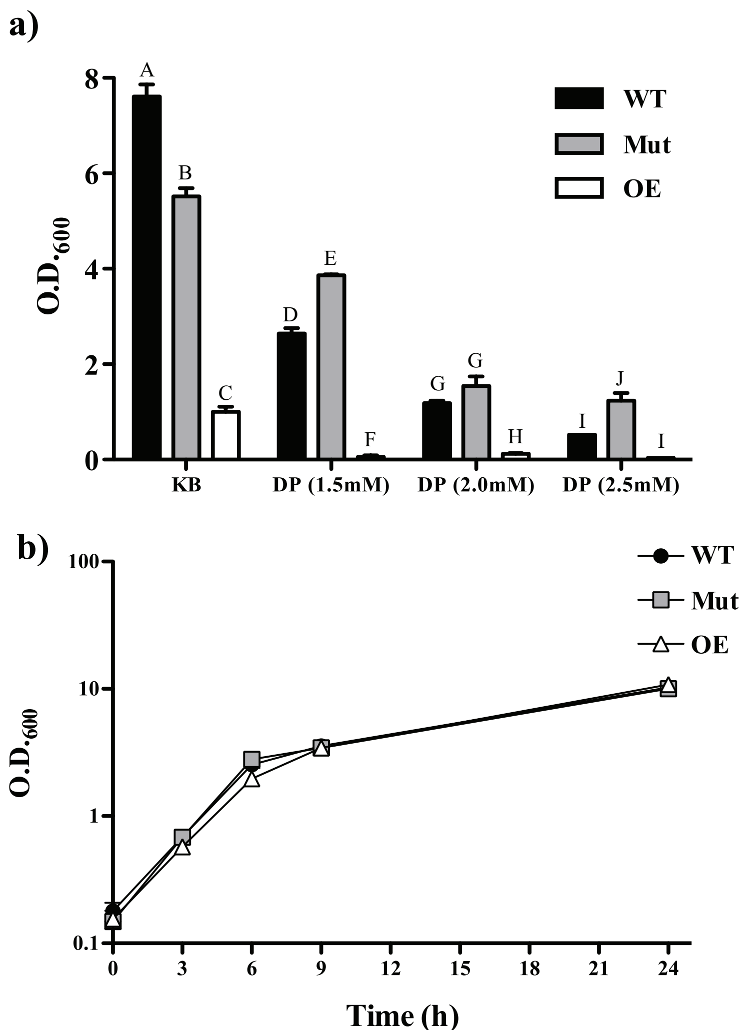

Figure 4-7. Effect of PsoR on siderophore production.
a) Optical densities reached after 24 hrs of growth by *P. fluorescens* wild type (WT), *oryR*::Km mutant (Mut) and *oryR*::Km (pBBRPsoR) complemented strain (OE) in different concentrations of iron chelator 2,2′-dipyridyl (DP) bars with different symbols are significantly different ($P < 0.05$; 2-way ANOVA). b) Growth of the same three strains in KB media supplemented with 100 mM iron sulfate. All values given are means from three independent replicates; error bars denote standard deviation (SD).

4.2.2 Promoter trapping

It was of interest to identify gene targets of the OryR-homolog of *P. fluorescens* called PsoR. In order to isolate gene promoters undergoing positive PsoR regulation, a promoter trapping method was set up in which *SauI* and *AluI* cut fragments of *P. fluorescens* genomic DNA were ligated in the promoterless pQF50 plasmid harboring the *psoR* gene in a heterologuous *E. coli* system as described in the Materials and Methods section (Figure 3-1). A screening of over 25,000 *E. coli* colonies harboring recombinant plasmids yielded 114 blue colonies, indicating putative targets of PsoR activation or constitutive promoters. Of these 114, only 8 were not constitutive promoters, since they produced white colonies, i.e. lacking transcriptional activity, in the absence of PsoR. Promoter activities for these 8 constructs were measured using the *ß*-galactosidase assay to measure the promoter activity of the putative promoter (in the plasmid pQF50) and comparing it to the *E. coli* strain harboring the empty pBBR plasmid, as control (Figure 4-8).

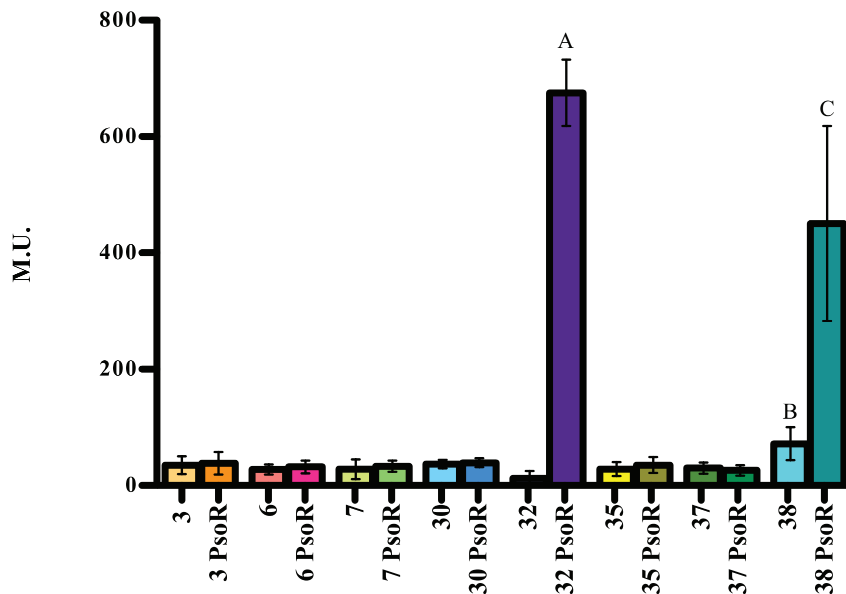

Figure 4-8. Promoter trapping *P. fluorescens*.
ß-galactosidase assay of positive colonies, number indicates the colony number (with the empty pBBR plasmid) and PsoR indicates the same colony with the presence of a plasmid-Bourne copy of PsoR (pBBRPsoR). Values given are means from three independent replicates, error bars denote standard deviation (SD); columns with different symbols are significantly different (P<0.05, one way ANOVA).

Only two clones, numbers 32 and 38, were found to show a high promoter activity in the presence of PsoR. Number 32 corresponds to large adhesive protein (*lapA*) in the locus PFL_0133 and colony 38 corresponds to a sensory box histidine

kinase/response regulator in the locus PFL_4057. Unfortunately, both of these sequences are within the annotated ORFs but since they have very strong promoter activities and *lux* box-like elements it's possible to assume a mistake in genome annotation (See Appendix 7-12).

4.3 Commonalities and differences between QS-LuxR and PAB-LuxR proteins

4.3.1 Promoter switching

In order to evaluate the degree of conservation between QS-LuxRs and PAB-LuxRs, gene promoter-switching experiments were performed; in the first experiment, the ability of QS-LuxR to activate the *Xoo* Pip promoter *pip* box (target of both OryR and PsoR) was evaluated.

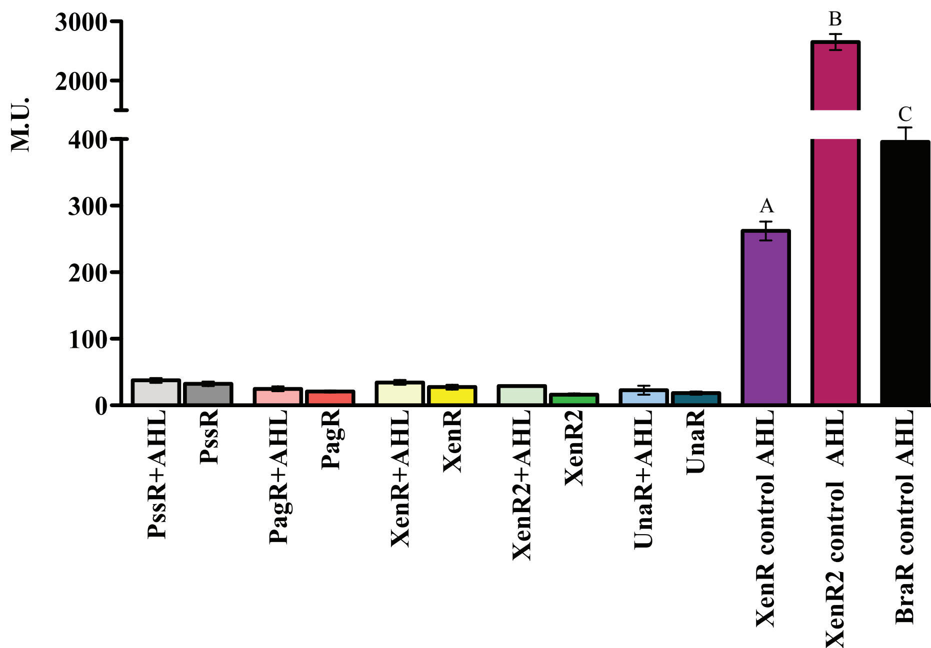

Figure 4-9. PAB promoter switching.
Activation of PAB *Xoo* PIP promoter by QS LuxR proteins in presence or absence of their cognate AHL. The last 3 columns represent the activation of the target *luxI* gene by the cognate LuxR and were used as positive controls. QS-LuxRs: PssR of *Pseudomonas savastanoi*, PagR of *P. agglomerans*, XenR and XenR2 of *B. xenovorans*, BraR of *B. brasiliensis* and UnaR of *B. unamae.* Values given are means from three independent replicates, error bars denote standard deviation (SD); columns with different symbols are significantly different (P<0.05, one way ANOVA), columns without symbols are not significantly different.

None of the QS-LuxR proteins that I tested, i.e. PssR, PagR, XenR, XenR2 and UnaR, were able to activate the *Xoo* Pip promoter (in pMP220) in an heterologous *E. coli* system either with or without their cognate AHL (See Materials and Methods for

experimental procedures) as is shown in Figure 4-9, while the positive controls showed high activities or their target *luxI* gene promoters (last 3 columns). These are LuxRs that respond to AHLs from various bacteria including *P. savastoanoi, Pantoea agglomerans* and *Burkholderia xenovorans*.

The second experiment evaluated the ability of PAB-LuxRs to activate the target *luxI* gene promoters of QS-LuxRs. In this case *luxI* promoters were cloned in the reporter plasmids pMP220 and the PAB-solos OryR and PsoR in the expression plasmid pQE30 and both plasmids were harbored in *E. coli*. *ß*-galactosidase activities were measured in *E. coli* M15 system after growth in LB media supplemented with rice-leaf macerate. The only *luxI* promoter that was significantly activated by both OryR and PsoR was the *lasI* promoter of *P. aeruginosa* (Figure 4-10). These results are significant, since *lux* boxes tend to be well conserved and PAB *pip* boxes are fairly similar to QS luxboxes (Figure 1-7). Mutational studies have shown that *lux* boxes have certain plasticity, most single substitutions had little effect on promoter activity. Notably, there are certain nucleotides, 3 to 5 and 16 to 18, which are very important because the make direct contact with the LuxR-type protein (Antunes et al., 2008, White and Winans, 2007). Furthermore, LasR *lux* boxes show little overall sequence conservation and no need for dyad symmetry (Schuster et al., 2004). Nonetheless, none of the QS LuxRs tested activated the PAB promoters (Figure 4-9), this could mean that these proteins are very specific to their own *pip* boxes. It therefore appears that event though there is some plasticity and similarity among *lux* boxes (i.e. the PAB *pip* boxes with the QS *lux* boxes; Figure 1-7), there is still a high degree of specificity as for example shown for the *lux* boxes of LasR and QscR from *P.aeruginosa,* the binding sites of these regulators shared 15 out of 20 bp but binding to the target gene was not interchangeable (Lee et al., 2006). Interestingly, the LasR target promoter, from the *lasI* gene, was activated by both OryR and PsoR (Figure 4-10), which could indicate not only a relaxed specificity by LasR but also by a high specificity of these *lux* boxes towards LuxR-type transcriptional regulators. It is important to mention that the results from first experiment testing the ability of QS LuxRs to activate promoter activity of PAB *pip* boxes, should be taken with caution due to the difficulties generally observed when analyzing *X. oryzae* gene promoters in heterologous *E. coli* systems.

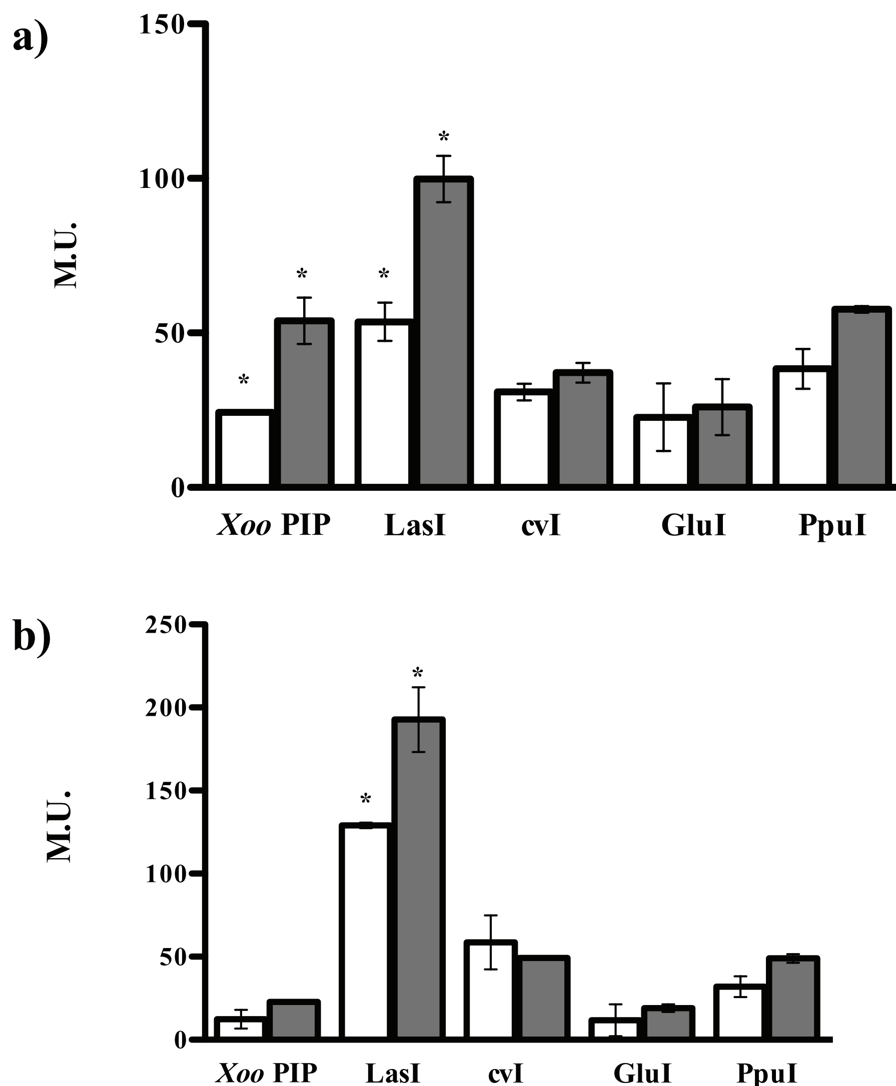

Figure 4-10. QS promoter switching.
Activation of QS LasI promoters by (a) OryR of *X. oryzae* and (b) PsoR of *P. fluorescens*. ☐ denotes LB meda and ■ LB supplemented with rice-leaf macerate in the heterologous *E. coli* system. *E. coli* M15 (pQEOryR) and (pQEPsoR) respectively. Values given are means from three independent replicates, error bars denote standard deviation (SD); * indicates a significantly different at P < 0.05 (*t*-test) compared with unsuplemented media values from the same experiment.

4.3.2 Point mutations

With the aim of testing whether it is possible make OryR and PsoR able to bind AHLs, point mutations that re-established the highly conserved amino acid residues typical of QS-LuxRs (Figure 1-4) were performed. When compared to QS LuxR family proteins, OryR and PosR were not absolutely conserved in the 6-key amino acids in their AHL-binding domain. For this reason point mutagenesis was

performed (i) in OryR M67W and W71Y: the methionine at positions 67 (corresponding to position 57 in TraR) was changed to a tryptophan and the tryptophan at position 71 (corresponding to position 61 in TraR) was substituted with a tyrosine. (ii) In PsoR W71Y: the tryptophan at position 66 (corresponding to position 61 in TraR) was substituted with a tyrosine (See Appendix 7-5).

