A novel mechanism of coupling quorum sensing systems in *Rhizobium leguminosarum* bv. *viciae* 3841

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September 2010

Thesis submitted to the University of East Anglia for the degree of Doctor of Philosophy

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Acknowledgments

My time here in Norwich has come to an end, and so this is a good time to reflect on what I have learnt the past four years. Working at the John Innes Centre has been a fantastic experience and I think I have learnt a great deal, both at a scientific and a personal level. This would not have been possible without the help and support of many people around me.

I am very grateful to my supervisor Allan Downie, for giving me the opportunity to come and work in his lab, useful advice and discussions and the freedom to explore this project. Working in the lab would not have been the same without the other lab members. Anne Edwards is probably the most colourful person I have met here in Norwich and with her around, life in the lab never had a chance of becoming boring. She started this project and I am sure I would not have been able to finish it without her expert advice and help. I am also grateful to the other people in the lab - Andrew Stanger, Alan Williams, Jenny Richens, Giulia Morieri and Fang Xie – for many useful discussions and practical help in the lab. I would also like to thank Anna Swiderska, for the initial studies on the *praR* mutant and other people, whose strains and plasmids I have used over the years - Maria Sanchez-Contreras and Craig McAnulla.

I would like to thank my supervisory committee, Mark Buttner and Ray Dixon for many useful discussions and advice. Philip Poole made it possible for me to do microarray analysis, under the expert guidance of Ramakrishnan Karunakaran. I am also grateful to the other people in the department, especially Richard Little for teaching me how to purify proteins and Tung Le for teaching me about protein-DNA interactions. For analysis of the CinS protein, Clare Stevenson helped me with the DLS spectrum and Phil Johnson with the CD spectrum. During this project I had the opportunity to visit the lab of Guofan Hong in Shanghai, to study interactions with sRNA's under the guidance of Bihe Hou, which was a unique experience.

The John Innes Centre is a fantastic place to work for many reasons. I realise just how much time I saved by the work of Shirley, the media kitchen girls and Paula and I am very grateful for that.

I would like to thank my parents and the rest of my family for giving me many chances in life. I would also like to thank my future parents-in-law for their interest and support. Finally, I would like to thank Jan for being there during my whole PhD, sharing the happiness of successful experiments and the disappointments of failed ones.

Marij Frederix, September 2010

Abstract

Rhizobia are Gram-negative bacteria that form a nitrogen-fixing symbiosis with legume plants, resulting in the formation of nodules. Many aspects of nodulation are regulated by quorum-sensing (QS), which is a mechanism by which bacteria regulate their gene expression in a population-density dependent manner. A typical QS system consists of an acylhomoserine lactone (AHL) synthase and a transcriptional regulator that responds to these AHLS.

Analysis of QS in *Rhizobium leguminosarum* revealed an unusual type of gene regulation that relies on the population-density-dependent accumulation of an antirepressor. The *cinS* gene is cotranscribed with the AHL synthase gene *cinI*. CinS couples the induction of the *cin* QS genes with the induction of the *rhi* and *rai* QS genes, by activating the expression of their respective *luxR*-type regulators. Purified CinS bound to the *R. leguminosarum* transcriptional regulator PraR, which represses its own expression and that of *rhiR*. PraR was shown to bind to the *rhiR* and *praR* promoters *in vitro* and CinS displaced PraR from these promoters. Thus, CinS acts as an antirepressor and as it accumulates in a population-density dependent manner, it induces the expression of *rhiR* by attenuating PraR-mediated repression. The LuxR-type regulator ExpR represses *praR* expression, thus leading to induction of *rhiR* and *raiR*.

A *praR* mutant attached more efficiently to pea root hairs, leading to increased competitiveness in the rhizosphere. Microarray analysis showed that amongst the PraR-targets, there are several proteins with a predicted function in root hair attachment (*Rhizobium* adhesion proteins and cadherin proteins). PraR also affected the expression of the transcriptional regulator *rosR,* which regulates exopolysaccharide production, and the extracellular glycanase *plyB*. Mutants in the PraR target genes were obtained and their role in nodulation competitiveness was studied.

Abbreviations

3-OH PAME	3-hydroxypalmitic acid methyl ester
A	Absorbance
aa-UTP	amino-allyl uridine triphosphate
AHL	*N*-acylhomoserine lactone
AI-2	autoinducer 2
Amp	Ampicillin
AMS	acid minimal salts
Apra	Apramycin
APS	ammonium persulphate
ATP	adenosine triphosphate
BSA	bovine serum albumine
bv.	Biovar
CAI-1	*cholerae* autoinducer 1
cAMP	cyclic adenosine monophosphate
CD	circular dichroism
CDF	cell density factor
cGMP	cyclic guanosine monophosphate
cNMP	cyclic nucleoside monophosphate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DF	diffusable factor
dGTP	deoxyguanosine triphosphate
DHP	4,5-dihydroxy-2,3-pentanedione
DKP	diketopiperazine
DLS	dynamic light scattering
DNA	deoxyribonucleic acid
DSF	diffusable signal factor
DTT	dithiothreitol
dTTP	deoxythimidine triphosphate
EDTA	ethylenediaminetetraacetic
EHEC	*enterohemorrhagic E. coli* serotype 0157H7
EMSA	electromobility shift assay
EPS	exopolysaccharide
EtOH	ethanol
FPLC	fast protein liquid chromatography
gfp	green fluorescent protein
Gm	gentamicin

GMP	guanosine monophosphate
HAI-1	*harveyi* autoinducer 1
HNN	hierarchical neural network
HSL	homoserine lactone
HTH	helix-turn-helix
IAA	indole acetic acid
IPTG	isopropyl b-D-1-thiogalactopyranoside
Km	kanamycin
Liv	lividomycin
LPS	lipopolysaccharide
MALDI-ToF	matrix-assisted laser desorption/ionisation time-of-flight
MBP	maltose binding protein
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger ribonucleic acid
MS	mass spectrometry
Neo	neomycin
nod	nodulation
OD	optical density
ONPG	2-nitrophenyl-β-D-galactopyranoside
ORF	open reading frame
PCR	polymerase chain reaction
PMT	photomultiplier tube
ppGpp	guanosine tetraphosphate
PQS	*Pseudomonas* quinolone signal
qrr	quorum regulatory RNA
QS	quorum-sensing
Q-ToF	quantitative time of flight
Rap	rhizobium adhesion protein
rCTP	ribosomal cytidine 5'-triphosphate
RNA	ribonucleic acid
rpm	rounds per minute
rRNA	ribosomal ribonucleic acid
RT	room temperature
SAM	S-adenosyl methionine
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
Spec	spectinomycin
sRNA	small ribonucleic acid
SSC	saline sodium citrate
Strep	streptomycin
TBE	tris/borate/EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
Tet	tetracycline
tRNA	transfer ribonucleic acid
TY	tryptone yeast
UV	ultraviolet

WT	wild type
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside
YEM	yeast extract mannitol

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Chapter 1: Introduction

1.1 The *Rhizobium*-legume symbiosis

1.1.1 Nodulation

Nitrogen is a very important nutrient for plants and is often a limiting factor for plant growth. Although nitrogen is abundant in the atmosphere in the form of N_2, plants can not use it until it is converted into a more readily accessible form like ammonium (NH_4^+) or nitrate (NO_3^-). Chemically this conversion is done using the Haber-Bosch process, which requires high temperatures and pressures and is therefore very energy-demanding. An alternative is biological N_2 fixation by bacteria. Rhizobia are Gram-negative bacteria that can form N_2-fixing symbioses with legume plants. During this symbiosis the legume plant forms a specialised nodule organ, in which the rhizobia differentiate into bacteroids. In these bacteroids the rhizobia convert atmospheric N_2 to NH_4^+ and in return they receive carbon from the plant. The symbioses between legumes and rhizobia are tightly regulated and require specific partnerships between rhizobia and legumes. For example, *Rhizobium leguminosarum* biovar *viciae* is able to form a symbiosis with pea or vetch, but not alfalfa or lotus. *Sinorhizobium meliloti* on the other hand can establish a symbiosis with alfalfa, but not with pea or lotus. The specificity between rhizobia and legumes is determined by an exchange of signal molecules between both symbionts and by surface interactions.

In the first step of nodulation, rhizobia are attracted to the nutrient-rich rhizosphere of the legume plants (Miller et al. 2007). The legume roots exude flavonoids, which diffuse into the bacteria (Recourt et al. 1989) and activate the rhizobial NodD transcriptional regulators (Spaink 2000), inducing the *nod* genes. These are responsible for the biosynthesis of rhizobial signalling molecules called Nod-factors

(for a review see D'Haeze and Holsters 2002; Geurts et al. 2005). Nod-factors are lipochitin oligomer signalling molecules, consisting of four to five β, 1-4 linked *N*-acetylglucosamine units with a lipid attached to the non-reducing end and host-specific modifications on the backbone**.** The type of Nod-factor produced is the primary determinant of the host specificity of the symbiosis between legumes and rhizobia.

Nod-factors are detected by specific plant receptors and induce a Ca^{2+} spiking-dependent signalling pathway in the legume plants (for a review see Oldroyd and Downie 2006). The amount of Nod-factor that is perceived initially by the plant is very low, in the picomolar concentration range. This induces the expression of nodulation-specific genes and cytoskeletal deformations, causing the root hairs to curl, thus entrapping the bacteria in an infection pocket. This causes the concentration of Nod-factor to increase, inducing further responses in the plant (see reviews by D'Haeze and Holsters 2002; Downie and Walker 1999). Infection pockets develop into infection threads by tubular invagination of the legume cell wall. At the tip of this infection thread the rhizobia divide, leading to the formation of a tunnel in the root hair. This tunnel grows through the root cortex until it reaches the nodule primordium, and then delivers the bacteria to the plant cells by endocytosis. This mechanism of infection usually results in a clonal infection, which means that the bacteria reaching the nodule are usually derived from a single infection event (see review by Gage 2004). If two infection threads invade a nodule, this can result in the mixed infection of a nodule. The released rhizobia are enclosed in a plant-derived peribacteroid membrane in which they undergo divisions and diffentiate into bacteroids. Depending on the host legume, different kinds of nodules can be formed (Franssen et al. 1992). Nodules from the indeterminate type are formed by legumes such as clover, pea or alfalfa and are cylindrical in shape, with a persistent apical meristem. Nodules from the determinate type are formed by legumes such as soybean or common bean and are spherical with a nonpersistent meristem.

The organelle-like structures that are formed in the plant nodule cells are called symbiosomes and the bacteroids in these symbiosomes reduce atmospheric N_2 to NH_4^+. This reaction is catalysed by the nitrogenase enzyme complex, the synthesis of which is encoded by the *fix* and *nif* genes (see reviews by Dixon and Kahn 2004; Rubio and Ludden 2005). In order to obtain a high rate of nitrogen fixation, the bacteria need substantial amounts of adenosinetriphosphate (ATP), which is provided by oxidative phosphorylation. However, free oxygen in the nodules could denature the

nitrogenase enzyme complex. To prevent this, the plant regulates oxygen flow into the nodule and also produces large amounts of a haem protein called leghaemoglobin, which acts as an oxygen buffer (Appleby et al. 1983; Downie 2005).

When the nodules start to senesce, many of the bacteria within the nodules lyse and are degraded by plant enzymes as an additional source of nutrients for the plant. Nevertheless, some of the bacteria that were still present in the infection threads survive and are released into the environment (Timmers et al. 2000). This increases the number of these rhizobia in the soil, providing a mechanism to optimise the selection of effective rhizobia by the legume plant.

1.1.2 Attachment of rhizobia to the root hairs

The attachment of rhizobia to the root hairs is the first step in the nodulation process and therefore very important. Although the addition of Nod-factor can induce deformation of root hairs, it is thought that the directional gradient of Nod-factor that is provided by root hair attached rhizobia is required for the curling of the root hairs to be able to entrap the rhizobia (for a review see Downie and Walker 1999). Rhizobial attachment to root hairs starts with a loose association, followed by the formation of a biofilm cap on the root hair.

The first loose attachment to root hairs has been studied in *R. leguminosarum* and is different depending on whether attachment occurs in acidic or alkaline conditions (Laus et al. 2006; Smit et al. 1992; Williams et al. 2008). The Ca^{2+}-binding adhesion protein rhicadhesin is produced by all tested members of the rhizobiaceae and is important for root hair attachment under slightly alkaline conditions, by binding to both the rhizobial surface and the root hair surface (Smit et al. 1989). Rhicadhesin-mediated attachment is not specific for the *Rhizobium*-legume symbiosis, as it was able to mediate binding of rhizobia to both host and non-host plants (Smit et al. 1989). It has been purified from *R. leguminosarum,* but there is no genetic evidence for its role in attachment as the gene encoding rhicadhesin has not been identified (Smit et al. 1989). The adhesion protein RapA1 was identified in *R. leguminosarum* bv. *trifolii* and this protein appears to be somewhat similar to rhicadhesin as it can also bind Ca^{2+} and is involved in rhizobial attachment (Ausmees et al. 2001). Nevertheless it is probably not the same protein as rhicadhesin, as the biochemical properties of both proteins were different and RapA1 is only produced by *R. leguminosarum* strains

(Ausmees et al. 2001). Overexpression of RapA1 caused stronger attachment to root hairs and increased nodulation competitiveness (Mongiardini et al. 2008; Mongiardini et al. 2009).