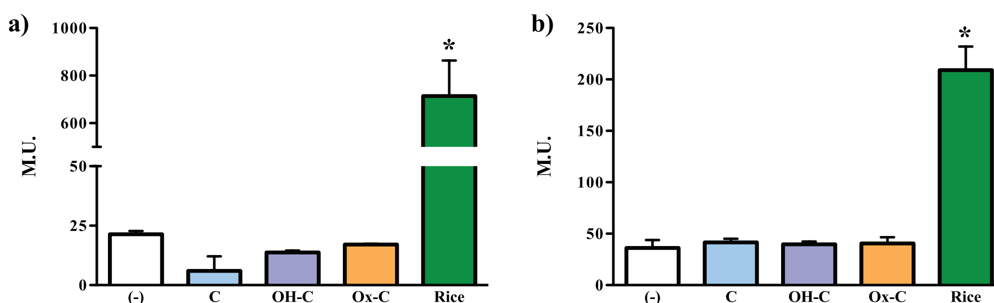

Figure 4-11. Point mutation experiments.
(a) **OryR: *Xoo oryR*⁻ (pPIP122pnt); the *oryR* gene, with point mutations restoring consensus amino acid sequence, was introduced into the reporter pPIP122, and cloned into an *oryR* mutant strain (b) PsoR: *P. fluorescens psoR*⁻ (pBBRPsoRpnt) (pXoopipprom); a plasmid-born copy of the *psoR* gene (pBBRpsoRpnt), with point mutations restoring consensus amino acid sequence, was cloned into *psoR* mutant strain with the reporter pXoopipprom. (-) LB media with solvent ethyl acetate, LB media supplemented with a cocktail mix of (C) unsubstituted AHL, (OH-C) hydroxy-AHLs, (Ox-C) oxy-AHLs. Values given are means from three independent replicates, error bars denote standard deviation (SD). * indicates a significant different ($P<0.05$, one way ANOVA), columns without symbols are not significantly different.**

These point mutations were unable to allow AHL-binding capacities to neither of the PAB-LuxRs tested as can be observed in Figure 4-11, reaching only basal activities when compared to the positive control and in the presence of RSM (rice signal molecule). This result is not entirely surprising, as was mentioned above, there are 9 highly conserved amino acids among QS-LuxR proteins, but there are other very important residues, among them the hydrophobic region that constitutes the AHL-binding pocket and the surrounding residues which also contribute to the stability of the pocket and the interactions with the signal molecule (Whitehead et al., 2001, Zhang et al., 2002).

4.3.3 Helix-turn-helix domain expression

It was previously shown that the C-terminus of LuxR from *V. fisheri* alone could activate transcription of target promoters (Choi and Greenberg, 1991). In order to find commonalities between QS-LuxRs and PAB-LuxRs, the helix-turn-helix domains of OryR and PsoR were expressed heterologously in *E. coli* M15 and their

ability to activate the target *Xoo pip* box was measured through a *ß*-glucuronidase assay.

Table 4-7. Activation of *pip* boxes by helix-turn-helix domains.

Strain	Avg M.U.	SD
E. coli M15 (pMPXoopip)	33.3	3.4
E. coli M15 (pMPXoopip) (pQEOryRHTH)	37.8	6.1
E. coli M15 (pMPXoopip)	26.6	5.5
E. coli M15 (pMPXoopip) (pQEPsoRHTH)	28.0	2.8

Avg M.U.:Average activity (Miller Units) of 3 independent experiments; SD: standard deviation

Neither the helix-turn-helix domain alone of OryR or PsoR could activate the target promoter (Table 4-7). A model for LuxR binding of DNA is that once the N-terminus binds the ligand it induces a conformational change that leads to the exposure of the DNA-binding site of the C-terminus (Nasser and Reverchon, 2007). The results of my experiments could indicate a different mechanism in PAB-LuxRs, in which both domains are needed in order to bind DNA. On the other hand, this could again reflect the above-mentioned incompatibility of *Xoo* promoters when expressed in heterologous *E. coli* systems.

4.4 *In planta* proteomics as a method for studying pathogen expression

4.4.1 Proteomics of *in planta* expressed *Xoo* proteins.

Infection of rice leaves with *Xoo* occurs along the central veins and typical bacterial leaf blight symptoms (Figure 4-12a) are visible after a few days after infection (Swings et al., 1990b). Rice plants were infected and xylem sap, which contained considerable levels of *Xoo*, was isolated from the leading edge of the lesion in rice leaves (Figure 4-12b). Three samples from three independent infection experiments were collected and analyzed by mass spectroscopy with the purpose of identifying secreted proteins expressed *in planta* by *Xoo*. In order to eliminate most of the bacterial cells and debris without affecting *Xoo* secreted proteins expressed *in planta*, the xylem sap samples were centrifuged to remove bacterial cells before they were analyzed by mass spectroscopy.

Proteomic analysis of the three xylem sap samples yielded 180 proteins in sample 1; 291 in sample 2 and 190 in sample 3: resulting in a total of sum of 661 proteins identified. Since most proteins were shared among samples, 324 unique proteins were identified and of these, 64 were shared among all three samples (Figure 4-12c, Table 4-8). Most of the 64 shared proteins were found on the upper part of the

abundance index in all three samples, suggesting a link between abundance and infection. As can be observed from this list, the majority of identified proteins were putatively classified as either secreted or membrane proteins. The reason for this is because the xylem sap samples were centrifuged in order to remove most *Xoo* cells prior to mass spectroscopy analysis. The cytoplasmic proteins identified are likely due to *in planta* lysis of the growing bacteria in the xylem sap.

Importantly many of the known secreted virulence factors of *Xoo* have been detected in the *in planta* proteomic experiment; these include type II secreted hydrolytic enzymes, modulators of the plant immune response, motility and adhesion associated proteins and iron transport factors. For example several hydrolytic enzymes involved in degradation of the plant cell walls such as 1,4-*b*-cellobiosidase (Q5GVI4), two cellulases (engXCA: Q5GVK0; egl: Q5H685), one cellulase S (Q5H3Z0), one putative pectinesterase (Q5GZC1), one rhamnogalacturonase B (rhgB: Q5H3Y9) and one lipase/esterase (EstA: Q5GXE7) were abundantly detected in the xylem sap. Several of these enzymes have also been reported as effectors capable of inducing basal defenses against *Xoo* (Jha et al., 2007). Similarly, several motility proteins were also found including pilus biogenesis protein (PilL; Q5H219), fimbrial assembly protein (PilQ; Q5H3Q6), flagellar hook protein (FlgE; Q5GZP5), flagellin (FliC; Q5GZN6), chemotaxis protein (Q5GZQ9) and methyl accepting chemotaxis protein (Q5H0R3); motility and chemotaxis have long been known to be important for virulence in many bacterial species. The ability to acquire iron in the host is important for *Xoo* virulence (Subramoni and Sonti, 2005b)-(Xu et al., 2010) and several proteins potentially associated with iron acquisition have been identified including TonB-like protein (Q5H5X6), TonB-dependent receptors (fecA: Q5GWI4; Q5H3F7; Q5H4G6; Q5GXB4) and three putative ferric enterobactin-like siderophore receptors (bfeA: Q5H2G3, Q5H351, Q5H353). On the basis of these results, it is likely that these three outer-membrane receptors are employed by *Xoo* to acquire iron from the host via endogenous and/or heterologous ferric siderophores present in the vicinity possibly synthesized by other bacterial species. Two catalases (srpA: Q5H5R9; katE: Q5GX94) have also been detected in all samples; the presence of KatE in infected leaves is significant, because it has been reported that in *Xanthomonas axonopodis* pv. *citri* this nonfunctional catalase is required for full virulence (Tondo et al., 2010).

Table 4-8. *In planta* proteomics.
Classification of putative function or cellular localization of *X. oryzae* proteins found *in planta* in all three biological replicates.

Accession	Putative function	Accession	Putative function
Hydrolytic enzymes		***Membrane/Transport/Periplasmic***	
gi122879315	1,4-beta-cellobiosidase	gi58581150	OmpA family protein
gi122879107	cysteine protease	gi58579873	OmpA-related protein
gi58582319	putative pectinesterase	gi122879349	TonB-dependent receptor
gi58582993	estA, lipase; esterase	gi58580013	TonB-like protein
		gi58580017	iroN, TonB-dependent receptor
Iron Transport		gi58581407	iroN, TonB-dependent receptor
gi122879094	putative siderophore receptor	gi58581474	mucD, periplasmic protease
gi122879095	putative siderophore receptor	gi58580739	oprO, polyphosphate-selective porin O
gi58581227	putative siderophore receptor	gi58581134	pcp, peptidoglycan-assoc. outer membr lipoprot
		gi122879039	xadA, outer membrane protein
Motility/chemiotaxis		gi58580465	xadA, outer membrane protein
gi58582825	fimT, pre-pilin like leader sequence	gi58580722	fyuA, TonB-dependent receptor
gi58582195	flgE, flagellar hook protein FlgE		
gi161898984	fliC, flagellin	***Cellular processes/Metabolism***	
gi58580534	pilH, PilH family regulatory protein	gi122879258	Alanyl dipeptidyl peptidase
gi58580784	pilQ, fimbrial assembly protein	gi58581347	DNA-binding related protein
gi58581827	tlpC, methyl-accepting chemotaxis protein	gi58580353	atpA, F0F1 ATP synthase subunit alpha
		gi122879043	atpD, F0F1 ATP synthase subunit beta
Hypothetical proteins		gi58583386	colR, two-component system regulatory protein
gi58580149	hypothetical protein XOO0526	gi58581612	dapE, succinyl-diaminopimelate desuccinylase
gi58580207	hypothetical protein XOO0584	gi58581654	dnaK, molecular chaperone DnaK
gi122879062	hypothetical protein XOO1004	gi58581688	fkpA, FKBP-type peptidyl-prolyl cis-trans isomerase; rotamase
gi58581053	hypothetical protein XOO1430	gi58584111	glnA, glutamine synthetase
gi58581354	hypothetical protein XOO1731	gi58583911	groEL, chaperonin GroEL
gi58581428	hypothetical protein XOO1805	gi122879256	katE, catalase
gi58581525	hypothetical protein XOO1902	gi58583219	rplK, 50S ribosomal protein L11
gi58582745	hypothetical protein XOO3122	gi58580951	rplS, 50S ribosomal protein L19
gi58582823	hypothetical protein XOO3200	gi58581803	rpsA, 30S ribosomal protein S1
gi58583149	hypothetical protein XOO3526	gi58583189	rpsE, 30S ribosomal protein S5
gi58583822	hypothetical protein XOO4199	gi58582451	sodM, superoxidase dismutase
gi58583826	hypothetical protein XOO4203	gi58581666	sucB, dihydrolipoamide succinyltransferase
gi58583889	hypothetical protein XOO4266	gi58583369	surA, peptidyl-prolyl cis-trans isomerase
gi58584049	hypothetical protein XOO4426	gi58582533	xylA, xylose isomerase
gi58584153	hypothetical protein XOO4530	gi58583210	tufB, elongation factor Tu
gi58584155	hypothetical protein XOO4532		
gi58579717	hrcC, hypothetical protein XOO0094	***Others***	
		gi58581605	pru, protein U
		gi58579754	virK, VirK

Hydrogen peroxide is one of the reactive oxygen species produced by the host plant as part of the innate immune response. The pathogen uses antioxidant enzymes such as catalases to overcome oxidative stress in order to establish and maintain infections (Green and Paget, 2004). Cell-cell signaling related proteins have also been found, including the regulator of pathogenicity factors RpfN (Q5GZ04) that is reported to have transport activity and to be integral to the cell membrane. The *Xoo rpf* operon is well studied and shown to regulate the production of virulence factors through a QS mechanism based on diffusible signal factor (DSF) (He and Zhang, 2008b, Dow, 2008, He et al., 2010). The XadA high-molecular weight outer-membrane protein with adhesin-like function and role in virulence (Ray et al., 2002) was also detected (Table 4-8).

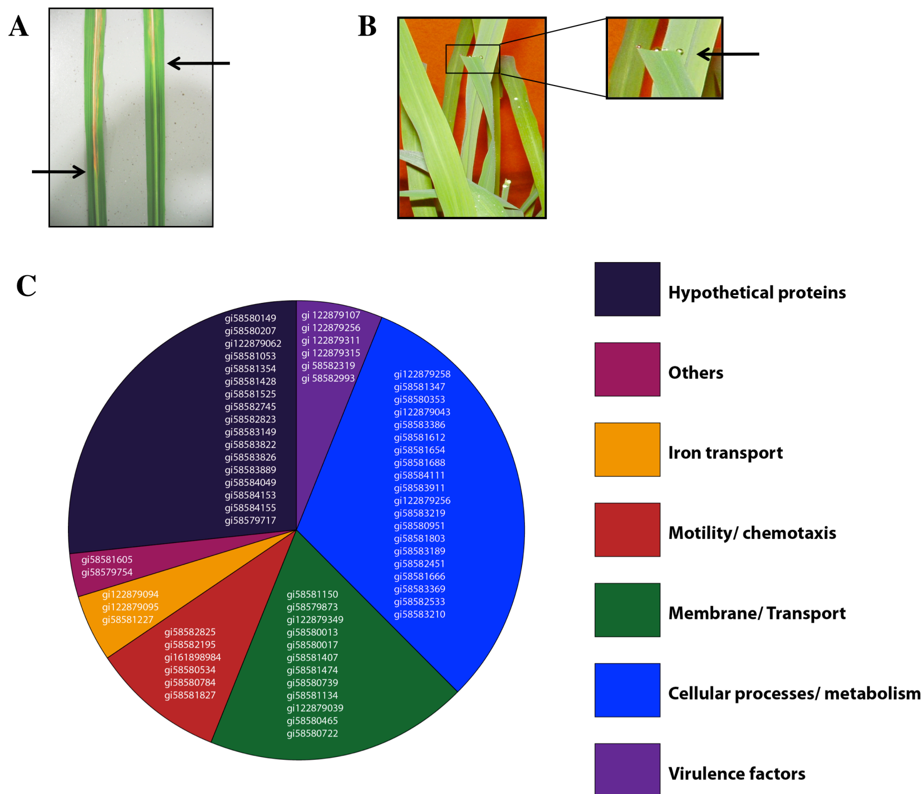

Figure 4-12. Proteomics of *in planta* expressed *Xoo* proteins.
A, lesion length of *X. oryzae* pv *oryzae* infected leaves, borderline of lesion length indicated by an arrow; B, xylem sap drops on cut infected leaves as indicated by an arrow; C, the 64 *Xoo* proteins found *in planta* in all three biological replicates categorized by proposed function or cellular localization. Accession numbers are shown within each category.

Several other known proteins have been found which have not yet been reported to be involved in virulence and/or plant colonization by *Xoo*. These include several outer-membrane proteins/membrane associated proteins like an OmpA family protein (Q5H2P0), OmpA related protein (Q5H6B6), three outer membrane proteins

(Q5H501; Q5GTY7; OmpW: Q5H4Z9) and a peptidoglycan-associated outer membrane lipoprotein (pcp: Q5H2Q6). Interestingly, three proteins with protease activity were found in all samples: a periplasmic protease (mucD: Q5H1R6), a peptidase (Q5H5S7) and a cysteine protease (Q5H2T0). The ortholog periplasmic protease MucD of *Xanthomonas campestris* pv. *campestris* was shown to be part of the signal transduction regulatory pathway together with the alternative sigma factor RpoE and RseA. This mechanism is involved in environmental stress response and pathogenicity in many bacterial species (Bordes et al., 2011). A recent report demonstrated that cysteine proteases play a role in the interaction with the host and could be type III secretion proteins/effectors (Gurlebeck et al., 2006). In *Xanthomonas campestris* pv *vesicatoria* a YopJ-like protein (YopJ-like proteins share structural homology with cysteine proteases) called AvrXv4, is secreted and translocated into the plant cell during infection in a type III-dependent manner. However the cysteine protease homolog of *Xoo* detected here *in planta* was found to be a putative type II secretion protein with a deduced signal peptide sequence at the N-terminus (Furutani et al., 2004b). The role of these proteases in *Xoo* is currently unknown. Two elongation factors, Tu (tufB: Q5GWR8) and P (efP: Q5GZB2), were also detected in all three samples. Interestingly the N-terminus of elongation factor Tu, the most abundant protein in bacteria, were shown to act as a plant-associated molecular pattern (PAMP) in *Arabidopsis thaliana* and other *Brasicaceae*, triggering innate immune response and resistance to subsequent infection with pathogenic bacteria (Kunze et al., 2004). Their putative role as modulators of the plant immune response by *Xoo* is currently unknown (Table 4-8).

In summary 324 unique proteins were found to be expressed *in planta* by *Xoo*, approximately 15% of which are hypothetical proteins. All the other proteins were tentatively categorized according to their putative cellular or metabolic function and divided accordingly, as shown in Figure 4-12c and Table 4-8 for proteins found in all three samples. Among the identified proteins, 36 are homologs or orthologs already found in other bacteria and with a known or putative function in virulence or plant-bacteria interaction.

4.4.2 *In planta* analysis of 10 gene knock-out mutants.

The genes of 10 of the corresponding *in planta* expressed proteins were inactivated in the genome by genomic insertion, as described in Materials and Methods (Section

3.4), and the mutants tested for their virulence in rice. Greater attention was focused on unstudied proteins identified in the *in planta* proteomic analysis with a potential role in pathogenicity and/or host-pathogen interaction; these included protein U, one of the cysteine proteases, some hydrolytic enzymes, membrane associated proteins and some randomly selected hypothetical proteins. Insertion mutants of these 10 genes were constructed (Table 3-1 and Table 3-3) and their virulence on rice was assessed by the clipping method (Figure 4-13 and 3-14).