At pH below 7, rhizobial attachment is thought to occur via an interaction between legume lectins and rhizobial polysaccharides. Legume lectins have carbohydrate-binding domains and lectins from different legumes have different carbohydrate specificities, which probably contributes to establishing host specificity between host legume and invading rhizobial species (Salahuddin 1992). Lectins localise to the tip of root hairs and bind simultaneously to the plant cell wall and the exopolysaccharides that are attached to the rhizobial surfaces (Dazzo 1981; Dazzo et al. 1976; Hirsch 1999; Laus et al. 2006). Transfer of lectin genes to non-host legumes can allow infection by heterologous rhizobia, as long as these synthesise the appropriate Nod-factor (Diaz et al. 1995; van Rhijn et al. 2001; van Rhijn et al. 1998). Lectins can also be produced by rhizobia, thus affecting root hair attachment. For example, *Bradyrhizobium japonicum* produces a unipolarly located lactose-binding lectin, which can bind to the soybean root surface (Ho et al. 1990a; Ho et al. 1990b; Ho et al. 1994; Loh et al. 1993). Apart from rhicadhesin and the rhizobial polysaccharide-lectin interaction, other factors (like the pili in *B. japonicum*) have been described that could play a role in the rhizobial attachment to root hairs (Vesper and Bauer 1986).

After the first weak binding, stronger binding occurs and this is dependent on the production of cellulose by the rhizobia. This stronger binding results in the formation of biofilm-like caps on the root hairs (Laus et al. 2005; Smit et al. 1987). These caps are not required for nodule formation and are not involved in competitiveness in lab conditions (Williams et al. 2008). They might however play a role under natural conditions. Secreted legume lectins might also play a role in the formation of these caps as it is thought to help the rhizobia bind to each other (Kijne et al. 1988).

1.2 Quorum-sensing gene regulation

1.2.1 Introduction to quorum-sensing

Quorum-sensing (QS) is a regulatory mechanism that allows bacteria to control their gene expression in response to the population density. To be able to sense the population density, the bacteria produce autoinducer molecules that accumulate in the environment. The QS signal is produced during specific stages of growth, although the production level is also influenced by the environmental conditions. When a threshold concentration is reached, the autoinducers activate a transcriptional regulator by binding to it and the activated regulator can induce or repress the expression of target genes. This leads to the activation of a cellular response that extends beyond physiological changes required to metabolize or detoxify the molecule (Winzer et al. 2002). Usually processes that are regulated by QS are not worthwhile when undertaken as an individual cell but they are beneficial when a group of bacteria acts together (for a review see Waters and Bassler 2005). QS was first described in the marine bacterium *Vibrio fischeri*, where it regulates luminescence in the squid light organ (Eberhard et al. 1981; Nealson et al. 1970). One individual cell producing luminescence would be a waste of energy, but when a whole community of cells works together, the resulting light production is worth the effort. Similarly, QS gene regulation can be used as a strategy to invade hosts successfully: when just one bacterium expresses its virulence genes, this bacterium would be easily detected and dealt with by the host's immune response. If the bacteria wait before attacking until they are present in sufficient numbers, they may be able to overwhelm an unexpecting host before it has a chance to defend itself. Many species of bacteria use QS for gene regulation and many aspects in bacterial life are QS regulated, like biofilm formation, bioluminescence, virulence, DNA exchange, sporulation, etc. (for reviews see Loh et al. 2002c; Parsek and Greenberg 2000; Whitehead et al. 2001; Williams et al. 2000; Winzer and Williams 2001).

An alternative explanation for the use of autoinducer molecules by bacteria has been proposed by Redfield (2002) to be a means of detecting diffusion-limited situations. Diffusion sensing would allow the bacteria to assess the cell's environment and prevent the possible loss of energy-demanding products by diffusion. For example, when virulence encompasses the secretion of extracellular enzymes, it is better to do

so in an environment that does not allow the produced enzymes to diffuse away. The concepts of QS as population density sensing and diffusion sensing have been unified in the concept of efficiency sensing (Hense et al. 2007).

1.2.2 Molecular mechanisms of quorum-sensing

QS in Gram-positive bacteria relies on the production of gamma-butyrolactones or small peptides and will not be discussed further (for a review see Lyon and Novick 2004; Novick and Geisinger 2008; Podbielski and Kreikemeyer 2004; Sturme et al. 2002). In Gram-negative bacteria, the two most commonly used autoinducers are *N*-acylhomoserine lactones (AHLs) and autoinducer 2 (AI-2), but others have also been identified. For an overview of the chemical structures of autoinducers used by Gram-negative bacteria, see Figure 1.1.

***N*-Acylhomoserine lactones**

AHL-based QS requires the presence of two genes. One codes for an AHL-synthase and the other encodes a LuxR-type regulator whose activity is modified by binding to the AHLs. AHL molecules from diverse species are chemically different, although their basic structures are similar. They consist of a homoserine lactone (HSL) ring, linked to a variable acyl side chain which can vary in length and degree of saturation. In addition, the third carbon atom can contain a hydrogen-, oxo- or hydroxyl-substitution. This variation, together with the ability of most bacteria to produce more than one type of AHLs, provides a mechanism for specificity in QS communication, and they can enable bacteria to distinguish between their own AHLs and the ones produced by other species.

There are three known protein families capable of synthesising AHL molecules. The first and largest family, the LuxI-type synthases, catalyzes the ligation of *S*-adenosylmethionine (SAM) with an acylated acyl-carrier protein from lipid metabolism (Parsek et al. 1999; Schaefer et al. 1996b; Val and Cronan 1998). LuxI-type synthases have been identified in more than 50 different species, including α-, β- and γ-proteobacteria (Gray and Garey 2001). The second family of AHL synthases has only been found in *Vibrio* species. It includes LuxM from *Vibrio harveyi,* AinS from *Vibrio fischeri* and VanM from *Vibrio anguillarum* (Gilson et al. 1995; Hanzelka et al. 1999; Milton et al. 2001). This family shows little sequence similarity with the LuxI-type synthases although it does seem to use the same reaction mechanism for the

synthesis of AHLs (Hanzelka et al. 1999). A third family of AHL synthases has also been found, comprising of HdtS in *Pseudomonas fluorescens* (Laue et al. 2000) and Act in the extreme acidophile *Acidithiobacillus ferrooxidans* (Rivas et al. 2007). HdtS and Act are related to the lysophosphatidic acid acyltransferase protein family, but the enzymatic mechanism they use to synthesise AHLs remains to be identified.

Most AHL response regulators belong to the LuxR-type response regulators and contain two conserved domains. The N-terminal domain contains a conserved cluster of residues to which the AHLs bind in a one-to-one stoechiometry. This binding leads to dimerisation and activation of the regulators (Choi and Greenberg 1992; Hanzelka and Greenberg 1995). The C-terminal domain contains a conserved helix-turn-helix (HTH) motif, which allows activated AHL response regulators to bind to *cis*-acting DNA sequences (the so-called '*lux* boxes') and thus activate DNA transcription. The crystal structures of the LuxR-type regulators LasR (*P. aeruginosa*) and TraR (*A. tumefaciens*) in complex with their cognate AHLs have been determined (Bottomley et al. 2007; Vannini et al. 2002; Zhang et al. 2002; Zou and Nair 2009). Although induction of gene expression upon activation by AHLs is the most common mechanism by which LuxR-type regulators regulate gene expression, other mechanisms have been described as well (see review by Nasser and Reverchon 2007). For example, LuxR-type regulators can bind to their target sequences in the absence of AHLs, thus blocking transcription. After binding to AHLs, the DNA binding affinity reduces, allowing other transcription regulators to activate gene transcription (Horng et al. 2002; Minogue et al. 2002). In addition to LuxR-type response regulators, AHL-responsive sensor kinases (e.g. LuxN) have been found in *Vibrio* species as part of a typical two-component signalling system (Bassler et al. 1994).

The transport of AHLs through the cell membrane to the environment appears to occur mainly by diffusion (Kaplan and Greenberg 1985), although the presence of specialised efflux pumps for long chain AHLs has also been reported (Pearson et al. 1999). AHL concentrations are also influenced by their degradation rates. Non-enzymatic degradation is increased by a high temperature and an alkaline pH (Byers et al. 2002). In addition, three classes of AHL-degrading enzymes have been identified: AHL lactonases inactivate AHLs by hydrolysis of the ester bond of the HSL ring, while AHL acylases hydrolyse the AHL amide bond between the fatty acid and HSL moieties and AHL oxidoreductases inactivate AHLs by a hydrolysis reaction of the 3-oxo group (Czajkowski and Jafra 2009; Dong et al. 2001; Dong et al. 2000; Dong and Zhang 2005).

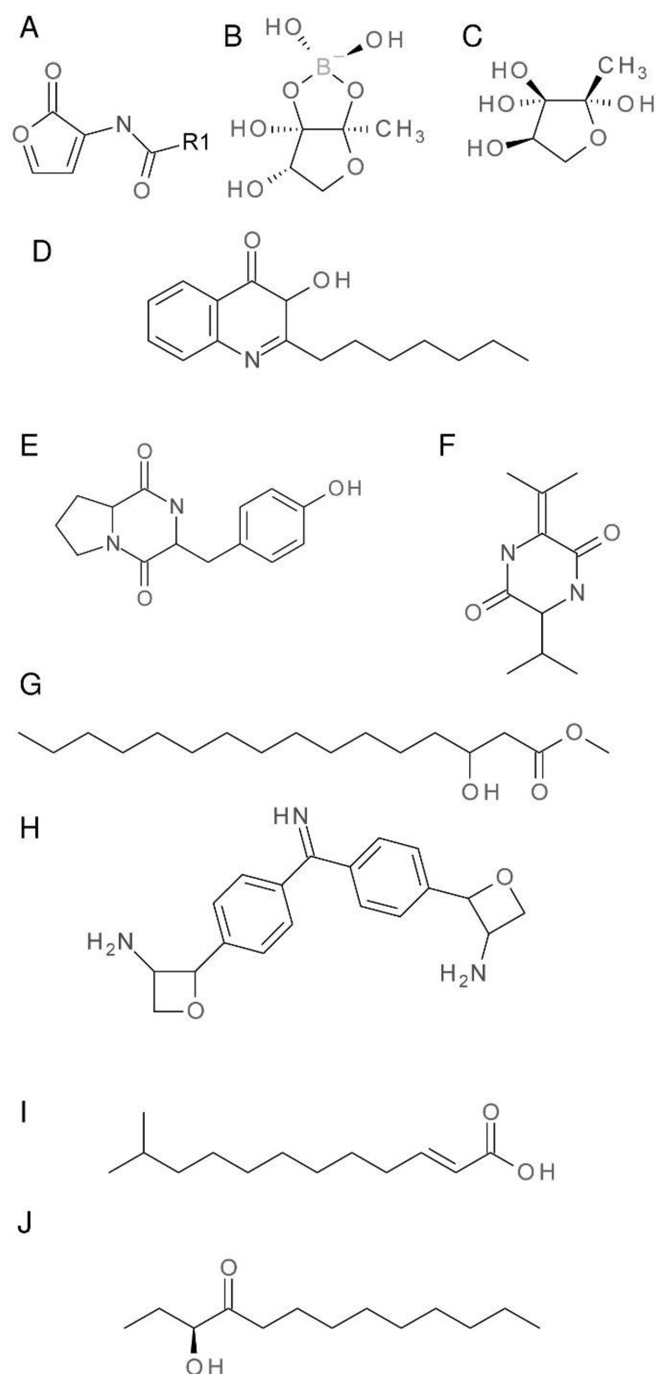


Figure 1.1: Different kinds of QS molecules in Gram-negative bacteria. A: AHLs. R-group is variable among different species, with changes in length and degree of saturation of the carbon chain. In addition, the third carbon atom can contain a hydrogen-, oxo- or hydroxyl-substitution. B: AI-2 produced by *Vibrio* species. C: AI-2 produced by *S. typhimurium.* D: PQS, E+F: DKPs, E: cyclo-(L-Pro-L-Tyr), F: cyclo-(Δala-L-Val), G: 3OH-PAME, H: bradyoxetin, I: DSF, J: CAI-1.