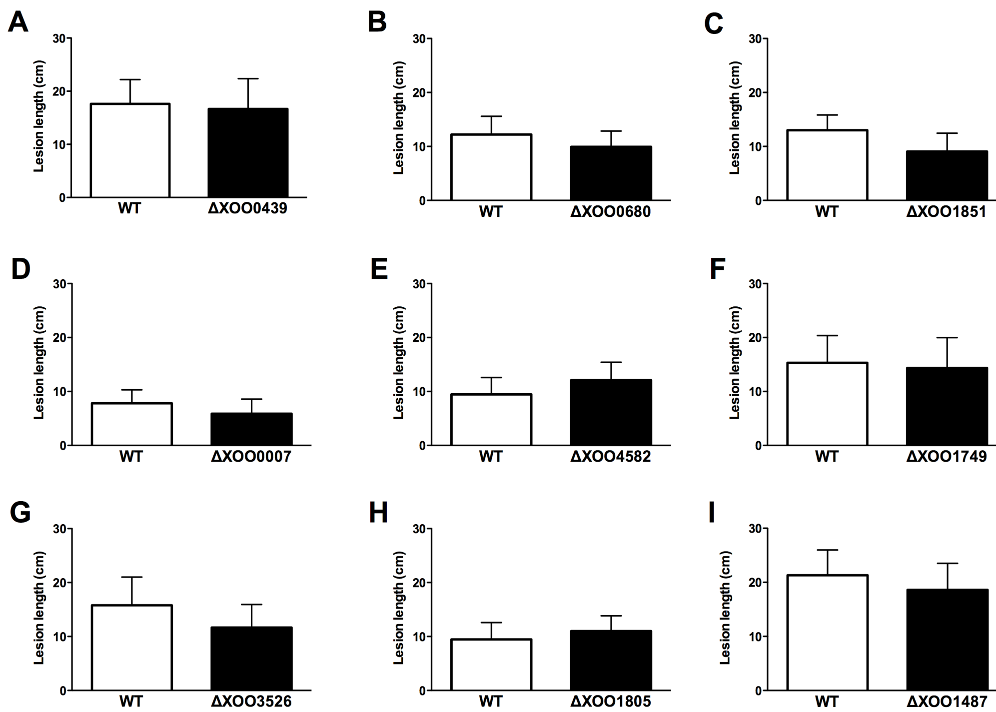

Figure 4-13. Rice infection assays with all *Xoo* mutants .
Rice infected with *Xoo* wild type and knock out mutants and virulence estimation through lesion lengths. Lesion length were measured on rice leaves infected with *Xoo* wild type (WT) and *Xoo* knock-out mutants of A, peptidase Xoo0439; B, protease Xoo0680; C, periplasmic protease Xoo1851; D, hypothetical protein Xoo0007; E, outer membrane protein OmpP1 Xoo4582; F, twitching motility protein PilJ Xoo1749; G, hypothetical protein Xoo3526; H, hypothetical protein Xoo1805. Values given are means from lesion lengths of approximately 30 leaves from 10 plants; error bars denote standard deviation (SD).

Only the genomic mutant for Protein U (Xoo1982) was found to cause a reduction in lesion formation with a statistical significance ($P<0.05$). The observed reduction was of 34.2% for Protein U with respect to the wild type (Figure 4-14). In order to determine whether the mutation affected *in planta* growth, the CFU/g of rice was calculated for the *Xoo* wild type and the Protein U mutant. It was established that the *Xoo* wild type titers reached $2.8\pm0.95 \times 10^{13}$ and the protein U mutant $2.2\pm0.45 \times 10^{13}$ CFU/g. When the *protein U* knock-out mutation was complemented using the wild-

type gene cloned *in trans* in a plasmid, the virulence was only partially restored (difference not statistically significant; Figure 4-14).

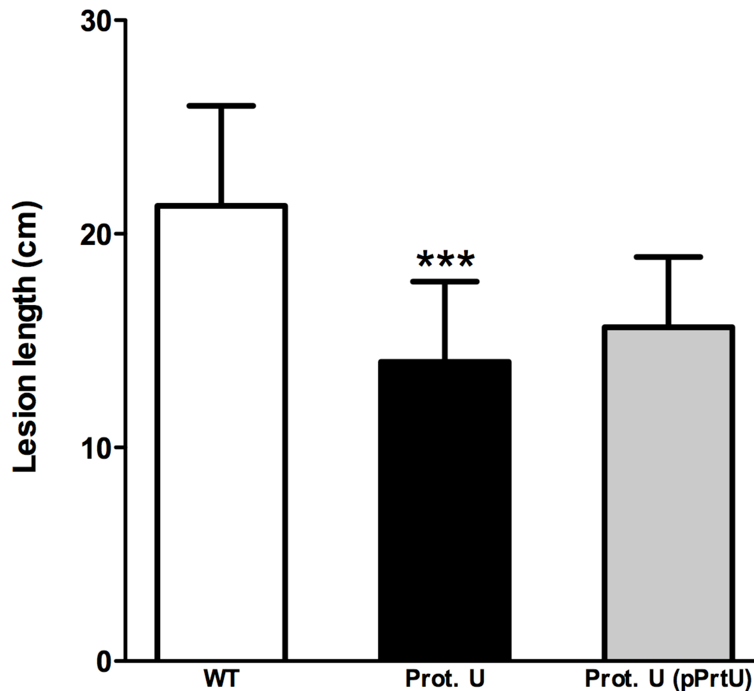

Figure 4-14. Rice infections with protein U mutant and complimented strain.
Rice infected with *Xoo* wild type and Protein U knock out mutants showing reduced virulence (lower lesion lengths). Statistical significance (P ≤ 0.05) of the difference in lesion length comparing with wild type is indicated by asterisks (Two-tailed Student's *t*-test). Lesion lengths were measured on approximately 30 leaves from 10 different plants leaves infected with *Xoo* wild type (WT), protein U (Prot.U; gi58581605) and the complemented mutant, Prot.U (pPrtU). Error bars denote standard deviation (SD). The software Prism 5 for Mac OS X was used for statistical analysis.

Consequently, retention of the plasmid *in planta* was tested as, plasmid loss could take place. After growth in planta, the resulting bacteria were titrated on PSA (total number of bacteria) and PSA plus gentamycin (bacteria which retained the pUFRPrU plasmid) and found that the CFU/g of the complemented strain grown in PSA and PSA plus gentamycin resulted in the values of $1.9 \pm 0.85 \times 10^{13}$ and less than 1×10^{8} CFU/g, respectively. This indicates that there is a significant loss of pUFRPrU plasmid during growth in the rice plant resulting from lack of antibiotic selection pressure. Given the rapid loss of the pUFRPrU plasmid *in planta*, it seemed reasonable to conclude that the rescue of virulence is robust.

4.4.3 Protein U expression

Further studies were undertaken to try to elucidate the expression of Protein U in *Xoo*. When the amino acid sequence of Protein U was analyzed for the presence of a

signal peptide and a cleavage site using the SignalP 4.0 Server software (Petersen et al., 2011), the result suggested that Protein U could be a T2S protein with a putative cleavage site between amino acids 43 and 44 (Appendix 7-10). In order to study the expression of the Protein U gene, I constructed a transcriptional fusion of the promoter to a promoterless *ß*-glucuronidase gene. The gene promoter for protein U was inactive in both rich and minimal media, however the promoter was activated by the presence of macerated rice in the medium (Figure 4-15). The production of Protein U was further confirmed the in the presence of macerated rice by the Western blot analysis of *Xoo* cultures grown in different conditions (Figure 4-16). These expression studies confirm the *in vivo* proteomic data indicating that Protein U was best expressed in the presence of macerated rice suggesting a role *in planta*. Apparently this role is not dependent on OryR, since Protein U did not appear in the genome-wide transcription analysis (Table 4-1 and Table 4-2).

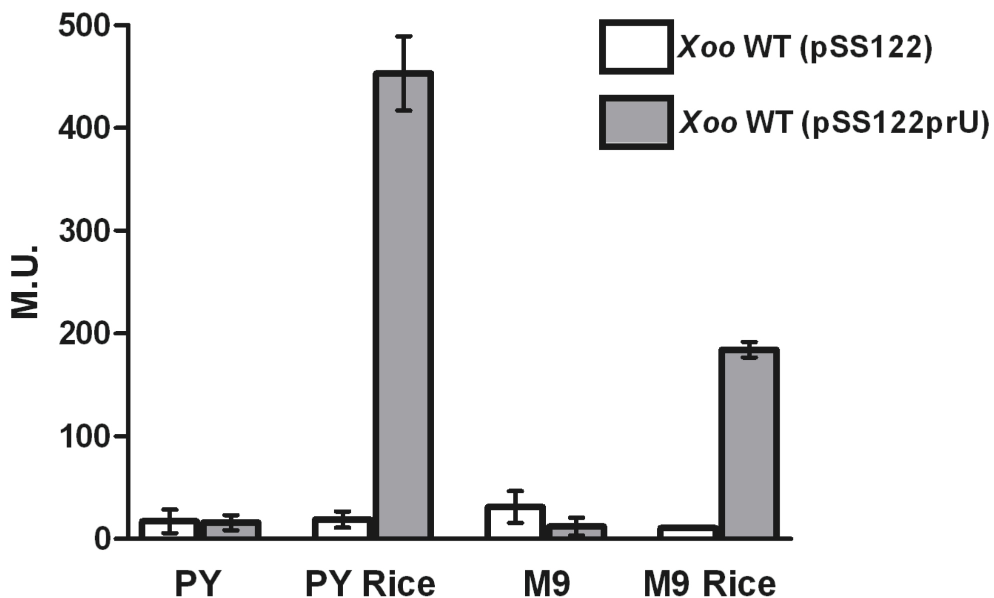

Figure 4-15. Protein U gene promoter transcriptional fusion studies.
β-glucuronidase assay of the protein U gene promoter in the reporter plasmid pSS122 in rich (PY) and minimal (M9) media with (PY+rice and M9+rice) or without (PY and M9) rice macerate, respectively. *Xoo* WT (pSS122) is *Xoo* wild type harboring the reporter plasmid without the promoter (negative control); *Xoo* WT (pSS122PrU) is *Xoo* wild type harboring the reporter plasmid with the β-glucuronidase reporter gene under the control of protein U promoter (for details see Experimental Session). Values given are means from three independent replicates; error bars denote standard deviation (SD).

Protein U (pru: Q5H1D5) was identified in significant amounts in all three xylem sap samples; at present there are no reports on the possible role of this protein in

Xanthomonas. It is 29% identical to Protein U of *Myxococcus xanthus* which plays a role in spore coat formation (Gollop et al., 1991); *Xoo* is a Gram-negative non-sporogenic bacterium, thus it will have a different role in *Xoo*. Most probably *Xoo* protein U is produced as a secretory precursor pre-protein U, then the signal peptide (predicted to be 42 amino acids in *Xoo*,) is cleaved and the protein secreted, most probably into the periplasm; it is not known if the protein is then exported in the extracellular medium. The presence of conserved domains was analyzed (http://hydra.icgeb.trieste.it/sbase/sbase.php) and three domains found: (i) DUF599, an unknown protein domain, (ii) a sugar fermentation stimulation protein domain, and (iii) spore coat U domain. This last domain was found first in a spore coat protein of the sporogenous *Myxococcus xanthus* (Gollop et al., 1991), but later also in other genes which are part of an operon coding for type I pili assembling system (Tomaras et al., 2003); these pili are involved in motility processes and in biofilm formation. One possible hypothesis concerning the role of Protein U in *M. xanthus* is that it is one of the 21 proteins forming the extracellular matrix or fibrils ("flaccid filaments" of 50 nm in diameter) together with carbohydrate, mainly extracellular exopolysaccharides (EPS) (Mauriello et al., 2010).

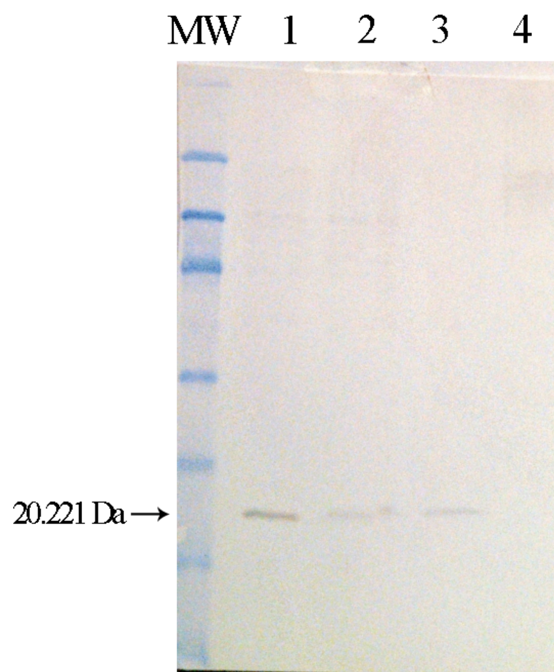

Figure 4-16. Western blot analysis.
Xoo **culture grown in (1) PY + rice extract; (2) PY; (3) M9CA + rice extract; (4) M9CA. Immunostaining was performed using mice polyclonal antibody anti-6xHis-tagged protein U antiserum (for experimental details see Experimental Session).**

5 SUMMARIZING DISCUSSION

5.5 OryR regulon

Transcriptome analysis revealed that OryR is a global regulator, influencing the transcription levels of 305 genes by two-fold or more, representing 7.5 % of the protein encoding genes in *Xoo*. OryR positively regulated 191 of these genes, while 114 were down regulated (Figure 4-2), keeping in mind that this set of genes include both direct and indirect regulation by OryR and further studies would be needed in order to find direct targets of involving direct OryR binding at their gene promoters. This set of regulated genes therefore most probably includes over-lapping by other regulators as has been observed in the regulon of the QscR solo in *P. aeruginosa*, which overlaps with its QS systems (Lequette et al., 2006, Ledgham et al., 2003, Fuqua, 2006). The high number of hypothetical proteins found in both the positively and negatively regulated gene sets. This is indicative of proteins of unknown function typically found in bacterial genomes, which can range between 30 and 40% of the total genome (Karaoz et al., 2004); this could be a potentially exciting direction for future studies linking OryR with targets of unidentified function. Additionally, many transposases were positively regulated indicating that OryR could play a role in the regulation of these IS elements which have been suggested to give the *Xoo* genome a great plasticity and rapid adaptability (Salzberg et al., 2008) allowing for its great diversity of genomes and pathovars (Leach et al., 1995). Interestingly, the *Xoo* DSF-based QS system seems to be completely independent of OryR function, since none of the genes in the *rpf* operon, responsible for DSF synthesis and detection, were differentially regulated in the transcriptome analysis. It is worth mentioning that rice macerate is very complex and includes a large number of compounds, including proteins, polysaccharides and low molecular weight secondary metalobolites, which could affect the behavior of *X. oryzae* beyond the regulation of OryR. Additionally, the microarray chip was designed using the genome of the strain KACC 10331, not the XKK12 strain under study here. Since an unusually high degree of variation has been observed between the sequenced strains of *Xoo* (Lee et al., 2005, Salzberg et al., 2008), this could add an extra degree of variation to the transcriptomics analysis.

The most highly represented single gene family in the transcriptome analysis was related to motility. 30 flagellar genes (Table 4-1), accounting for 16% of the up-

regulated genes above the 2-fold cut-off value (P < 0.01) were positively regulated, along with 9 chemotaxis and 5 pili-related genes indicating that OryR plays a major role in the regulation of motility genes. This finding is also interesting because it reconfirms the role of the plant signal molecule that activates the OryR regulator at the transcriptional level and also at the phenotypical level. Plate motility assays showed that rice macerate induces motility in *Xoo* and that this induction is OryR-dependant, since the OryR insertion mutant showed a significant reduction in motility (Figure 4-4Figure 4-5). Additionally, in this study I have shown that the flagellar regulator *flhF* contains a lux box just like the *pip* gene and is regulated by OryR. FlhF has been shown to be required for the correct placement of polar flagella in *P. putida* (Pandza et al., 2000), in addition FlhF together with FlhG regulates the number of flagella in *Vibrio alginolyticus* (Kusumoto et al., 2006). In *P. aeruginosa*, FlhF is required for swimming and swarming and together with FleN orchestrate the correct placement and number of the flagellum (Dasgupta et al., 2000, Murray and Kazmierczak, 2006). The exact role of FlhF has not been elucidated in xanthomonads; although the correct placement of the flagellum could explain the deficiency in motility observed in the OryR mutant. Finally, the *in vitro* transcriptome analysis was partially validated *in planta*, through the use of a powerful proteomics approach that showed a highly significant decrease in flagellin production by the OryR insertion mutant compared to the wild type strain during plant infection (Appendix 7-11).

5.5.1 A model for OryR function

It has been observed previously that bacteria have “intensity switches” that allow them to sense the environment in order to ensure the correct activation of flagellar and TTSS-encoding genes (Blocker et al., 2003). This could indicate that *Xoo* needs to ‘wait’ for the correct conditions, close proximity to the hydathode for example, in order to initiate the energy expensive process of flagellation, through the regulation of FlhF and other flagellar genes by OryR *in planta*. This is also evidenced from motility assays, which show limited swimming and no swarming in normal motility plates but when rice macerate is added to the media both phenotypes are greatly enhanced (Figure 4-4 and 3-5) indicating the need of this external stimulus to start the process. This highlights that these forms of motility are triggered by compound(s) present in the rice plant; OryR plays a part in swarming and swimming

under these conditions since motility is reduced but not eliminated. It must therefore be noted that other mechanisms also exist in *Xoo,* which respond to plant signals and trigger these forms of movement (Figure 5-1). The introduction of *oryR* in a plasmid in the *Xoo oryR* mutant did not result in complementation of these phenotypes; the reason for this is currently unknown. It has been however previously reported that introduction of multiple copies of the *oryR* homolog *psoR* of *P. fluorescens* resulted in unexpected behavior/phenotypes (Subramoni et al., 2011). Finally, the role of OryR in motility is also evidenced by the significant reduction of flagellin content in the OryR mutant observed in the *in planta* proteomic studies. This could mean that the OryR mutant is "deaf" to the signal and thus cannot sense that the correct conditions for motility to take place. As *Xoo* is a vascular pathogen, the *in planta* proteomic approach used here using infected xylem sap samples proved to be an effective technique to study proteins are expressed *in vivo*.