Autoinducer 2

A second common autoinducer used by Gram-negative bacteria is furanosyl borate diester (AI-2) and is produced by a wide range of Gram-positive and Gram-negative bacteria (for a review see Federle 2009). The structure of *V. harveyi* AI-2 has been determined as the boron ester of (2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydro-furan (Chen et al. 2002), while AI-2 from *S. typhimurium* was found to lack the borate (Miller et al. 2004). LuxS is responsible for the production of AI-2 by cleaving *S*-ribosyl-*L*-homocysteine to generate homocysteine and the AI-2 precursor 4,5-dihydroxy-2,3-pentanedione (DHP). DHP spontaneously cyclises, thus forming AI-2 (Schauder et al. 2001). LuxS in *S. typhimurium* can be post-translationally modified and is transported across the cytoplasmic membrane, despite the lack of an obvious signalling motif. This indicates that the function of LuxS is potentially not limited to synthesising AI-2 (Kint et al. 2009).

AI-2 is produced by a great variety of bacterial species, but there is discussion about the precise role of AI-2 as a signalling molecule. In *Vibrio* species a receptor complex, LuxPQ, for AI-2 has been identified (Henke and Bassler 2004b; Miller et al. 2002; Sun et al. 2004). In *Salmonella typhimurium* and *E. coli* AI-2 is perceived by an ABC transporter (Lsr) that phosphorylates AI-2 upon uptake (Xavier et al. 2007). The phophorylated AI-2 molecule is thought to bind to the transcriptional regulator LsrR that activates further transcription of the *lsrACDBFGE* operon (Taga et al. 2003; Taga et al. 2001; Xavier et al. 2007). Reports on other species have suggested that AI-2 has no signalling function but merely serves as a metabolite formed by LuxS in the recycling of methionine from *S*-adenosyl-*L*-homocysteine (Rezzonico and Duffy 2008; Vendeville et al. 2005). In several studies, it was observed that adding chemically synthesised AI-2 did not restore the phenotype of *luxS* mutants and it was concluded that the changes in gene expression that occur upon mutation of *luxS* are a consequence of metabolic changes (Holmes et al. 2009; Vendeville et al. 2005; Wang et al. 2005b; Winzer et al. 2003).

Pseudomonas* quinolone signal and diketopiperazines in *Pseudomonas aeruginosa

Besides AHLs, *Pseudomonas* also produces *Pseudomonas* quinolone signal (PQS) (Pesci et al. 1999) and diketopiperazine (DKP) autoinducers (Holden et al. 1999). The PQS molecules (3,4-hydroxy-2-heptylquinolines) (Deziel et al. 2004) belong to the family of 4-hydroxy-2-alkylquinolines (HAQ) and are synthesised by the enzymes encoded by *pqsABCD* and *pqsH,* via the condensation of anthranilic acid with β-keto fatty acids. The PQS precursor, 4-hydroxy-2-heptylquinoline (HHQ), is converted to

PQS by an oxidation step catalysed by PqsH (Bredenbruch et al. 2005). Both PQS and HHQ function as autoinducers as they can bind to the transcriptional regulator PqsR (MvfR) and activate expression from the *pqsA* promoter (Cao et al. 2001; Xiao et al. 2006a; Xiao et al. 2006b). It has been suggested that PQS might be dispensable as *pqsH* mutants display normal PqsR-dependent gene regulation (except for pyocyanin production), although HHQ is 100-fold less potent than PQS (Xiao et al. 2006a). In addition to PqsR, two other regulatory mechanisms for PQS signalling have been proposed. PqsE functions as a PqsR-independent response effector and requires the LuxR-type regulator RhlR for function. A *pqsE* mutant is not capable of producing PQS-controlled virulence factors although this phenotype can be suppressed by addition of RhlI-made AHLs (Farrow et al. 2008). PQS molecules have iron-chelating properties and this can contribute to the regulation of genes involved in iron scavenging and siderophore biosynthesis by trapping iron at the cell surface (Bredenbruch et al. 2006; Diggle et al. 2007). The PQS-iron complex is toxic for the host (Zaborin et al. 2009). Although PQS-dependent gene regulation has been mainly studied in *Pseudomonas,* other bacteria like *Burkholderia pseudomallei* have also been shown to produce HAQ molecules (Diggle et al. 2006).

DKPs are cyclic dipeptides that have activated AHL-dependent reporter constructs. The DKP concentration needed for activation of these constructs is much higher than the AHL concentrations. DKPs have been suggested to function as QS molecules (Degrassi et al. 2002; Holden et al. 1999) but other reports contradict this (Campbell et al. 2009). DKP molecules have also been identified in *Burkholderica cepacia* (Wang et al. 2010).

Two component system based QS

The other QS molecules that have been described all use two component sensor kinases to detect these molecules. Examples include 3-hydroxypalmitic acid methyl ester (3-OH PAME) in *Ralstonia solanacearum,* cell density factor (CDF) in *B. japonicum,* autoinducer 3 (AI-3) in enterohemorrhagic *E. coli* serotype 0157H7 (EHEC) and diffusible signal factor (DSF) in *Xanthomonas campestris.*

a) 3-OH-PAME in *R. solanacearum*

The plant pathogen *R. solanacearum* uses 3-OH PAME to regulate its virulence factors in a population dependent manner. 3-OH PAME is synthesised by PhcB, which catalyses the conversion of a fatty acid to its methyl ester (Clough et al. 1997b). 3-OH

PAME is sensed by the sensor kinase PhcS and the response regulator PhcR relays the information to the regulator PhcA. PhcA is the actual regulator that induces the expression of the virulence genes at high population densities (Clough et al. 1997a; Clough et al. 1997b; Flavier et al. 1997). The chemolithoautotroph *Ralstonia eutropha* regulates expression of motility and siderophore synthesis by a similar mechanism (Garg et al. 2000).

b) CDF in *B. japonicum*

In *B. japonicum* bradyoxetin or CDF accumulates at high population density (Loh et al. 2002a). CDF affects the expression of *nolA* and *nodD2* and by doing so represses the expression of the nodulation genes at high population densities (Loh et al. 2001). CDF activates the two-component response regulator NwsB (Loh et al. 2002b). Bradyoxetin activity has been detected in extracts of all tested α-proteobacteria (Loh et al. 2002a).

c) AI-3 in EHEC

In the human pathogen EHEC a new kind of autoinducer, AI-3, was discovered. AI-3 is thought to resemble the mammal hormones epinephrine and norepinephrine, thus providing a means of communication with the eukaryotic host in addition to its role as a QS molecule (Sperandio et al. 2003). Production of AI-3 was reported to depend on a *luxS* gene (Sperandio et al. 2003), but this was later shown to be due to an indirect effect (Walters et al. 2006). AI-3 is perceived by the sensor kinase QseC and its cognate response regulator QseB (Clarke et al. 2006; Clarke and Sperandio 2005).

d) DSF in *X. campestris*

The plant pathogen *X. campestris* produces the autoinducer *cis*-11-methyl-2-dodecenoic acid or DSF, which is involved in the regulation of virulence factors (Torres et al. 2007; Wang et al. 2004). Production of cis-2-dodecenoic acid (BDSF) was also shown in *Burkholeria cenocepacia* (Boon et al. 2008; Ryan et al. 2009).

DSF is produced by RpfF and is sensed by the two-component sensor kinase RpfC, which transmits the signal to the HD-GYP protein RpfG (Torres et al. 2007). RpfG is not a DNA-binding protein as is usually the case for a two-component response regulator, but relies on its HD-GYP domain for its regulatory activity (Ryan et al.

2006; Ryan et al. 2010). This HD-GYP domain has phosphodiesterase activity and hydrolyses cyclic-di-guanosine monophosphate (cyclic-di-GMP) to cyclic guanosine monophosphate (cGMP). The levels of cyclic-di-GMP and cGMP in the cell are monitored by the DNA-binding regulator Clp, which has a putative cyclic nucleoside monophosphate (cNMP) binding domain (Chin et al. 2010; He et al. 2007). It is this protein that is responsible for mediating the transcriptional response when DSF is sensed by RpfC.

A second diffusible signal DF is produced by *X. campestris* and is involved in the regulation of EPS biosynthesis. DF is chemically different from DSF and has been tentatively identified as a butyrolactone, which is used by *Streptomyces* species for QS gene regulation (Poplawsky and Chun 1997). Biosynthesis of DF requires the presence of the *pigB* and dioxygenase *xanB2* genes (Poplawsky et al. 2005). The exact structure of DF and the regulatory mechanism used remain to be identified.

1.2.3 Quorum-sensing and the *Rhizobium*-legume symbiosis

Rhizobia and legumes communicate with each other by means of signalling molecules like flavonoids and Nod-factors. Apart from this, rhizobia also use QS to communicate with each other. Most rhizobial species appear to contain one or more AHL-based QS system and different aspects of the *Rhizbobium*-legume symbiosis have been shown to be regulated by QS, such as nodulation efficiency (Cubo et al. 1992; Gao et al. 2006; Yang et al. 2009; Zheng et al. 2006), nodule formation (Cao et al. 2009; Zheng et al. 2006), symbiosome development (Daniels et al. 2002), exopolysaccharide production (Marketon and Gonzalez 2002), symbiotic plasmid transfer (Danino et al. 2003) and nitrogen fixation (Daniels et al. 2002). Nevertheless, many rhizobia seem to be able to establish effective symbioses with their legume hosts after mutation of their QS genes, indicating that their role is mainly to optimize the interactions between the bacteria and their host. The role of QS in the *Rhizobium* legume symbiosis has been studied extensively in many species. Many reviews regarding this subject have been written (Downie and Gonzalez 2008; Gonzalez and Marketon 2003; Sanchez-Contreras et al. 2007; Wisniewski-Dye and Downie 2002). For a short overview, the QS genes that have been identified in different rhizobial species are summarised in Table 1.1.

There is evidence that AHL molecules might also be a way for the rhizobia to communicate with their legume host. Higher plants, including legumes, can synthesise

AHL mimic compounds, which could activate or disrupt rhizobial communication and thus influence the symbiosis (Degrassi et al. 2007; Gao et al. 2003; Sanchez-Contreras et al. 2007; Teplitski et al. 2000). Conversely, *Medicago truncatula* can perceive rhizobial AHL signals, inducing changes in gene expression in the plants (Mathesius et al. 2003).

Strain	Gene (location)	Signal	Phenotypes	Reference
A. tumefaciens	*traR/traI* (pTi)	3-O-C_8-HSL	plasmid transfer	(Fuqua & Winans, 1994)
R. leguminosarum A34	*cinR/cinI* (chromosome)	3-OH-$C_{14:1}$-HSL	growth inhibition, polysaccharide degradation	(Edwards *et al.*, 2009; Lithgow *et al.*, 2000)
	rhiR/rhiI (pRL1JI)	C_6-HSL, C_7-HSL, C_8-HSL	nodulation efficiency	(Cubo *et al.*, 1992; Rodelas *et al.*, 1999)
	traR/traI (pRL1JI)	3-O-C_8-HSL, C_8-HSL	plasmid transfer	(Danino *et al.*, 2003; Wilkinson *et al.*, 2002)
	raiR/raiI (non-symbiotic plasmid)	3-OH-C_8-HSL, C_8-HSL	unknown	(Wisniewski-Dye *et al.*, 2002)
	expR	unknown	polysaccharide degradation	(Edwards *et al.*, 2009)
R. etli				
CNPAF512	*cinR/cinI* (chromosome)	3-OH-slc-HSL	nitrogen fixation, symbiosome development, growth inhibition	(Daniels *et al.*, 2002)
	raiR/raiI (chromosome)	short-chain AHLs	nitrogen fixation, growth inhibition	(Rosemeyer *et al.*, 1998)
CNP42	*traR/traI* (p42a)	3-O-C_8-HSL, 3-OH-C_8-HSL	plasmid transfer	(Tun-Garrido *et al.*, 2003)
S. meliloti				
Rm1021	*sinR/sinI* (chromosome)	3-O-C_{14}-HSL, $C_{16:1}$-HSL, 3-O-$C_{16:1}$-HSL, 3-O-C_{16}-HSL, C_{18}-HSL, C_{12}-HSL	EPSII production, swarming	(Gao *et al.*, 2005; Hoang *et al.*, 2004; Hoang *et al.*, 2008; Marketon & Gonzalez, 2002; Marketon *et al.*, 2002; Teplitski *et al.*, 2003)
	expR (chromosome)	$C_{16:1}$-HSL	EPSII production, swarming	(Gao *et al.*, 2005; Hoang *et al.*, 2004; Hoang *et al.*, 2008; Pellock *et al.*, 2002)
	nesR (chromosome)	unknown	stress adaptation, competition for nodulation	(Patankar & Gonzalez, 2009)
Rm41	*traR/traI* (pRm41a)	3-O-C_8-HSL	plasmid transfer	(Marketon & Gonzalez, 2002)
RU10/406	*visN/visR* (chromosome)	unknown	motility	(Sourjik *et al.*, 2000)