Xanthomonas oryzae pv. *oryzae* being a vascular pathogen of the rice plant, for a successful infection *Xoo* must: (1) move towards the host, (2) establish contact with the host (3) penetrate the plant and (4) proliferate once inside (Gnanamanickam et al., 1999). Most studies have focused to understand steps 3 and 4; *Xoo* is known to invade rice plants by entering through hydathodes, as shown by electron microscope studies (Horino, 1984, Mew et al., 1984) or wounds; the latter is common during periods of high precipitation when plants are frequently damaged. Once inside, *Xoo* activates its artillery of virulence factors (Niño-Liu et al., 2006). On the other hand, the ecology of *Xoo* is poorly understood, very little is known about the functions that contribute to epiphytic growth, preference for portal of entry and modes of dissemination (Ryan et al., 2011). OryR homolog PsoR of *P. fluorescens* is also involved in inter-kingdom signaling, probably allowing this beneficial bacterium to "sense" its proximity to plants in order to produce anti-microbial compounds in the place where they are useful (Subramoni et al., 2011). *Xoo* could use a similar mechanism during the initial steps of infection. Once *Xoo* reaches the plant it initially grows epiphytically (Ryan et al., 2011), then it must move in order to find a point of entry; *Xoo* is known to have a chemotactic response towards water drops exudated by the rice plant (Feng and Guo, 1975). Water drops are found in wounds or hydathodes, the latter are involved in guttation, the process by which plants exudate water in order to move nutrients up from the roots. It is reasonable to assume that these drops, coming from the vascular system of the plant, contain a great number of

plant secondary metabolites, including the molecule that OryR binds/responds to. By sensing this molecule, *Xoo* can activate hyper-motility via OryR regulation, in order to successfully find a point of entry and colonize the plant, instead of wasting resources when not needed (Figure 5-1). In the later stages of infection, once inside the plant, *Xoo* duplicates and secretes large quantities of EPS, important in initiating biofilms (Ryan et al., 2011) and probably making flagellar motility less relevant. Importantly, and in line with our group's previous findings, my results show that OryR is activated and acts only in the presence of rice macerate, further highlighting its role in inter-kingdom signaling. Future studies are focused on the identification of the plant signal molecule(s), which interacts with OryR as this will considerably help in the understanding of the role of OryR and related proteins in plant associated bacteria.

5.6 PsoR regulation

It was previously found that the PAB LuxR solo PsoR of *P. fluorescens* plays an important role in inter-kingdom communication system between the soil bacterium and plants. This regulator, homologous to quorum sensing R transcriptional proteins, binds an unidentified plant signal molecule. This solo is part of a new class of inter-kingdom signaling LuxR-type transcriptional regulators found in plant associated bacteria that is reported here. Gene expression profiles revealed that PsoR affected various genes involved in inhibition of plant pathogens. These include a chitinase and biosynthetic genes of anti-fungal compounds. 2,4-diacetylphloroglucinol (DAPG) production is PsoR dependent both *in vitro* and *in vivo*. *psoR* mutants were significantly reduced for their ability to protect wheat plants from root rot and damping-off caused by Pythium ultimum infection. PsoR positively regulates genes involved in biocontrol most likely sensing a molecule(s) and interestingly this response seems to be plant-specific (Subramoni et al., 2011). Here I have found that PsoR is also important for iron metabolism, an additional trait that makes *P. fluorescens* an attractive candidate as a bio-control strain (Figure 5-1). Similarly to OryR, it is most likely that PsoR responds to a plant molecule exudated in the rhizosphere informing of its whereabouts and in turn activate expression of genes which are beneficial to the plant. Performing *in vivo* studies will further validate this model.

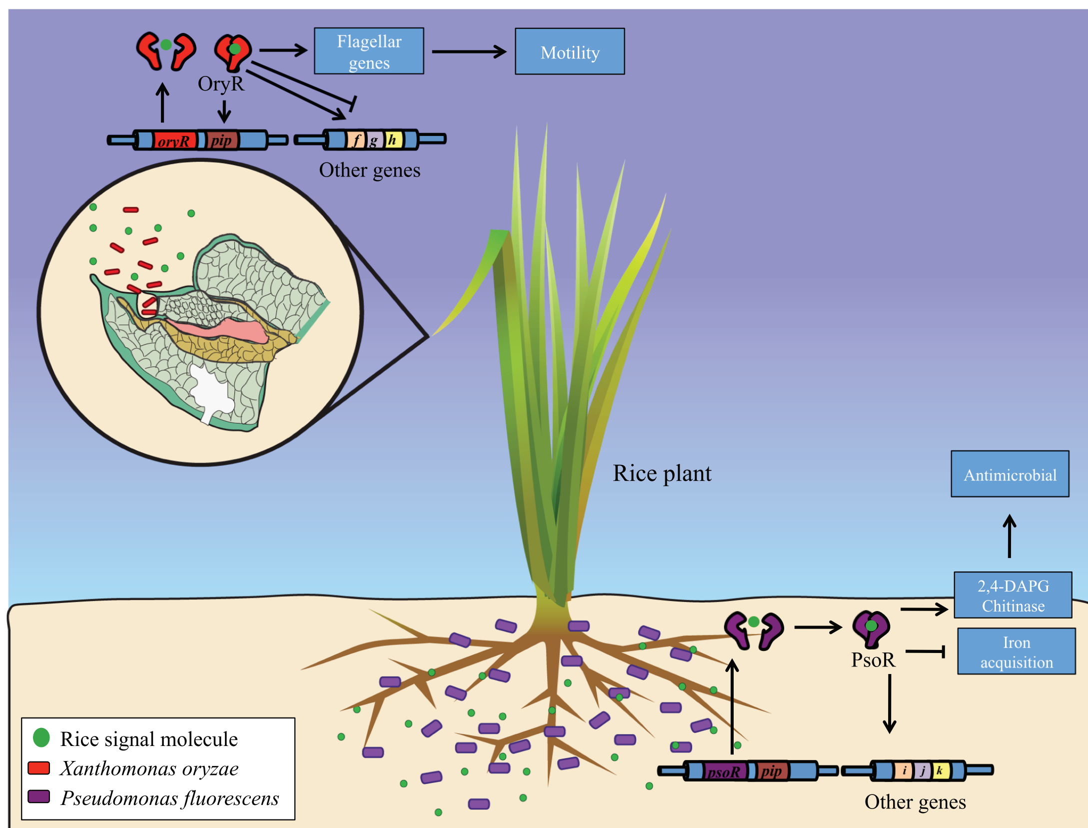

Figure 5-1. Working model of inter-kingdom signaling systems.
Both *X. oryzae* (red cells) and *P. fluorescens* (purple cells) have a an inter-kingdom signaling system with the rice plant. The pathogen uses this system to sense proximity to the plant and activate the Pip virulence factor and hyper motility to enter the plant through the hydathode (magnified circle). The beneficial soil bacteria use this system to activate antimicrobial agents and modulate iron acquisition factors.

5.7 Commonalities and differences between QS-LuxR and PAB-LuxR proteins

LuxR-type transcriptional regulators share various characteristics: (1) They are comprised of two domains, an autoinducer-binding domain at the N-terminus and a DNA-binding helix-turn-helix domain at the C-terminus. (2) They bind *N*-acyl homoserine lactones with a high degree of specificity to the autoinducer produced by their cognate LuxI synthase. (3) Crystallization studies of these proteins show that they share a similar configuration, having a hydrophobic pocket that recognizes and binds the autoinducer. (4) Binding of autoinducer stimulates dimerization. (5) The DNA-binding domain recognizes a 20 bp inverted repeat sequence called a *lux* box (Nasser and Reverchon, 2007, Fuqua et al., 2001, Whitehead et al., 2001, Waters and Bassler, 2005). Nonetheless, QS LuxR proteins have a surprising degree of diversity in their primary structure. Among them they show a low degree of sequence

conservation that would presumably allow them to discern among different AHLs; for example LuxR of *V. fischeri* requires acyl chains of at least 5 carbons, showing optimal binding of those with six or eight carbons (Schaefer et al., 1996), while TraR can tolerate acyl groups one carbon shorter and up to four carbons longer than cognate 8OC8-HLS (Zhu et al., 1998) and LasR shows a similar behavior (Passador et al., 1996). Similarly, different QS LuxR proteins bind their autoinducer with different affinities; TraR makes an irreversible complex (Zhu and Winans, 2001), while LuxR's complex is reversible by dilution (Urbanowski et al., 2004) and LasR is at an intermediate situation (Schuster et al., 2004). There are a number of highly conserved amino acids among most QS LuxR proteins, especially those that interact with the autoinducer. In the AHL-binding cavity, as previously mentioned, but even among residues that interact with the molecule there can be some variations such as F_{62} of TraR, which closes the binding cavity at the acyl side and is responsible for the irreversibility of molecule binding; in most other QS LuxR proteins it corresponds to a residue smaller than phenylalanine (Vannini et al., 2002). Additionally, the amino acids involved in QS LuxR protein dimerization can also vary, such is the case of CarR, which can exist as a stable preformed dimer and can interact with target promoters in the absence of AHLs, while binding of autoinducer results in multimers (Welch et al., 2000). Additionally, QS LuxR-type proteins can act either as an activator or a repressor (Nasser and Reverchon, 2007).

PAB-LuxR solo proteins studied here share the main characteristics of QS-LuxR proteins but since these regulators are a recent subject of study there are still many pieces of information not available. Perhaps the biggest drawback we have is that the structure of the molecule bound by these proteins is unknown. The fact that they have substitutions in some of the highly conserved amino acid residues of the binding pocket (W_{57} and Y_{61}) could indicate that we are dealing with a molecule significantly different form AHLs. This led us to the perform point mutation experiments in which these residues were mutated to establish the QS consensus sequence (M57W and W61Y for OryR and W61Y for PsoR). Unfortunately, these point mutations could not restore AHL-binding capacity for either PAB-R studied here. This is not surprising because, as mentioned above, QS LuxR proteins have slight variations that allow them to have high affinities for their cognate AHLs and not others, and slight variations in protein sequence can significantly alter their affinity, furthermore, there are other residues that interact with the autoinducer but

are not always conserved (Nasser and Reverchon, 2007). Without any information about the nature of the plant signal molecule ligated by PAB-R proteins, it is difficult to predict any interactions with the receptor or which amino acid residues play an important role for binding. Identification of the plant low molecular weight molecule will therefore of primary importance in future studies.

While none of the QS-LuxR was able to activate the *pip* box of *X. oryzae* it was interesting to find that both OryR and PsoR were able to activate the target *lux* box of the *lasI* gene (target of LasR). There does not seem to be a higher similarity between the *lasI lux* box and the other *lux* boxes tested but it has been reported that LasR does not need a highly conserved *lux* box to activate target promoters (Schuster et al., 2004), which could indicate a high affinity of these *lux* boxes towards LuxR proteins. Expression of the OryR and PsoR HTH domains alone did not activate transcription of target *Xoo pip* box. This could indicate a different behavior by PAB-R proteins over QS-Rs since the latter have been known to bind DNA and activate transcription without the need of the autoinducer-binding domain (Choi and Greenberg, 1991).

In conclusion, PAB-R proteins share many of the characteristics of QS-LuxR proteins but have diverged enough to not be interchangeable. By establishing the consensus of highly conserved amino acids of QS-Rs did not permit PAB-Rs to bind AHLs indicating that the latter probably bind a plant molecule that is most probably significantly different from AHLs. Furthermore, in the promoter switching experiment only the *lasI lux* box was activated by the PAB-Rs indicating a clear significant difference between *pip* and *lux* box binding or the way in which these proteins activate transcription. Additionally, the PAB-R HTH binding domain alone did not activate transcription. As mentioned above, these experiments are indications to the workings of PAB-Rs and should be observed with caution, especially since the nature of the plant signal molecule that these transcriptional regulators bind remains unknown.

5.8 *In planta* proteomics

In this study many proteins expressed by *Xoo* during infection of rice leaves have been identified. Mass spectroscopy analysis of proteins present in the xylem sap taken from symptomatic infected leaves was a very effective method for identifying *in planta* expressed *Xoo* proteins, minimizing the interference of the host proteins. The recovery of *Xoo* infected xylem could therefore be of great potential in

understanding the dynamics of *Xoo* infection in rice. My approach focused on putative secreted proteins; future studies can better delineate protein compartmentalization in parallel with the identification procedure. This approach can also be adapted to the many vascular pathogens, which infect a large variety of different plants.

A total of 324 unique proteins were detected and 64 were shared among all three infected independent xylem sap isolations. These 64 proteins are probably the most abundant in *Xoo* during the rice infections, as suggested by the abundance index of these putative virulence-associated factors. Many of the known or putative virulence factors were already reported as such in other plant pathogenic bacteria and were present in at least one sample indicating that the procedure followed is effective in determining proteins that are important for virulence.

Inactivating a set of 10 genes, encoding for 10 proteins identified in at least two of the samples analyzed, showed that one of the mutant, Protein U, caused a reduced average lesion length if compared with the wild type. The Protein U knock out mutant was less able to cause symptoms *in planta* and the correspondent protein was present in the xylem sap of infected leaves of all three samples. I found that expression of Protein U is induced by the presence of macerated rice in the media confirming that it is preferentially expressed *in planta*; future work will need to focus on establishing the role of Protein U in *in planta* infections. On a more general note, a wide approach should probably also be followed to study, initially via generation of insertion mutants, of all secreted proteins in vivo. This could reveal if any of the secreted factors plays a pivotal role in the infection process and could therefore be a potential target for controlling Xoo virulence.

5.9 Future research directions

All these results represent an important advancement in the understanding of the role of solo LuxR-type proteins, up to now most work on solos focused on QscR and SdiA of *P. aeruginosa* and *E. coli* respectively, which respond to AHLs. These findings reported here on this new sub-family of plant associated solos, open the ground for exciting further research, for example:

(i) The most evident question remaining is the structure of the plant signal molecule(s). Once identified it will allow answering questions such as the

nature of the interaction with the solo. It will also reveal whether the molecule(s) or family of molecule(s) is widespread or specific to a certain group of plants. Additionally, it would be interesting to find if there is a specificity of PAB LuxR proteins towards different molecules as it has been observed that different bacteria use this signaling system to regulate different phenotypes.

(ii) It would also be interesting to find additional targets of OryR and PsoR, possibly in different conditions, more representative of the interactions between the plant and the bacteria (ie: *in vivo*). In addition the role of the hypothetical proteins regulated by OryR and PsoR could reveal new loci involved in plant-bacterial interactions.

(iii)A more complete picture of OryR and PsoR regulation could be obtained by further studies in respect to the nature of this regulation, wether it is direct or indirect with the use of protein/DNA approaches like gel-mobility shift experiments.

(iv) Additionally, it would be interesting to follow the action of OryR and its targets (such as *pip*) *in planta* through the use of techniques like as fluorescence microscopy in order to elucidate the time/place of maximum PAB-solo activity.

(v) Electron microscopy could be used to elucidate the function of protein U by observing possible changes in surface structure.

(vi) Finally, once could study in order to establish how plant-associated bacteria from different phylogenetic groups use this novel inter-kingdom signaling system.

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7 APENDIX

Appendix 7-1. Media and solutions

All media were prepared in distilled water, and pH was adjusted. Media were sterilized at 121°C for 15 minutes.