Strain	Gene (location)	Signal	Phenotypes	Reference
Rhizobium sp.				
NGR234	*traR/traI* (pNGR234a)	3-O-C_8-HSL	plasmid transfer	(He *et al.*, 2003)
	Unknown genes (chromosome)	unknown AHLs	growth inhibition	(He *et al.*, 2003)
Mesorhizobium				
loti R7A	*traR/traI1, traI2*	unknown AHLs	symbiosis island transfer	(Ramsay *et al.*, 2009)
loti NZP2213	*mrlI1, mrlI2, mrlI3*	3-O-C_6-HSL, C_8-HSL, C_{10}-HSL, C_{12}-HSL	nodulation efficiency	(Yang *et al.*, 2009)
tianshanense CCBAU060A	*mtqR/mtqI*	unknown AHLs	nodulation, growth	(Cao *et al.*, 2009)
tianshanense CCBAU3306	*mrtR/mrtI*	unknown AHLs	legume nodulation and root hair attachment	(Zheng *et al.*, 2006)
huakii	unknown	unknown	biofilm formation, nodulation	(Gao *et al.*, 2006; Wang *et al.*, 2004)
Bradyrhizobium				
japonicum USDA110	unknown	bradyoxetin	*nod* gene control	(Loh *et al.*, 2002; Loh & Stacey, 2003)
japonicum USA 10/290	unknown	unknown AHLs	unknown	(Brelles-Marino & Bedmar, 2001; Pongsilp *et al.*, 2005)
elkanii	unknown	unknown AHLs	unknown	(Brelles-Marino & Bedmar, 2001; Pongsilp *et al.*, 2005)

Table 1.1: Overview of rhizobial QS systems.

1.3 Regulation of quorum-sensing systems

To elicit an appropriate response, bacteria must integrate the QS signal with other environmental queues. This is especially important for pathogens, as they reside in a hostile environment, and so activating the virulence genes at the right time can be essential for survival (de Kievit and Iglewski 2000; Williams et al. 2000). Therefore in many bacteria the expression of the QS genes itself is under the control of other regulatory circuits. In the next sections these will be explained in more detail.

1.3.1 Multiple QS systems

Gram-negative bacteria are capable of producing different kind of autoinducers and in several species more than one QS system has been found. Most research has focussed on *R. leguminosarum, S. meliloti, P. aeruginosa* and different *Vibrio* species, but hierarchical organisation of QS systems has also been described in other species.

R. leguminosarum

R. leguminosarum A34 contains the *cin*, *rai*, *rhi* and *tra* QS genes. The *cinI* and *cinR* genes are located on the chromosome and are on top of a regulatory cascade, inducing the production of RaiI-, RhiI- and TraI-made AHLs (Lithgow et al. 2000; Wisniewski-Dye et al. 2002). CinI-made 3-hydroxy-$C_{14:1}$-HSL was originally identified as 'small bacteriocin', because it inhibited growth of *Rhizobium* strains carrying the symbiotic plasmid pRL1JI (Hirsch 1979; Schripsema et al. 1996; Wijffelman et al. 1983). They also play a role in the adaptation to stationary phase, as cultures entering stationary phase at high population densities showed no loss of viability over long periods, while cultures entering stationary phase at low population densities did. Adding 3-hydroxy-$C_{14:1}$-HSL to cultures at low population densities could restore this loss of viability (Thorne and Williams 1999). Mutation of the *cinI* or *cinR* genes did not cause any growth difficulties in laboratory conditions and pea nodulation was normal (Lithgow et al. 2000). QS genes similar to the *cinI/R* genes were identified in *Rhizobium etli* and *Mesorhizobium tianshanense* (respectively the *cinI/R* and *mrtI/R* genes). Despite the high sequence similarities, the roles of the *R. etli cin* and *M. tianshanense mrt* genes are different from that in *R. leguminosarum.* In *R. etli,* a *cinI* mutation increased the lag-phase and slowed growth, and symbiosome development and nitrogen fixation was abnormal (Daniels et al. 2002). However it is possible that the observed symbiotic

phenotypes are caused by the growth problems of the mutants. The *R. etli cin* locus was required for normal swarming (Daniels et al. 2004). In *M. tianshanense* mutation of the *mrtI/R* genes reduced the efficiency of root hair adherence and blocked nodule formation on its host *Glycyrrhiza uralensis* (Zheng et al. 2006).

The *traI* and *traR* genes on the symbiotic plasmid pRL1JI, are homologous to those found in *Agrobacterium tumefaciens* and are responsible for the induction of the plasmid transfer genes. Expression of the *traR* and *traI* genes is induced by CinI-made AHLs and results in recipient-induced plasmid transfer (Figure 1.2). The key to this is the presence of a LuxR-type regulator encoded on pRL1JI, BisR, which can act both as an inducer and as a repressor (Danino et al. 2003). In strains carrying pRL1JI (donor strains), BisR represses expression of *cinI*, thus preventing the synthesis of CinI-made 3-hydroxy-$C_{14:1}$-HSLs (Wilkinson et al. 2002). In strains that do not carry pRL1JI (recipient strains), this repression does not occur and therefore CinI produces 3-hydroxy-$C_{14:1}$-HSLs. When a recipient strain and donor strain come into close proximity, BisR in the donor strain will sense the 3-hydroxy-$C_{14:1}$-HSLs produced by the recipient strain. The activated BisR then induces the expression of *traR* (Wilkinson et al. 2002). TraR is then activated by TraI-made AHLs and induces the expression of the plasmid transfer genes, thus initiating the conjugation of the symbiotic plasmid to the recipient strain (Danino et al. 2003; McAnulla et al. 2007). The bivalent mode of action of BisR (both as an activator and a repressor) is therefore responsible for a regulatory mechanism that allows the recipient strains to induce plasmid transfer in the presence of a possible donor strain. This regulatory mechanism leads to very high conjugation frequencies and prevents the waste of energy that would occur if unnecessary plasmid transfer would take place, because plasmid transfer is only initiated in the presence of recipient strains that do not yet carry a plasmid containing BisR (Danino et al. 2003). pRL1JI also expresses TraM, which acts as an anti-activator of TraR at low concentrations of TraI-made AHLs (Danino et al. 2003).