LB-Luria Broth (Sambrook, 1989)

Ingredient	g/L
Tryptone	10
Yeast Extract	5
NaCl	10
Agar	15

pH 6.7

PYS (Tsuchiya et al., 1982)

Ingredient	g/L
Peptone	8
Yeast extract	2
K_2HPO_4	2
KH_2PO_4	0.5
pH 7.0, Autoclaved and added:	
$MgSO_4$ (1M)	1 ml
Glucose (10%)	50 ml

PS(A) (Tsuchiya et al., 1982)

Ingredient	g/L
Peptone	10
Sucrose	10
Agar	15

pH 7.0

KING's B (King *et al.,* 1954)

Ingredient	g/L
Proteose peptone No. 3	20
$MgSO_4$	1.5
Glycerol	10
KH_2PO_4	1.2
Agar	13

pH 7

M9C glucose (Sambrook, 1989)

Ingredient	g/L
Na_2HPO_4	6
KH_2PO_4	3
NaCl	0.5
NH_4Cl	1
Casaminoacids	2

pH 7.4. Autoclaved and added:

$MgSO_4$ 1 M	2 ml
Glucose 20%	10 ml
$CaCl_2$ 1M	0.1 ml

Antibiotic stocks

Antibiotic	Solvent	Concentration used (µg/mL)		
		Xanthomonas	***Pseudomonas***	***E. coli***
Ampicillin	Water	75	100	100
Kanamycin	Water	50	100	100
Gentamicin	Water	40	40	10
Choramphenicol	Ethanol	-	10	-
Tetracycline	Ethanol 50%	15	125	10
Rifampicin	N,N dimethylformamide	50	-	50

Stock solutions of each antibiotic were made at 1000x concentration (ie: mg/ml)

SDS- Polyacrylamide Gel Electrophoresis (PAGE) solutions (Sambrook, 1989)

SDS PAGE gel 12 % separating gel

Ingredient	15 mL
H_2O	4.9
30% Acrylamide Mix	6.0
1.5 M TRIS (pH 8.8)	3.8
10 % SDS	0.15
10% ammonium persulfate	0.15
TEMED	0.006

SDS PAGE gel 4 % stacking

Ingredient	5 mL
H_2O	3.4
30% Acrylamide Mix	0.83
1.5 M TRIS (pH 6.8)	0.63
10 % SDS	0.05
10% ammonium persulfate	0.05
TEMED	0.005

Tris Glycine Electrophoresis Buffer

	L
Tris	25 mM
Glycine	250 mM
SDS 10%	10 mL

SDS PAGE Loading Buffer 5X

250 mM Tris. HCl (pH6.8)
0.5 M Dithiothreitol
10% SDS
0.5 % Bromophenol Blue
50% Glycerol

Appendix 7-2. ß-glucuronidase assay

- Grows *Xoo* at 30°C either in PYS liquid media or PS solid medium until the desired optical density is reached.
- Measure optical density of the culture (OD_{600}). Pellet 1 ml of cells
- Resuspended in 600 µl of GUS buffer.
- Add 23 µl of 3% Triton X-100 in GUS buffer and 23 µl of 3% sodium lauryl sarcosinate in GUS to the samples.
- Incubate at 30°C for 10 min
- Add 100 µl of 25 mM *p-nitrophenyl-ß-D-glucuronic* acid (PNPG) (Sigma). Incubate until yellow color develops (5-10 min, taking note of the time)
- Stop the reaction by adding 280 µl of 1 M Na_2CO_3.
- Spin at maximun speed for 10 to remove cellular debris and EPS
- Measure OD_{415} of the yellow color that developed after the glucuronidase reaction

Calculate activity using the following fomula:

1 Miller unit = $1000 * \{[OD_{415} - (1.75 * ODOD_{600})]/(t * v * OD_{600})\}$

Where t is the time of the reaction (in minutes), v is the volume of the culture assayed (in milliliters), OD_{600} is the cell density just before the assay, and 1.75 is the correction factor.

Gus Buffer:

50 mM sodium phosphate [pH 7.0]

1 mM EDTA

14.3 mM 2-mercaptoethanol

Appendix 7-3. ß-galactosidase activity measurement.

- Pellet 1mL of overnight grown culture,and resuspend in the same volume of pre-chilled Z buffer.
- Resuspend 100 μL of cells in 900 μL of Z buffer and determine the OD600
- Permeabilize 100 μL of cells by adding 20 μL of SDS 0.05%, 20 μL of chloroform and 500 μL of Z buffer. Vortex vigorously for 20 seconds. Incubate at 30 oC for 20 minutes
- Add 100 μL of 0.4 % ONPG (Ortho-nitrophenyl-β-D-galactopyranoside), and allow the reaction to take place, note the time (*t*).
- Stop the reaction after sufficient yellow color has developed by adding 250μL 1M Na2CO3.
- Spin each sample for 10 min. at maximum speed to remove debris and chloroform and record the optical density of the supernatant at 420 nm Calculate the Miller units of activity:

Miller Units= (OD_{420} x 1000)/ (OD_{600} x *t)*

Where *t* is the time of ONPG reaction

Z Buffer:

Na2HPO4	0.06M
NaH2PO4	0.04M
KCl	0.01M
MgSO4	0.001M
ß-mercaptoethanol	0.05M

Appendix 7-4. Mutant verification

HflK

```
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U19
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CGCGGCCTGCTCCAGCACCAGATCGGCATTCCGCGAGCCGAACAGGTATTTGCGCGGGTCGCTGATCTGGTACTGCACGTTGAGC
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CCGACTCGATCGGCCAGGGCAGCTTGAAGTTCGGGCCAGGCTGCAGGATGCGCGAGAACTGGCCGAAGCGCAGCACCACACCACG
CTGCTGCTCGCCGATGAGCTGGAAGCTGGAGAACAGCACCATCAGCACCACCGCGATCAGGATCCAGCGCCCCACGCCGCCATCG
AACAGCTCTTTCAACGGGGCGGGCAGATTGCCCCAACCGCCACCGTTGCCACCGCCGCGCGGCCCCCAGGACCTGCGACGGTTGG
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CCGAGACCATCACNGC
Hflk ver primer: GCAACAAGGGCGGAGACGG
```

Analysis Report

Req#	22	Read Length(Normal)	806
Label	21-HFLKver_HFLK_PRIMER	Read Length(Q16)	805
Sample Name	21-HFLKver	Read Length(Q20)	805
Primer Name	HFLK_PRIMER	Signal strength(A)	843
		Signal strength(T)	1098
Instrument	Macrogen3730XL6-1518-009	Signal strength(G)	1304
Analysis	KB 1.4.0	Signal strength(C)	1267
Dyeset/Primer	KB_3730_POP7_BDTv3.mob	GC content	65.0
Lane	7		
Run strarted	2009/5/23 13:35:11		
Run ended	2009/5/23 15:45:41		
Spacing	16.429464		

Blastn Report

Query Download

Name	090522-35_I01_21-HFLKver-HFLK_PRIMER.ab1	Length	753

Start	18	End	753

Subject

DB	gb	AC	CP000967.1
Ref.	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=Nucleotide&list_uids=188518722&dopt=GenBank		
Gene	Xanthomonas oryzae pv. oryzae PXO99A, complete genome		
Start	4489288	End	4488553

Score

Bit	1459	Raw	736
EValue	0.0		

Identities

Match	736	Total	736
Pct.(%)	100		

Strand	Plus / Minus

Blastx Report

Download

Query

Name	090522-35_I01_21-HFLKver-HFLK_PRIMER.ab1	Length	753
Start	76	End	753

Subject

DB	ref	AC	YP_199551.1
Ref.	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=58580535&dopt=GenPept		
Gene	integral membrane protease subunit [Xanthomonas oryzae pv. oryzae KACC10331]		
Start	58	End	283

Score

Bit	441	Raw	1134
EValue	1e-122		

Identities

Match	226	Total	226
Pct.(%)	100		

Positives

Match	226	Total	226
Pct.(%)	100		

Frame	+1

Analysis Report

Req#	21	Read Length(Normal)	792
Label	21-HFLKver_M13F	Read Length(Q16)	791
Sample Name	21-HFLKver	Read Length(Q20)	791
Primer Name	M13F	Signal strength(A)	1906
		Signal strength(T)	2552
Instrument	Macrogen3730XL6-1518-009	Signal strength(G)	3760
Analysis	KB 1.4.0	Signal strength(C)	2956

Dyeset/Primer	KB_3730_POP7_BDTv3.mob	GC content	65.0
Lane	42		
Run strarted	2009/5/23 19:36:41		
Run ended	2009/5/23 21:31:53		
Spacing	15.729715		

Blastn Report

Download

Query

Name	090522-35_H12_21-HFLKver-M13F.ab1	Length	866
Start	14	End	792

Subject

DB	gb	AC	CP000967.1
Ref.	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=Nucleotide&list_uids=188518722&dopt=GenBank		
Gene	Xanthomonas oryzae pv. oryzae PXO99A, complete genome		
Start	4488553	End	4489331

Score

Bit	1544	Raw	779
EValue	0.0		

Identities

Match	779	Total	779
Pct.(%)	100		
Strand	Plus / Plus		

Blastx Report

Download

Query

Name	090522-35_H12_21-HFLKver-M13F.ab1	Length	866
Start	790	End	14

Subject

DB	ref	AC	YP_199551.1
Ref.	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=58580535&dopt=GenPept		
Gene	integral membrane protease subunit [Xanthomonas oryzae pv. oryzae KACC10331]		
Start	25	End	283

Score

Bit	462	Raw	1188
EValue	1e-128		

Identities

Match	240	Total	259
Pct.(%)	92		

Positives

Match	240	Total	259
Pct.(%)	92		

Frame	-2

OmpP1

T7 primer

| Analysis Report

Req#	2	Read Length(Normal)	696
Label	10-Omp1fragment_T7	Read Length(Q16)	695
Sample Name	10-Omp1fragment	Read Length(Q20)	695
Primer Name	T7	Signal strength(A)	949
		Signal strength(T)	961
Instrument	Macrogen3730XL7-16112-010	Signal strength(G)	1829
Analysis	KB 1.2	Signal strength(C)	2077
Dyeset/Primer	KB_3730_POP7_BDTv3.mob	GC content	66.0
Lane	84		
Run strarted	2009/6/27 4:26:18		
Run ended	2009/6/27 6:15:2		
Spacing	15.211394		

| Sequence

If sample type is plasmid, vector screening will be proceeded. Red-highlighted sequence represents strong match with Macrogen Vector DB.

```
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GGTTCCAGCCTGAGTCGGCCAACTACGCCAACGGCGCCCCGGTCTCCGGC
GGTAACGGCGGCGATGCCGGCATGATCGCGCCGGTGCCGGCGATGTACTT
CCACGTGCCGTTCGGCGAGAACGACAACATGCACCTGGGCACCTCGCTGA
CCGTGCCGTTCGGCTTCAAGACCGAATACGACCGCGACTGGGTCGGCCGC
TACCACGGCACCAAGACCGAGCTGCAGGCGATCGACTTCAACGTCGCGTT
CTCCTATGACGTGAACCCGTACGTGTCCTTCGGCGCCTCGGTGTTCGCCG
AGCGTCTGGATATCGACCTGGCCAACGCCGTCGATTTTGGCAGCGTGCTG
GCTGCGCGCCGCGTGCCGGGCTTCGCGCCGGGCAGCGCCGATGGCTACTC
GCGCATCAAGGGCGACAGCACTGAAGTGGGTTCACCTCANNGGGAA
```

OmpP1 Way

| Analysis Report

Req#	1	Read Length(Normal)	715
Label	10-Omp1fragment_omp1	Read Length(Q16)	714
Sample Name	10-Omp1fragment	Read Length(Q20)	714
Primer Name	omp1	Signal strength(A)	490
		Signal strength(T)	419
Instrument	Macrogen3730XL7-16112-010	Signal strength(G)	811
Analysis	KB 1.2	Signal strength(C)	781
Dyeset/Primer	KB_3730_POP7_BDTv3.mob	GC content	66.0
Lane	84		
Run strarted	2009/6/27 0:49:8		
Run ended	2009/6/27 2:37:53		

Spacing	15.201595	

Sequence

If sample type is plasmid, vector screening will be proceeded. Red-highlighted sequence represent with Macrogen Vector DB.

```
>090626-16_M24_10-Omp1fragment-omp1.ab1                                    739
NNNGCNTNCNTGAGCGCGAGTAGCCATCGGCGCTGCCCGGCGCGAAGCCC
GGCACGCGGCGCGCAGCCAGCACGCTGCCAAAATCGACGGCGTTGGCCAG
GTCGATATCCAGACGCTCGGCGAACACCGAGGCGCCGAAGGACACGTACG
GGTTCACGTCATAGGAGAACGCGACGTTGAAGTCGATCGCCTGCAGCTCG
GTCTTGGTGCCGTGGTAGCGGCCGACCCAGTCGCGGTCGTATTCGGTCTT
GAAGCCGAACGGCACGGTCAGCGAGGTGCCCAGGTGCATGTTGTCGTTCT
CGCCGAACGGCACGTGGAAGTACATCGCCGGCACCGGCGCGATCATGCCG
GCATCGCCGCCGTTACCGCCGGAGACCGGGGCGCCGTTGGCGTAGTTGGC
CGACTCAGGCTGGAACCTGGCCGAGAAGCTGATGGCGCTGACGTCGGCCT
GGAACAGGCGACCGTCCAACTGGCGCATACCGGCCGGGTTGTTGACCATG
ATGGAGGCATCGTCCGGGGCGCTGCCCGAACCTGCGAAGGCGCGGCCGAG
GCCCTTGGCGCTGTTTTCCTTCAGCTGGAACGCGGCGCCGTGCGCCTGAC
CAACGGCCAGCACGCCGGCGATACCGACGGCCAGCAGGGTGGCGCGGCTG
AGAATGGAAGCGGTAGAATCCATATGACTAGTAGATCCTCTAGAGTCGAC
CTGCAGGCATGCAAGCTTTGCCTATAGTGAGTNCGTATT
                          T7 Primer
```

Appendix 7-5. Point mutation verification.

OryR point mutation

Alignment between sequencing result of OryR with introduced point mutations (Seq 1-1) and annotated wild type OryR from *Xoo* KACC 10331 (seq 2-1).

Position	Original	Change to
67	M	W
268-270	**ATG**	**TGG**
71	W	Y
280-282	**TGG**	**TAC**

Original sequence is labeled in green and point mutations are red

```
Seq1=Conti of sequence results from macroen (reverse compliment)
Seq2=OryR + promoter sequence

Alignment of Sequence_1:  [contig oryR mut8] with  Sequence_2: [OryR+promoter.txt]

Similarity : 1019/1058 (96.31 %)

Seq_1  1      -------------------------TAAAATTCCGATGCATGGTAACCAGCCCGTAGATC  35
                                             || |   |||||  |||||||||||||||
Seq_2  1      AGACGCCGCCGAAGATCGAGAACGGCGCCG-TCGGCACCATGGACACCAGCCCGTAGATC  59


Seq_1  36     AACAGATCCTTGAGCGTGAGCTGCCGCTTGAGCTCTTGTGCGTAGCCGAAGCGTTCCAGC  95
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  60     AACAGATCCTTGAGCGTGAGCTGCCGCTTGAGCTCTTGTGCGTAGCCGAAGCGTTCCAGC  119


Seq_1  96     GTGGCCGCATCGTCGCGGCGTGGTGTGTCGGTCATGCAGGTGTCCGGGGAAAGGGATGCC  155
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  120    GTGGCCGCATCGTCGCGGCGTGGTGTGTCGGTCATGCAGGTGTCCGGGGAAAGGGATGCC  179


Seq_1  156    GACGCGCAGCGCAGATGCTGAAGGGACCTGAACGCCCGTTCTGCGCTGCAGTCATGTGAC  215
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  180    GACGCGCAGCGCAGATGCTGAAGGGACCTGAACGCCCGTTCTGCGCTGCAGTCATGTGAC  239


Seq_1  216    CGGCATTGTGCCGATAATGTGGCCCACACACCCATCCATTGGCAAAAGTGCTAGGTTTTC  275
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  240    CGGCATTGTGCCGATAATGTGGCCCACACACCCATCCATTGGCAAAAGTGCTAGGTTTTC  299


Seq_1  276    CCTGCTGGTTGTCGCTTGTGGGCAACCACCGGTACCGAGGAATCACCATGTTCGAAATTC  335
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  300    CCTGCTGGTTGTCGCTTGTGGGCAACCACCGGTACCGAGGAATCACCATGTTCGAAATTC  359


Seq_1  336    TAGCCAGCCTGGGCCGCGATCTGCAGGCGTCGCAAACGGTTAATAGCTGCCTGGATCGGG  395
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  360    TAGCCAGCCTGGGCCGCGATCTGCAGGCGTCGCAAACGGTTAATAGCTGCCTGGATCGGG  419


Seq_1  396    TGTTTCGCGATGTCTGTGCGCTCGGCTTCCAGTCGTTGGTCTACGACTACGCACCGGTGC  455
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  420    TGTTTCGCGATGTCTGTGCGCTCGGCTTCCAGTCGTTGGTCTACGACTACGCACCGGTGC  479


Seq_1  456    CGCTGAGCATGGAGGGCGCGCTGATCACGCCAACGGTGTTCATGCAGCGCAATGCGCCAG  515
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  480    CGCTGAGCATGGAGGGCGCGCTGATCACGCCAACGGTGTTCATGCAGCGCAATGCGCCAG  539


Seq_1  516    GCGAT-TGGCAGCATGTCTACTGCGAGCACGGGTACTACCAACATGACCCCGTTCAGCAG  574
              ||||| | |||||||||||  |||||||||||||||||||||||||||||||||||||||
Seq_2  540    GCGATAT-GCAGCATGTCTGGTGCGAGCACGGGTACTACCAACATGACCCCGTTCAGCAG  598


Seq_1  575    CGTGCAACGCGACGTAACACCCCGTTCGTATGGTCGTACCGCACCGACGGCGATTGCGCT  634
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  599    CGTGCAACGCGACGTAACACCCCGTTCGTATGGTCGTACCGCACCGACGGCGATTGCGCT  658


Seq_1  635    GGGGTGGAATATGTGGGTGGACAGCACCGGCAAGTCACGCGTTACTTGTGCGATAGCGGC  694
```

```
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  659   GGGGTGGAATATGTGGGTGGACAGCACCGGCAAGTCACGCGTTACTTGTGCGATAGCGGC  718

Seq_1  695   ATGGGTACCGGTGTCACCGTGCCGCTGCATCTGCCCGGTGGCGCGTTCGCCACCTTTAGC  754
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  719   ATGGGTACCGGTGTCACCGTGCCGCTGCATCTGCCCGGTGGCGCGTTCGCCACCTTTAGC  778

Seq_1  755   GCTGCGATTGATGCCGTGGCTGCGGAAGCGCTGCGTCTGGCCGAGTCGCAGTTATTGCCC  814
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  779   GCTGCGATTGATGCCGTGGCTGCGGAAGCGCTGCGTCTGGCCGAGTCGCAGTTATTGCCC  838

Seq_1  815   TTCTTGCTGCTGGCACATGCTTTCCAGGCGCGTGCGCAGGAATTGCTGGACCCGCAGGAA  874
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  839   TTCTTGCTGCTGGCACATGCTTTCCAGGCGCGTGCGCAGGAATTGCTGGACCCGCAGGAA  898

Seq_1  875   CGCCGCTGCCACCACATTCCATTGACCCGTCGCGAGCGGGAATGCCTGCAGAATTCGGCC  934
             |||||||||||||||||||||||||||||||||||||| |||||||||||| ||||||||
Seq_2  899   CGCCGCTGCCACCACATTCCATTGACCCGTCGCGAGCGCGAATGCCTGCAGTATTCGGCC  958

Seq_1  935   AAAGGCCTGACCTCCAAACGTATCGCCGCGGCGCTCAACCGCTCCACCGGCACGGTGAAC  994
             ||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Seq_2  959   AAAGGCCTGACCTCCAAACGTATCGCCGCGGCGCTCAACCGCTCCACCGCCACGGTGAAC  1018

Seq_1  995   CTGCATCTGAATTCGGCTGCCCGCAAACTGGGTG-CACGTAACAGCGTGAAGCGGGACGT  1053
             |||||||||||||||||||||||||||||||| |      | |     |||||||
Seq_2  1019  CTGCATCTGAATTCGGCTGCCCGCAAACTGGGGGCACGTAACCGCGTGGAAGCGGTGGTG  1078

Seq_1  1054  GAAAAGA--------------------------  1060
                   |
Seq_2  1079  CGTGGTATGCACTATCGGTTGCTGGAGCCATAA  1111
```

PsoR point mutation

Alignment between sequencing result of PsoR with introduced point mutations (PsoR_pnt) and annotated wild type PsoR from *P. fluorescens* Pf-5 (PsoR). ClustalW (Larkin et al., 2007).

Position	Original	Change to
66	W	Y
208-210	TGG	TAC

```
PsoR_pnt          ANNNGGGAACGGGGGNCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCG 60
PsoR_Pfl_Pf-5     ------------------------------------------------------------

PsoR_pnt          GATCAACAGTAAGGAAGCGGCAAGGATGCAGAGCAGGCTCGCGGAATTTGATACCCATCT 120
PsoR_Pfl_Pf-5     -------------------------ATGCAGAGCAGGCTCGCGGAATTTGATACCCATCT 35
                                           ***********************************

PsoR_pnt          TTTGTCCGGAAACTCGCTGCATGAGCGAATGACGGGAACCATGACACTGGCCTCGGAACT 180
PsoR_Pfl_Pf-5     TTTGTCCGGAAACTCGCTGCATGAGCGAATGACGGGAACCATGACACTGGCCTCGGAACT 95
                  ************************************************************

PsoR_pnt          GGGTTTCGATGCCCTGGTTTATGACTACAGCCCGGTGCCTTTCGATCAGGCAGGCGAGCT 240
PsoR_Pfl_Pf-5     GGGTTTCGATGCCCTGGTTTATGACTACAGCCCGGTGCCTTTCGATCAGGCAGGCGAGCT 155
                  ************************************************************

PsoR_pnt          GATCATCCCCAGTGCCATGGTCTGCAATACCCCCAGGGACTGGCTGGAGCTCTACTGCGA 300
PsoR_Pfl_Pf-5     GATCATCCCCAGTGCCATGGTCTGCAATACCCCCAGGGACTGGCTGGAGCTCTGGTGCGA 215
                  ****************************************************. *****

PsoR_pnt          TCAGGGTTACTACCATATCGACCCGGTCCAGCAGGTTGCTCTGGACAGTTCCTCGCCCTT 360
PsoR_Pfl_Pf-5     TCAGGGTTACTACCATATCGACCCGGTCCAGCAGGTTGCTCTGGACAGCTCCTCGCCCTT 275
                  ************************************************ ***********

PsoR_pnt          TATCTGGTCCTACAAGCCCGAGGCGGAAACCGTCCTGCGTCAGGCCCTCGGGCAGCAGCA 420
PsoR_Pfl_Pf-5     TATCTGGTCCTACAAGCCCGAGGCGGAAACCGTCCTGCGTCAGGCCCTCGGGCAGCAGCA 335
                  ************************************************************

PsoR_pnt          CGCTCCGGTATCCAGTTACCTGCACCATCATCAAATGGCCCACGGCATGACCGTGCCCAT 480
PsoR_Pfl_Pf-5     CGCTCCGGTATCCAGTTACCTGCACCATCATCAAATGGCCCACGGCATGACCGTGCCCAT 395
                  ************************************************************

PsoR_pnt          CCATTTACCCAGGGGCGGTTTCGCCAGCCTCACCGGTTTGCGCTCCGGCAATGCCACGGT 540
PsoR_Pfl_Pf-5     CCATTTACCCAGGGGCGGTTTCGCCAGCCTCACCGGTTTGCGCTCCGGCAATGCCACGGT 455
                  ************************************************************

PsoR_pnt          GACATTACAGGACTTGCAACAGGTTCAGGGCCACTTCACCCTGCTTGCTCATGCGTTGCA 600
PsoR_Pfl_Pf-5     GGCATTACAGGACTTGCAACAGGTTCAGGGCCACTTCACCCTGCTTGCTCATGCGTTGCA 515
                  *.**********************************************************
```

```
PsoR_pnt          GGAGCATCTCTATCCACAGCTCAGCAAATGCGTTCGCAGTTATCCGGTTGATCTGACCCG 660
PsoR_Pfl_Pf-5     GGAGCATCTCTATCCACAGCTCAGCAAATGCGTTCGCAGTTATCCGGTTGATCTGACCCG 575
                  ************************************************************

PsoR_pnt          GCGTGAACGCGAGTGCCTGAAATGGGCTGCAGAGGGCATGACTTCCGGCGAGATTGCCGA 720
PsoR_Pfl_Pf-5     GCGTGAACGCGAGTGCCTGAAATGGGCTGCAGAGGGCATGACTTCCGGCGAGATTGCCGA 635
                  ************************************************************

PsoR_pnt          GCGCCTGCAGCGTTCGCAGGCCACGATCAACCTGCACCTGACCTCGGCGATGCACAAGCT 780
PsoR_Pfl_Pf-5     GCGCCTGCAGCGTTCGCAGGCCACGATCAACCTGCACCTGACCTCGGCGATGCACAAGCT 695
                  ************************************************************

PsoR_pnt          GGGCGCCAGGAATCGGGTTCAGGCGGTGGTTCGCGCGATTCACTATCGCCTGCTGGGTAA 840
PsoR_Pfl_Pf-5     GGGGGCCAGGAATCGGGTTCAGGCGGTGGTTCGCGCGGTTCACTATCGCCTGCTGGGTAA 755
                  *** *********************************.**********************

PsoR_pnt          CTGAGGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAATTCGCCCTATA 900
PsoR_Pfl_Pf-5     CTGA-------------------------------------------------------- 759
                  ****

PsoR_pnt          GTGAGTCGTATTACGCGCGCTCACTGGCCGTCGTTTTACAACGTCGTGAC 950
PsoR_Pfl_Pf-5     --------------------------------------------------
```

Appendix 7-6. Preparation of *E. coli* competent cells

Based on methodology from Hanahan (Hanahan, Jessee et al., 1991).

- Take the frozen stock of cell type and streak out on an LB media plate. Incubate overnight at 37 C.
- Pick a colony off of the fresh streak plate and inoculate 10 mL of LB media. Grow overnight
- Inoculate 1 mL of the overnight culture into 100 mL of prewarmed LB media and grow at 37 C in a shaker until the OD_{600} reaches 0.6
- Transfer the cells to pre-chilled falcon tubes and pellet the cells at 4,000 rpm at 4 ºC for 10 -15 minutes. Drain thoroughly by inverting and tapping on paper towels to remove all traces of media.
- Resuspend cells by pipetting in 1/3 the original culture volume in $CaCl_2$ 0.1 M, and incubate on ice for at least 3 hours.
- Pellet the cells by centrifuging at 4000 rpm at 4. Resuspend cells by pipetting in 8 mL of RF2. Incubate on ice for 15 minutes.
- Pipet cells into 100 µL of cells into sterile prechilled tubes .
- Flash freeze cells in dry ice or liquid nitrogen and transfer to -80 °C.

RF2 Solution
0.5M MOPS (pH 6.8) $RbCl_2$
$CaCl_2 \cdot 2H_2O$
Glycerol
Sterilize by filtration through 22 µm
L 20 mL 1.2 11 118 mL
Final Concentration 10 mM 10 mM 75 mM 15 %

Appendix 7-7. Preparation of *Xoo* competent cells

- Inoculate a single colony of *Xanthomonas* in 2 ml of Peptone Sucrose medium and incubate at 30°C and 180 rpm for 24 hours.
- Transfer 1% of this culture (preinoculum) to PS and grow to optical density (OD_{600}) of 0.6-0.8.
- Once the desired OD is reached incubate the flask in ice for 20 min.
- Centrifuge (4°C, 6K, 10 min) and re-suspended in equal volume (volume of original culture) of ice-cold sterile MilliQ (MQ)
- Repeat last step three times.
- Re-suspended in ¼ of the original culture volume of 10% ice-cold glycerol and centrifuge (4°C, 6K, 10 min) twice.
- Finally, re-suspend in 1/500 of the initial culture volume of 10% glycerol and dispensed in aliquots of 40 µl in 1.5 mL microfuge tubes.
- If required, flash freeze cells in dry ice or liquid nitrogen and transfer to -80 °C.

Appendix 7-8. Electroporation of Xanthomonas cells

(T.J. White, 1991)

1. Around 300 ng of plasmid(s) is mixed with electrocompetent *xanthomonas* cells prepared using the above protocol and kept in ice.
2. This mixture is then transferred to 2mm gap cuvette (at the bottom). The cuvette should be kept in ice before and after adding the mixture of cells and DNA.
3. Electroporate at a voltage of 2.42 kV for 5 msec.
4. Immediately after electroporation add 1 ml of PS directly into the cuvette, mix and pipettte out to a fresh test tube so that cells can be grown subsequently with adequate aeration.
5. The transformed Xanthomonas cultures are grown for 3 hours at 28ºC at 180 rpm before plating on PSA (peptone sucrose agar; 12gms agar per litre) plates containing the desired antibiotic and incubate at 28ºC.
6. *Xanthomonas* colonies will begin to appear approximately 4-6 days after plating.

Appendix 7-9. Bi-parental conjugation protocol.

Xanthomonas oryzae:

- Grow *Xoo* strains in 50ml of PS media with appropriate antibiotics (if required) at 30 ºC degrees for 48 hrs. Grow *E. coli* S17-1λ-pir strains in LB + appropriate antibiotic at 37 ºC overnight
- Wash cells twice in phosphate buffered saline (PBS).
- Pellet cells, concentrate 40-fold.
- Mix *Xoo* and *E. coli* cells in a 1:1 ratio
- Spot mixture in PSA plates overlaid with a membrane (Millipore).
- Incubate overnight at 30 ºC
- Plate on PSA plates with appropriate antibiotic and kept at 30 degrees. Also plate original *Xoo* and *E. coli* colonies as controls.
- Exconjugants should grow in 4-5 days.

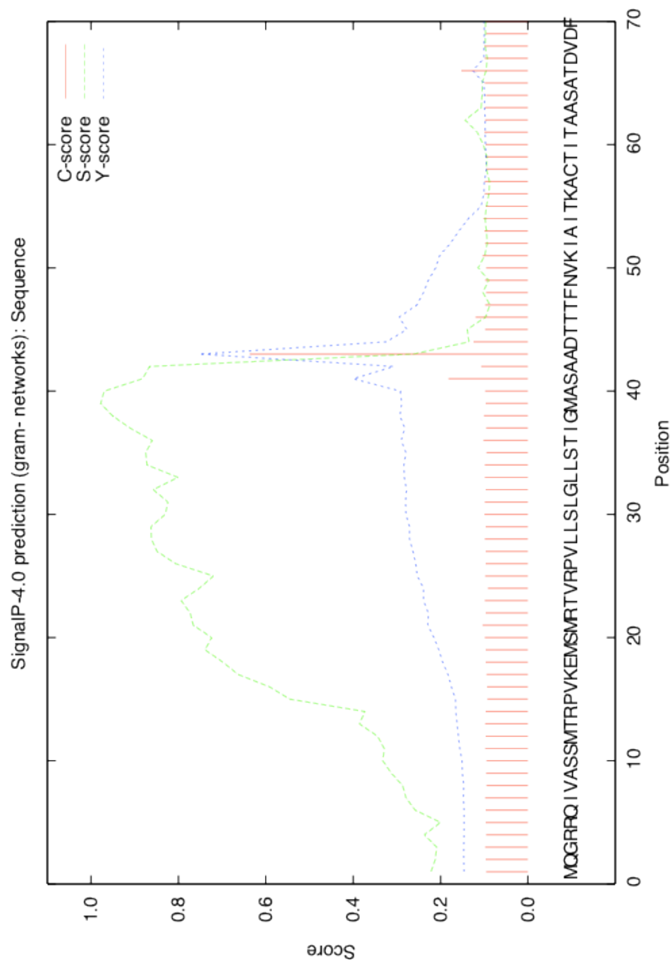
Pseudomonas fluorescens:

- *Grow P. fluorescens* in 20ml LB with appropriate antibiotics (if required) without shaking at 35 ºC degrees over night. Grow *E. coli* S17-1λ-pir strains in LB + appropriate antibiotic at 37 ºC overnight.
- Wash cells twice in phosphate buffered saline (PBS).
- Pellet cells, concentrate 40-fold.
- Mix *P. fluorescens* and *E. coli* cells in a 1:1 ratio
- Spot mixture in LB plates overlaid with a membrane (Millipore).
- Incubate for 3 hours at 37 ºC.
- Plate on LB solid media with appropriate antibiotics. Also plate original *P. fluorescens* and *E. coli* colonies as controls.
- Exconjugants should grow within 2 days.

Appendix 7-10. Protein U

Sequence:

```
MQGRRQIVASSMTRPVKEMSMRTVRPVLLSLGLLSTIGMASAADTTTFNVKIAITKACTITAASATDV
DFGSVLSTSTANVDANGSVTAQCTALTPYNIALSAGDNAATANDVTTRRMKNTDPLVTTNNFIAYQLY
QDIARSTVWGSTTGTNTLSRTATGINQVYPVYGRVTNPAASNAATGSYQDTITATIV
```



# Measure	Position	Value	Cutoff	signal peptide?
max. C	43	0.637		
max. Y	43	0.752		
max. S	39	0.978		
mean S	1-42	0.633		
D	1-42	0.696	0.570	YES

Name=Sequence SP='YES' Cleavage site between pos. 42 and 43: ASA-AD D=0.696 D-cutoff=0.570 Networks=SignalP-noTM

Appendix 7-11. *In planta* proteomics results

Xoo in *planta* proteomics analysis. Relative abundance of expressed proteins in wild type and OryR- knockout mutant

Wild Type

Accession	Description	PAI
gi58583319	outer membrane protein	3864.7
gi161898984	**flagellin**	**2970.3**
gi58581227	ferric enterobactin receptor	2185.8
gi58583210	elongation factor Tu	2088.2
gi58581525	hypothetical protein XOO1902	1829.3
gi58584079	organic hydroperoxide resistance protein	1630.4
gi58579754	VirK	1621.6
gi58583889	hypothetical protein XOO4266	1606.4
gi58581407	TonB-dependent receptor	1519.8
gi122879315	1,4-beta-cellobiosidase	1512.6
gi58583822	hypothetical protein XOO4199	1369.9
gi58581654	molecular chaperone DnaK	1310.0
gi58583911	chaperonin GroEL	1225.9
gi121632125	30S ribosomal protein S7	1162.8
gi58579632	biopolymer transport ExbB protein	1123.6
gi58580951	50S ribosomal protein L19	1111.1
gi58583102	hypothetical protein XOO3479	1111.1
gi58580739	polyphosphate-selective porin O	1068.4
gi161899006	30S ribosomal protein S16	1052.6
gi58584205	outer membrane protein	1030.9
gi122879107	cysteine protease	1030.9
gi58583197	30S ribosomal protein S17	990.1
gi58581605	protein U	985.2
gi58582823	hypothetical protein XOO3200	980.4
gi58580357	chorismate mutase	975.6
gi58582993	lipase; esterase	944.9
gi58582335	superoxidase dismutase	881.1
gi58579930	toluene tolerance protein	854.7
gi58580149	hypothetical protein XOO0526	851.1
gi58580465	outer membrane protein	834.0
gi58580013	TonB-like protein	833.3
gi58584049	hypothetical protein XOO4426	810.8
gi58583215	50S ribosomal protein L7/L12	793.7
gi161898968	50S ribosomal protein L18	781.3
gi58584111	glutamine synthetase	772.2
gi122879220	chemotaxis protein	751.9
gi58583826	hypothetical protein XOO4203	732.6
gi122879275	30S ribosomal protein S12	729.9
gi58581666	dihydrolipoamide succinyltransferase	712.6
gi58582451	superoxidase dismutase	711.7
gi58580207	hypothetical protein XOO0584	709.2
gi58581418	glycine cleavage system protein H	709.2
gi58580534	PilH family regulatory protein	699.3
gi58583775	30S ribosomal protein S9	689.7
gi58581103	cysteine protease	687.3
gi58583219	50S ribosomal protein L11	671.1
gi58581354	hypothetical protein XOO1731	660.1
gi58581139	hypothetical protein XOO1516	653.6
gi58584155	hypothetical protein XOO4532	649.4
gi161898969	50S ribosomal protein L15	649.4
gi58581428	hypothetical protein XOO1805	581.4
gi58582913	peptidyl-prolyl cis-trans isomerase	574.7
gi58582825	pre-pilin like leader sequence	558.7
gi122879058	ketol-acid reductoisomerase	555.6
gi58583181	DNA-directed RNA polymerase subunit alpha	551.0
gi58583216	50S ribosomal protein L10	549.5
gi58580352	F0F1 ATP synthase subunit delta	549.5
gi58583977	gas vesicle protein	546.4
gi58583191	50S ribosomal protein L6	543.5
gi58580353	F0F1 ATP synthase subunit alpha	542.5
gi58581134	peptidoglycan-associated outer membrane lipoprotein	540.5
gi58583036	pyruvate kinase	538.6
gi58581228	hypothetical protein XOO1605	523.6

OryR- mutant

Accession	Description	PAI
gi58583319	outer membrane protein	3864.7
gi58581525	hypothetical protein XOO1902	3658.5
gi58583210	elongation factor Tu	2552.2
gi58580722	TonB-dependent receptor	2168.7
gi58583775	30S ribosomal protein S9	2069
gi58583889	hypothetical protein XOO4266	2008
gi58583822	hypothetical protein XOO4199	1826.5
gi58581227	ferric enterobactin receptor	1748.6
gi58583102	hypothetical protein XOO3479	1666.7
gi58584079	organic hydroperoxide resistance protein	1630.4
gi58579754	VirK	1621.6
gi58583911	chaperonin GroEL	1576.2
gi121632042		1388.9
gi121632071:reversed		1333.3
gi58579952	polyvinylalcohol dehydrogenase	1306
gi58584155	hypothetical protein XOO4532	1298.7
gi58580149	hypothetical protein XOO0526	1276.6
gi58581493	cold shock protein	1219.5
gi58581666	dihydrolipoamide succinyltransferase	1187.6
gi122879315	1,4-beta-cellobiosidase	1176.5
gi121632125	30S ribosomal protein S7	1162.8
gi58582504	hypothetical protein XOO2881	1129.9
gi58583181	DNA-directed RNA polymerase subunit alpha	1101.9
gi58581244		1098.9
gi161899006	30S ribosomal protein S16	1052.6
gi122879107	cysteine protease	1030.9
gi58583202		1020.4
gi161898974		990.1
gi58581605	protein U	985.2
gi58583149	hypothetical protein XOO3526	925.9
gi122879256	catalase	912.6
gi58581347	DNA-binding related protein	905
gi58582335	superoxidase dismutase	881.1
gi58581654	molecular chaperone DnaK	873.4
gi58580739	polyphosphate-selective porin O	854.7
gi58579930	toluene tolerance protein	854.7
gi58580698	peptide deformylase	840.3
gi58580007		833.3
gi122879255	fructose-bisphosphate aldolase	826.4
gi58584205	outer membrane protein	824.7
gi58584049	hypothetical protein XOO4426	810.8
gi58582675	hypothetical protein XOO3052	793.7
gi58583215	50S ribosomal protein L7/L12	793.7
gi58582993	lipase; esterase	787.4
gi58583560	hypothetical protein XOO3937	787.4
gi58581150	OmpA family protein	781.3
gi58583308	hypothetical protein XOO3685	772.2
gi58579632	biopolymer transport ExbB protein	749.1
gi58582344		746.3
gi161898984	**flagellin**	**742.6**
gi58580420	phosphoglucomutase; phosphomannomutase	731.3
gi122879275	30S ribosomal protein S12	729.9
gi122879067	cellulase S	714.3
gi304342476	30S ribosomal protein S8	704.2
gi58580534	PilH family regulatory protein	699.3
gi58584124	partition protein	692
gi58581612	succinyl-diaminopimelate desuccinylase	678.7
gi122879150	30S ribosomal protein S2	678
gi58583203	50S ribosomal protein L2	671.1
gi58583219	50S ribosomal protein L11	671.1
gi58580465	outer membrane protein	667.2
gi58584247:reversed		653.6
gi58584159	2-hydroxyhepta-2,4-diene-1, 7-dioateisomerase/5-carboxymethyl-2-oxo-hex-3-ene-1, 7-dioatedecarboxylase	651.5

gi58581062	single-stranded DNA-binding protein	523.6
gi58583189	30S ribosomal protein S5	520.8
gi58580909	hypothetical protein XOO1286	512.8
gi58581292	outer membrane protein P6 precursor	510.2
gi58582130	hypothetical protein XOO2507	505.1
gi58581147	hypothetical protein XOO1524	500.0
gi58580586	adenylate kinase	500.0
gi58581596	ribosome recycling factor	492.6
gi58581803	30S ribosomal protein S1	486.2
gi122879332	hypothetical protein XOO4267	476.2
gi58582195	flagellar hook protein FlgE	468.4
gi58583738	dehydrogenase	459.8
gi122879274	50S ribosomal protein L4	458.7
gi58581612	succinyl-diaminopimelate desuccinylase	452.5
gi58581347	DNA-binding related protein	452.5
gi58582427	multidrug resistance protein	444.4
gi58581827	methyl-accepting chemotaxis protein	433.5
gi58583386	two-component system regulatory protein	425.5
gi58580784	fimbrial assembly protein	419.0
gi58582533	xylose isomerase	410.7
gi58583369	peptidyl-prolyl cis-trans isomerase	400.0
gi122879349	TonB-dependent receptor	395.3
gi122879043	F0F1 ATP synthase subunit beta	392.9
gi58580601	colicin I receptor	391.6
gi122879256	catalase	391.1
gi58581150	OmpA family protein	390.6
gi58582328	elongation factor P	380.2
gi122879062	hypothetical protein XOO1004	375.2
gi58580781	fimbrial assembly membrane protein	370.4
gi58583790	isocitrate dehydrogenase	367.6
gi58581690	hypothetical protein XOO2067	366.3
gi58580836	serine/threonine protein kinase	366.3
gi58583505	cytochrome C4	363.6
gi58580722	TonB-dependent receptor	361.4
gi58581083	phosphoglyceromutase	357.1
gi161898961	indole-3-glycerol-phosphate synthase	348.4
gi122879266:reversed		347.2
gi58582322	polygalacturonase	341.9
gi122879150	30S ribosomal protein S2	339.0
gi58581293	hypothetical protein XOO1670	337.8
gi58583203	50S ribosomal protein L2	335.6
gi122879347	hypothetical protein XOO4355	330.0
gi58581474	periplasmic protease	328.9
gi58584159	2-hydroxyhepta-2,4-diene-1, 7-dioateisomerase/5-carboxymethyl-2-oxo-hex-3-ene-1, 7-dioatedecarboxylase	325.7
gi58581053	hypothetical protein XOO1430	310.6
gi58580713	glutaredoxin-like protein	304.0
gi58583562	6-phosphogluconate dehydrogenase-like protein	303.0
gi122879306	sugar kinase	290.7
gi58584153	hypothetical protein XOO4530	288.2
gi58581688	FKBP-type peptidyl-prolyl cis-trans isomerase; rotamase	281.7
gi58584208	D-3-phosphoglycerate dehydrogenase	280.9
gi58580826	glyceraldehyde-3-phosphate dehydrogenase	277.0
gi58582006	hypothetical protein XOO2383	271.7
gi58580017	TonB-dependent receptor	269.5
gi58581694	fructokinase	268.1
gi58580790	methanol dehydrogenase regulatory protein	266.0
gi58581490	beta-hexosaminidase	265.3
gi122879258	alanyl dipeptidyl peptidase	259.4
gi58582011	phosphoserine aminotransferase	258.4
gi58580070	catalase	256.4
gi122879004	cellulase	255.1
gi58584070	oxidoreductase	253.2
gi58580780	fimbrial assembly membrane protein	251.9
gi58583405	voltage-gated potassium channel beta subunit	250.0
gi58582467	chemotaxis protein	243.0
gi58580293	DNA polymerase related protein	241.0
gi58582161	acetyl-CoA acetyltransferase	239.2
gi58583345:reversed		238.1
gi58582319	putative pectinesterase	236.4
gi58583149	hypothetical protein XOO3526	231.5
gi58579630	hypothetical protein XOO0007	230.9

gi161898969	50S ribosomal protein L15	649.4
gi58582037	50S ribosomal protein L9	636.9
gi58582586	phosphopyruvate hydratase	613.5
gi58580120:reversed		613.5
gi58582039	30S ribosomal protein S6	609.8
gi58581407	TonB-dependent receptor	607.9
gi58580713	glutaredoxin-like protein	607.9
gi58583562	6-phosphogluconate dehydrogenase-like protein	606.1
gi58581643	hypothetical protein XOO2020	595.2
gi122879043	F0F1 ATP synthase subunit beta	589.4
gi58580013	TonB-like protein	555.6
gi122879058	ketol-acid reductoisomerase	555.6
gi58580951	50S ribosomal protein L19	555.6
gi58583216	50S ribosomal protein L10	549.5
gi58583977	gas vesicle protein	546.4
gi58580721		545.7
gi58583191	50S ribosomal protein L6	543.5
gi58581134	peptidoglycan-associated outer membrane lipoprotein	540.5
gi58583036	pyruvate kinase	538.6
gi58581694	fructokinase	536.2
gi58580563	3-isopropylmalate dehydrogenase	523.6
gi58581228	hypothetical protein XOO1605	523.6
gi58581062	single-stranded DNA-binding protein	523.6
gi58580691	outer membrane protein Slp	518.1
gi58582322	polygalacturonase	512.8
gi58580070	catalase	512.8
gi58580909	hypothetical protein XOO1286	512.8
gi58581292	outer membrane protein P6 precursor	510.2
gi58582130	hypothetical protein XOO2507	505.1
gi58581147	hypothetical protein XOO1524	500
gi122879002		497.5
gi58581474	periplasmic protease	493.4
gi58581596	ribosome recycling factor	492.6
gi58583268	alkyl hydroperoxide reductase subunit C	490.2
gi58581803	30S ribosomal protein S1	486.2
gi58581428	hypothetical protein XOO1805	484.5
gi58581216	succinyl-CoA synthetase subunit beta	483.1
gi122879332	hypothetical protein XOO4267	476.2
gi58580207	hypothetical protein XOO0584	472.8
gi58582319	putative pectinesterase	472.8
gi58582427	multidrug resistance protein	444.4
gi58580656	ATP-dependent Clp protease proteolytic subunit	438.6
gi58583220		431
gi58583386	two-component system regulatory protein	425.5
gi58580551		403.2
gi58582819	PilY1	402.6
gi58583369	peptidyl-prolyl cis-trans isomerase	400
gi58580601	colicin I receptor	391.6
gi122879258	alanyl dipeptidyl peptidase	389.1
gi58584111	glutamine synthetase	386.1
gi58582328	elongation factor P	380.2
gi122879062	hypothetical protein XOO1004	375.2
gi58584160		371.7
gi58580781	fimbrial assembly membrane protein	370.4
gi58583790	isocitrate dehydrogenase	367.6
gi58583505	cytochrome C4	363.6
gi58581083	phosphoglyceromutase	357.1
gi58582451	superoxidase dismutase	355.9
gi122879094	ferric enterobactin receptor	353.8
gi122879093	ferric enterobactin receptor	350.5
gi161898961	indole-3-glycerol-phosphate synthase	348.4
gi122879266:reversed		347.2
gi58581217	succinyl-CoA synthetase subunit alpha	339
gi58581293	hypothetical protein XOO1670	337.8
gi58580347		335.6
gi122879347	hypothetical protein XOO4355	330
gi58580701	rhamnogalacturonase B	328.9
gi58583154		327.9
gi58581809	UTP-glucose-1-phosphate uridylyltransferase	311.5
gi58580492	hypothetical protein XOO0869	309.6
gi58580903		303

gi58583483	serine hydroxymethyltransferase	222.7
gi58581229	hypothetical protein XOO1606	221.2
gi58582181	chemotaxis protein	215.1
gi58581695	hypothetical protein XOO2072	214.6
gi58582213	nucleotide sugar transaminase	209.6
gi58581505	serine protease	206.2
gi58582436	regulator of pathogenicity factors	206.2
gi58582586	phosphopyruvate hydratase	204.5
gi58580777	type II citrate synthase	203.3
gi58582856	NADH dehydrogenase subunit D	202.4
gi122879039	outer membrane protein	201.6
gi122879157	adenylosuccinate lyase	201.2
gi58584111:reversed		193.1
gi122879311	cellulase	191.2
gi58580538	adenylosuccinate synthetase	189.8
gi122879102	response regulator	185.5
gi58579873	OmpA-related protein	183.7
gi58580420	phosphoglucomutase; phosphomannomutase	182.8
gi58582842	transcription elongation factor NusA	181.8
gi58582247	chemotaxis related protein	168.6
gi122879019	peptidase	167.2
gi58583461	hypothetical protein XOO3838	166.9
gi58580701	rhamnogalacturonase B	164.5
gi58583659	cellulase	161.6
gi58579717	hypothetical protein XOO0094	156.3
gi58580596	GTP-binding elongation factor protein	149.0
gi58583100:reversed		147.5
gi58580022	hypothetical protein XOO0399	146.8
gi58579915	phytochrome-like protein	142.0
gi58581372	pilus biogenesis protein	139.3
gi58580660	peptidyl-prolyl cis-trans isomerase	136.4
gi58583612	aminopeptidase	132.8
gi58583535	HmsF	132.6
gi58584021	glycyl-tRNA synthetase subunit beta	125.5
gi122879174	beta-glucosidase	124.8
gi58583534	HmsH	123.5
gi58583304	hypothetical protein XOO3681	122.4
gi122879095	ferric enterobactin receptor	118.6
gi122879094	ferric enterobactin receptor	117.9
gi58582160	3-hydroxyacyl-CoA dehydrogenase	117.1
gi122879243:reversed		114.5
gi58582433:reversed		111.0
gi58581591	outer membrane antigen	109.8
gi58584095	hypothetical protein XOO4472	101.8
gi58582488	aconitate hydratase	100.9
gi58582745	hypothetical protein XOO3122	65.0

gi58581050	aspartate carbamoyltransferase catalytic subunit	297.6
gi58581827	methyl-accepting chemotaxis protein	289
gi58584153	hypothetical protein XOO4530	288.2
gi58581688	FKBP-type peptidyl-prolyl cis-trans isomerase; rotamase	281.7
gi122879015	delta-aminolevulinic acid dehydratase	274
gi58582882		272.5
gi58582006	hypothetical protein XOO2383	271.7
gi58580790	methanol dehydrogenase regulatory protein	266
gi58583535	HmsF	265.3
gi58581490	beta-hexosaminidase	265.3
gi58580780	fimbrial assembly membrane protein	251.9
gi58583038	phosphoglycerate kinase	245.1
gi58582823	hypothetical protein XOO3200	245.1
gi58582161	acetyl-CoA acetyltransferase	239.2
gi58582195	flagellar hook protein FlgE	234.2
gi58579630	hypothetical protein XOO0007	230.9
gi122879113		226.8
gi58583483	serine hydroxymethyltransferase	222.7
gi58581229	hypothetical protein XOO1606	221.2
gi58583709		218.3
gi58582181	chemotaxis protein	215.1
gi58581695	hypothetical protein XOO2072	214.6
gi58582436	regulator of pathogenicity factors	206.2
gi58582533	xylose isomerase	205.3
gi58581689		204.5
gi58580777	type II citrate synthase	203.3
gi58582856	NADH dehydrogenase subunit D	202.4
gi58581291		202
gi58580550	hypothetical protein XOO0927	201.6
gi58582926	hypothetical protein XOO3303	201.6
gi122879157	adenylosuccinate lyase	201.2
gi58581680	fumarate hydratase	200.8
gi122879023		199.2
gi58580419	phosphomannose isomerase; GDP-mannose pyrophosphorylase	196.1
gi58580457		196.1
gi122879061	family II 2-keto-3-deoxy-D-arabino-heptulosonate 7-phosphate synthase	195.7
gi122879311	cellulase	191.2
gi58582110		186.6
gi58582212		184.2
gi58579873	OmpA-related protein	183.7
gi58580655		182.8
gi58582842	transcription elongation factor NusA	181.8
gi58580353	F0F1 ATP synthase subunit alpha	180.8
gi58580040	catalase precursor	177.3
gi58582674	hypothetical protein XOO3051	171.2
gi58582247	chemotaxis related protein	168.6
gi58583461	hypothetical protein XOO3838	166.9
gi58581354	hypothetical protein XOO1731	165
gi58583659	cellulase	161.6
gi58579737	maltooligosyltrehalose trehalohydrolase	156.5
gi58579717	hypothetical protein XOO0094	156.3
gi58581212		154.1
gi58583751		153.1
gi58580596	GTP-binding elongation factor protein	149
gi58581279	aspartyl-tRNA synthetase	147.9
gi58580022	hypothetical protein XOO0399	146.8
gi58580784	fimbrial assembly protein	139.7
gi58581372	pilus biogenesis protein	139.3
gi58580660	peptidyl-prolyl cis-trans isomerase	136.4
gi58583612	aminopeptidase	132.8
gi58581547		132.6
gi58582745	hypothetical protein XOO3122	130
gi58580249	ferrichrome-iron receptor 3	128.4
gi58584021	glycyl-tRNA synthetase subunit beta	125.5
gi122879174	beta-glucosidase	124.8
gi58583306:reversed		114.2
gi58582433	multiphosphoryl transfer protein	111
gi58582488	aconitate hydratase	100.9
gi58580960	ATP-dependent Clp protease subunit	100.7
gi58580017	TonB-dependent receptor	89.8

Appendix 7-12. Promoter trapping

```
32A             GCTGTACAGCGAAGAGCTGAAGAACTACCTCAACCGCTTCAGCCCCAACGATCAGCGCGA 60
Pfl_0133        -----------------------------------------------ATGAGCAGTGT-- 11
                                                               * ** *** *    

32A             CGGCAAGATCCGCACCGTCCTGCCCACCGACCTGGACCGCTGCTTTGCCATCGAGACCCT 120
Pfl_0133        --------------------TG----------------------TTGCCATCGT------ 23
                                    **                      *********        

32A             GGCCTACGAAGGCGACGAGGCCGCGACCCGGGACAAGATCGCGAGGCCTGGGTCGAGGGG 180
Pfl_0133        ------CAAAAGCA---------------------------------------------- 31
                      * ** **                                                

32A             CTGCTGGCCAGCGGCGGGCGCGGTTTCGCTGTGCCGACATAGGGCTGTTGATCCGACGCG 240
Pfl_0133        TTGTCGGCCAA--GTTTTCGTGGTTTCCCCG----GA---AGG------GATCCGACGCG 76
                 **  *****   *    ** ****** * *    **   ***      ***********

32A             TACTCATTGAAGGGGACCGCCTGTTCGTGGGTGACGAGGTAGACACCGGCCCGGCCGGGG 300
Pfl_0133        TACTCATTGAAGGGGACCGCCTGTTCGTGGGTGACCAGGTAGACACCGGCCCGGCCGGGG 136
                ********************************** *************************

32A             CCGTCACCCTGGAGTTGACCGACGGGCGTACTCTGGACCTGGGGCGTGACACCCAGTGGA 360
Pfl_0133        CCGTCACCCTGGAGTTGACCGACGGGCGCACTCTGGACCTGGGGCGTGACACCCAGTGGA 196
                **************************** *******************************

32A             GCGGTGCCGCACCGGACTCCAGCACGGACCTGGCACAGGCCACCGCTCAGGCCGCCCCTT 420
Pfl_0133        GCGGTGCCGCACCGGACTCCAGCACGGACCTGGCACAGGCCACCGCTCAGGCCGCCCCTT 256
                ************************************************************

32A             CGGTGGCAGAACTGCAGCAGGCCATCGCCGCCGGCGCCGACCCGACCACCGACCTGGAAG 480
Pfl_0133        CGGTGGCAGAACTGCAGCAGGCCATCGCCGCCGGCGCCGACCCGACCACCGACCTGGAAG 316
                ************************************************************

32A             CCACCGCCGCCGGCCCGACCGCCGCGGGGAATGGCGGCGCGGCCGGTGGTGGCCACAGTT 540
Pfl_0133        CCACCGCCGCCGGCCCGACCGCCGCGGGGAATGGCGGCGCGGCCGGTGGTGGCCACAGTT 376
                ************************************************************

32A             TTGTGCAGTTGGAAGAGACTGCGGGCCGGGTCGACCCGACCATCGGTTTCCCCACCGCTG 600
Pfl_0133        TTGTGCAGTTGGAAGAGACTGCGGGCCGGGTCGACCCGACCATCGGTTTCCCCACCGCTG 436
                ************************************************************

32A             GCCTGGGGCTGGCCAATTCGACCCAGGACAACACCTTTGGCGGCCTGACCAACAATGGCG 660
Pfl_0133        GCCTGGGGCTGGCCAATTCGACCCAGGACAACACCTTTGGCGGCCTGACCAACAATGGCG 496
                ************************************************************

32A             GCCTGGGCAATGGCAACGGCAATGGCAGCGGGAGTGACTCCCTGCGCCCGGCCACCCTGA 720
Pfl_0133        GCCTGGGCAATGGCAACGGCAATGGCAGCGGGAGTGACTCCCTGCGCCCGGCCACCCTGA 556
                ************************************************************

32A             CCCTGGGCGCCACCCCAACCATTACCGAAGCCGGTGGCACCCTGGTGTACACCGCGACCC 780
Pfl_0133        CCCTGGGCGCCACCCCAACCATTACCGAAGCCGGTGGCACCCTGGTGTACACCGCGACCC 616
                ************************************************************

32A             TGACCCAGGCGCCACTTACCGACCTGACTGTGACCCTGTCCAATGGCGCGGTCATCGTCA 840
Pfl_0133        TGACCCAGGCGCCACTTACCGACCTGACTGTGACCCTGTCCAATGGCGCGGTCATCGTCA 676
                ************************************************************

32A             TCAGCGCCGGCCAGACCACTGGCAGCGTGCAGGTGCCCCTGGCGCCCCAGGACAACGTCT 900
Pfl_0133        TCAGCGCCGGCCAGACCACTGGCAGCGTGCAGGTGCCCCTGGCGCCCCAGGACAACGTCT 736
                ************************************************************

32A             ATATCGACCCGGGCCAGATCTGC-TTAGCA----------------------------GC 931
Pfl_0133        ATATCGACCCGGGCCAGATCAGCGTCAGCGTGACCGGCACCACCGGCGGTGGCGGCCTGC 796
                ******************** ** * ***                            **

32A             T-----------------CCCCNG------------------------------------ 938
Pfl_0133        TGGTAACGGTCAACCCGACCCCGGCGGTCACCCAGGTCACCGACACCATCGACACCACCA 856
                *                 **** *                                    

32A             -------------------------------------------GNTTNNCTNTNN----- 950
Pfl_0133        CCGTCAGCCTGACCGCTGATGCCAGCGTTACCGAGGGCGGGCAGATCACCTACACCGCCA 916
                                                           * *   **          

32A             ------------------------------------------------------------
Pfl_0133        CCCTCACCAACCCGGCACAGACTCCGGTGACCATCACCCTGTCCAACGGCTCGGTGATCA 976

32A             ------------------------------------------------------------
Pfl_0133        CTATCGAAGCGGGCAAGTCCAGTGGCTCGATCGACGTCCCGGCGCCAGCCAATGACGTCT 1036

32A             ------------------------------------------------------------
Pfl_0133        ACAAGAACGGCGACACCATCGAAGTCGGTATCACCAAGGCTGAAGGCGGCAACTTCGAGA 1096

32A             ------------------------------------------------------------
Pfl_0133        ACCTGCAACCCAGCCCAGACAAAGCGGTCACCACCGTGACCGACAGCATCGACGACACCG 1156

32A             ------------------------------------------------------------
Pfl_0133        GCCTGAGCCTGTCGGCCACCGATACCGTGGCCGAGGGTGGCTCCATCGTCTACACCGCCA 1216

32A             ------------------------------------------------------------
Pfl_0133        CTCTGACCAACCCTGCTGGCACCCCGGTCACCGTGACCCTGAGCAACGGCGCGGTAATCA 1276

32A             ------------------------------------------------------------
Pfl_0133        CCATCGCTGCCGGTGCCACTTCCGGTACTGCCACCGTCGCCGCCCCTGCCGATGATGTCT 1336

32A             ------------------------------------------------------------
Pfl_0133        ATAAAGACGCGGGCAAGGTTGAAGTCACCATCACCAAGGCCGATGGCGGCAATTTCGAAA 1396

32A             ------------------------------------------------------------
Pfl_0133        ACCTGGTCACCGACCCGGCTCCGGCTGTGACCGATGTCACTGACACGATCGATACTTCGA 1456
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Colony 38

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Colony_38       -----------------NNNNNGN---------------NCCTGCTGC------ATCG-- 20
Pfl_4057        ATGACTACTTCCCCACCTCGTGGTGCAGCGCCCAAGACCTCCCGCGGCGGCGAGATCGGT 60
                                 . ...*.               .** ** **      ****
Colony_38       -CTG--------CACTTCAACTGGTCGCAGACCTCGTTGGGCCCTCGTGAAGGCTGGCCC 71
Pfl_4057        GCTGCAATCCGTCGCTTCGACTGGTCGCAGACCGCGGTGGGCCCTCGTGAAGGCTGGCCC 120
                 ***        *.****.************** ** ***********************
Colony_38       GGCCTGCTGCCCAATGCCTTGAGCCAAATGCTCGAGTCCCCGGAGAGCCTGTACCTGTTG 131
Pfl_4057        GGCCTGCTGCGCAGTACCTTGAGCCAGATGCTCGAGTCCCCGGAGAGCCTGTACCTGCTG 180
                ********** **.*.**********.****************************** **
Colony_38       TGGGGGCCGCACATGCAGTTCTTCTTCGGCGATGGCTACCGGCCGGCCCTCTGCCCGCGC 191
Pfl_4057        TGGGGGCCGCAGATGCAGTTCTTCTTCAACGATGCCTACCGCCCGGTCCTCGGCCCGCGC 240
                *********** ***************..***** ****** **** **** ********
Colony_38       CTGGCCCATGCCCTGGGGCAGACCATCCAGACCCTCTGGGCCGAGGCCTGGTCGGATGTG 251
Pfl_4057        CTGGCCAATGCCCTGGGGCAGACCATCCAGACCCTCTGGGCCGATGCCTGGCCGGATGTC 300
                ******.************************************* ****** *******
Colony_38       CAGCCGATGGTGGAGCAAGTGTTGCTCGGTTACTCCTGCCGCCACGAAGACCTGCCGGTG 311
Pfl_4057        CAGCCGATGGTGGAGCAGGCGTTTCTCGGTTACTCCTGCCGCCACGAAGACCTGCCGGTG 360
                *****************.* *** ************************************
Colony_38       AACATGCACCGCTACCCTGGCCAAGA-ATGACCTGGTGGTCGTTCTCCATGAGCCCGGTG 370
Pfl_4057        AACATGCAGCGCTACCCTGGCGAAGAGCTGACCTGGTGGTCGTTCTCCATGAGCCCGGTG 420
                ******** ************ **** .********************************
Colony_38       TATGACGAGGACAACTAACAGGTGCTCGGAGTGCTGCGCCACTGTGTGAAGACCCCCCAT 430
Pfl_4057        TATGACGAGGACGACGAACAGGTGCTCGGAGTGCTGTGCCACACCGTGGAGACCACCGAT 480
                ************.** ******************** *****:  ***.*****.** **
Colony_38       CGC-----------CGGGTACCCCCG-------------CATCT---------------- 450
Pfl_4057        CGCGTGCTGCTGGCCCGGCACGCCCGGCAGACCGAGGCGCATCTGCGCGAAACCCGCGAG 540
                ***           * ** ** ****             *****
Colony_38       ----------------------CTAGAAACTGCT---------------ACCTAGGTGG- 472
Pfl_4057        CTCAATACCCGGGTGCTGGCCGCCAGCAACGACTGCATCAAGGTGCTGGACCTCGATGGC 600
                                      * **.*** .**               ****.*.***
Colony_38       ----TATTAATAATGGGA------------------------------------------ 486
Pfl_4057        CAGCTGTCGTTCATGAGCGAAGGTGGCCTGCGGGTCATGGAGGTCAGTGATTTCAATGCG 660
                    *.* .:*.***.*.
Colony_38       ------------------------------------------------------------
Pfl_4057        GTGCGGGGCTGTCCCTGGCCGGACTTCTGGCAGGGCCAGGGCCACCAGGAGGCCCGGGAT 720

Colony_38       ------------------------------------------------------------
Pfl_4057        GCCATTGCTGCCGCGCGCCAGGGTCGCTCGGCGCGGTTCCAGGGCGCCGCCGATACCCTC 780

Colony_38       ------------------------------------------------------------
Pfl_4057        CAGGGGCGCACCAAGTGGTGGGATGTGCAGGTTACGCCGATGCTCGACGCGGCCGGCCAG 840

Colony_38       ------------------------------------------------------------
Pfl_4057        CCGGAGAAGATCCTCTGCATTTCCCGGGACATCAGCGCCACCCGCGAGGCGCAGGAGGAA 900

Colony_38       ------------------------------------------------------------
Pfl_4057        CTGCGGCGGCTCAATGAGTCTCTGGAGCAGCGGGTCCAGGAGCGTACCCAGGAGCGCGAC 960

Colony_38       ------------------------------------------------------------
Pfl_4057        CGGATCTGGCAGCTGTCCACGGACCTGATGCTGGTGGCGGACTTCAATGGCCGCATCGTC 1020

Colony_38       ------------------------------------------------------------
Pfl_4057        GCCGTCAACCCGGCCTGGACCCAGCTTCTGGGCTGGGAGCGCGAGGAACTGGAAGGCAGC 1080

Colony_38       ------------------------------------------------------------
Pfl_4057        AGTTTCTACGATTTCATCCACCCCCAGGACAGCCTGGCCGCAGCGGGGGCGGCGGCCGAT 1140

Colony_38       ------------------------------------------------------------
Pfl_4057        CTGTCCCGGGGCGAGAGCTTTTCGCGCTTCGAGAACCGCTTTCGCCACCGCGACGGTTCC 1200

Colony_38       ------------------------------------------------------------
Pfl_4057        TATCGCTGGATGGCCTGGAGCGCTGTGCCGGACCCGCACCACATTCACGCCGTGGGCCGT 1260

Colony_38       ------------------------------------------------------------
Pfl_4057        GACATGCAGGCCGAGCGCGAGGCCGCCCAGGCGCTGCGCAAGACCGAGGACATCCTGCGC 1320

Colony_38       ------------------------------------------------------------
Pfl_4057        CAGGCGCAGAAGATGGAGGCGGTGGGGCAACTGACCGGTGGCGTGGCCCACGACTTCAAC 1380

Colony_38       ------------------------------------------------------------
Pfl_4057        AACCTGCTGACGGTGATCCGCTCTTCCACCGACCTGTTGCGCCGCAACCTCAGCGAAGAG 1440

Colony_38       ------------------------------------------------------------
Pfl_4057        CGCCGGCAGCTGTACATCAAGGCGATTTCCGACACCGTCGACCGCGCCAGCAAGCTCACC 1500

Colony_38       ------------------------------------------------------------
Pfl_4057        GGCCAGCTCCTGGCGTATGCCCGCCAGCAGAACCTGCGCCCGGAGACCTTCGACGTCGGC 1560

Colony_38       ------------------------------------------------------------
Pfl_4057        GCCAGCGTGCAGTTGCTGGCGCAGATGATCAATCCACTGATCGGCGCCTGCATCGAACTG 1620

Colony_38       ------------------------------------------------------------
Pfl_4057        CGTATCGAGTTGCCGCAGCAGCCTTGCTACATCCACGCCGATGCCGGCCAGTTCGACACG 1680
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Colony_38        ------------------------------------------------------------
Pfl_4057         GCGCTGATCAATATGGCGGTCAATGCCCGGGATGCCATGCAGGGCGAAGGCTTGCTGGTG 1740

Colony_38        ------------------------------------------------------------
Pfl_4057         GTGGCTGTGGAGCGGGTGGCGCAGATTCCCCCCAGTGCCAGCCAGGGTGCCCAGGCCGGG 1800

Colony_38        ------------------------------------------------------------
Pfl_4057         GACTTTGTCGCGGTATCCCTGACTGATACCGGCGCCGGCATCGACCCCCAGCAGATCGAA 1860

Colony_38        ------------------------------------------------------------
Pfl_4057         CGGATCTTCGAACCCTTCTTCACCACCAAGGAGGTGGGCAAGGGCACCGGCCTGGGCTTG 1920

Colony_38        ------------------------------------------------------------
Pfl_4057         TCCCAGGTGTTCGGTTTCGCCCAGCAATCGGGCGGCGGCCTGGCGGTGCACAGCCAGCCG 1980

Colony_38        ------------------------------------------------------------
Pfl_4057         GGGCAGGGCACGTGCTTTACCCTGTACCTGCCGCGCGCGGAGGCCCTGGCCGGCTTGCCG 2040

Colony_38        ------------------------------------------------------------
Pfl_4057         ACGCCAACCCAGGCGGTGGTGCCCATGATCGACGGCCAGGGCATGACCCTGCTGGTGGTG 2100

Colony_38        ------------------------------------------------------------
Pfl_4057         GAGGACAATCCCGAAGTCGGCGTGCTGCTGGCCCAGTCCCTGAACGAGCTGGGCTACCAG 2160

Colony_38        ------------------------------------------------------------
Pfl_4057         ACCGAATGGGCCACCAGCGCGGCGGAGGCCCTGCGCCGCCTGGGCGAGGAGCGGACCCGA 2220

Colony_38        ------------------------------------------------------------
Pfl_4057         TTCCAGGCGGTGTTTTCCGATGTGGTGATGCCGGGCATGAGCGGTATCGAACTGGCCAGC 2280

Colony_38        ------------------------------------------------------------
Pfl_4057         CGGATCCGTTGCGACTACCCGCGGTTGCCGGTCATCCTCACCAGCGGCTACAGCCCGGCC 2340

Colony_38        ------------------------------------------------------------
Pfl_4057         CTGGCCCAGGGCCAGACCCGGGAGTTCGTGTTCCTGCAAAAGCCCTACTCGGTGGCGGAA 2400

Colony_38        ------------------------------------------------
Pfl_4057         CTGGCCCTGGCCCTGAGCGCGGCGATCGGCCAGCCCGAGGTGCCGTAG 2448
```