Induction of the *cinI* and *cinR* genes also leads to induction of the expression of RaiI-made AHLs (Wisniewski-Dye et al. 2002). RaiR induces *raiI* expression in response to RaiI-made AHLs (3-hydroxy-C_8-HSL as its major product and C_6-HSL, C_7-HSL and C_8-HSL as minor products). In addition, RaiR is weakly activated by CinI-made 3-hydroxy-$C_{14:1}$-HSL and TraI-made 3-oxo-C_8-HSL (Wisniewski-Dye et al. 2002). It is currently unknown which genes are regulated by RaiR in *R. leguminosarum*, but in *R. etli* RaiR was involved in the restriction of nodule number. *In vitro* mutation of *raiI* led to an increase in nodulation numbers and nitrogenase activity, although *in planta,*

no significant increase in nitrogen fixation could be demonstrated (Rosemeyer et al. 1998). Interestingly, mutation of *raiR* had no effect on nodulation. It has recently been shown that regulation of *rai* by *cin* is mediated by a small regulatory protein encoded by *cinS,* immediately downstream of *cinI.* CinS-dependent gene regulation required the presence of the LuxR-type regulator ExpR (Edwards et al. 2009), but the molecular mechanism by which this regulation occurs, had not yet been determined.

CinR was also shown to induce the production of RhiI-made AHLs (Gray et al. 1996; Lithgow et al. 2000), which is present on the symbiotic plasmid. The molecular mechanism by which this happens has not yet been identified. The *rhiI* and *rhiR* genes were first identified in *R. leguminosarum*, because of the high expression level of the RhiA protein, which was not produced by strains lacking the *nod-nif* gene region (Dibb et al. 1984). RhiR regulates the expression of the *rhiABC* genes in response to RhiI-made C_6-, C_7- and C_8-HSLs. *rhiA* encodes a protein of unknown function that is highly expressed in the rhizosphere (Cubo et al. 1992; Dibb et al. 1984; Economou et al. 1989). Mutation of *rhiA* or *rhiR* caused a decrease in the number of nodules in strains that were already compromised for nodulation (Cubo et al. 1992). Expression of the *rhi* genes was inhibited by the presence of flavonoids, which is a *nodD*-dependent effect mediated via *rhiR* expression (Economou et al. 1989). RhiA is present in all strains of biovar *viciae*, but is absent in the other biovars *trifolii* and *phaseoli*, suggesting that it might function to optimize interactions between *R. leguminosarum* bv. *viciae* and pea or vetch.

Sinorhizobium meliloti

In *S. meliloti* two LuxR-type regulators SinR and ExpR have been identified that can respond to SinI-made AHLs (ranging in size from C_{12}-HSL to C_{18}-HSL) (Hoang et al. 2004; Marketon et al. 2002; Pellock et al. 2002) (Figure 1.3). Mutation of *sinI* or *sinR* delayed nodule formation and reduced the total number of nodules (Gao et al. 2005; Marketon et al. 2002). Based on microarray experiments it appears that most gene regulation in response to SinI-made AHLs is mediated via ExpR and not via SinR. ExpR regulates the biosynthesis of the symbiotically important EPSII and succinoglycan, as well as motility and other processes (Gao et al. 2005; Hoang et al. 2004; Hoang et al. 2008; Marketon et al. 2003; Pellock et al. 2002). Gene regulation by ExpR is particularly unusual, because it is capable of influencing gene expression in a versatile way: it can be both dependent and independent of SinI-made AHLs and it can have both positive and negative effects on gene expression (Hoang et al. 2004).

Figure 1.2: Recipient induced plasmid transfer in *R. leguminosarum.* When BisR is present (in donor strains), it represses the expression of *cinI,* thus preventing the synthesis of CinI-made AHLs. Recipient strains do not express BisR and can therefore produce CinI-made AHLs. These then diffuse into a donor strain in close proximity, to activate BisR. BisR induces the expression of *traR* and TraR induces the expression of the plasmid transfer genes in response to TraI-made AHLs. TraM represses expression of the plasmid transfer genes at low levels of TraI-made AHLs by forming an antiactivator complex with TraR. The symbiotic plasmid pRL1JI also contains the *rhiI* and *rhiR* genes. RhiR activates the expression of the *rhiABC* genes in response to RhiI made AHLs.

Expression of *sinI* is absolutely dependent on SinR and SinR induces moderate transcription of *sinI* even in the absence of SinI-made AHLs (McIntosh et al. 2008). ExpR also regulates expression of the *sinI* and *sinR* genes and it does so at two levels, resulting in both a positive and a negative feedback loop. ExpR induces *sinI* in response to SinI-made AHLs by binding to a sequence upstream of *sinI* (Bartels et al. 2007; McIntosh et al. 2008), while it represses the expression of *sinR* (McIntosh et al. 2009). An ExpR binding site was identified in front of *sinR,* but this binding site was not required for the repression of *sinR* by ExpR, showing that the observed reduction in expression might be due to an indirect effect. The amount of AHLs in the environment probably determine whether the positive or the negative feedback mechanism has the upper hand, eventually resulting in an equilibrium state between both at higher population densities (McIntosh et al. 2009) .

Pseudomonas aeruginosa

P. aeruginosa is an opportunistic, bronchial human pathogen, associated with infection of immuno-compromised patients. Pathogenicity in *P. aeruginosa* is caused by secretion of multiple extracellular virulence factors, such as proteases, haemolysins, exotoxinA, exoenzyme S and pyocyanin that cause extensive tissue damage. The regulation of the expression of these virulence factors is tightly regulated by QS (Passador et al. 1993; Willcox et al. 2008; Winstanley and Fothergill 2009), allowing the bacteria to evade the host defence response, until a sufficiently high population density is reached. Because of its importance in pathogenicity, QS has been studied extensively in *P. aeruginosa* PAO1. In this species, at least three QS systems are present and their expression is organised in a hierarchical fashion (Figure 1.4).

P. aeruginosa PAO1 contains the *rhlI* and *rhlR* (Ochsner et al. 1994; Ochsner and Reiser 1995; Pearson et al. 1995) and the *lasI* and *lasR* QS genes (Gambello and Iglewski 1991; Pearson et al. 1994). RhlI synthesises C_4-HSL and LasI synthesises 3-oxo-C_{12}-HSL. Together, the *rhl* and *las* genes regulate, either directly or indirectly, the expression of about 6% of the *P. aeruginosa* genome (Schuster et al. 2003). LasR induces the expression of *lasI* in response to LasI-made AHLs. A second transcriptional regulator RsaL is encoded between *lasI* and *lasR* and represses transcription of *lasI* (de Kievit et al. 1999; Rampioni et al. 2006; Rampioni et al. 2007). LasR and RsaL bind to adjacent sites in the *lasI* promoter and the repressor activity of RsaL is dominant over the inducer activity of activated LasR (Rampioni et al. 2007). In addition, RsaL affects the QS response by binding directly some of the promoters of genes that are controlled by QS (Rampioni et al. 2007).

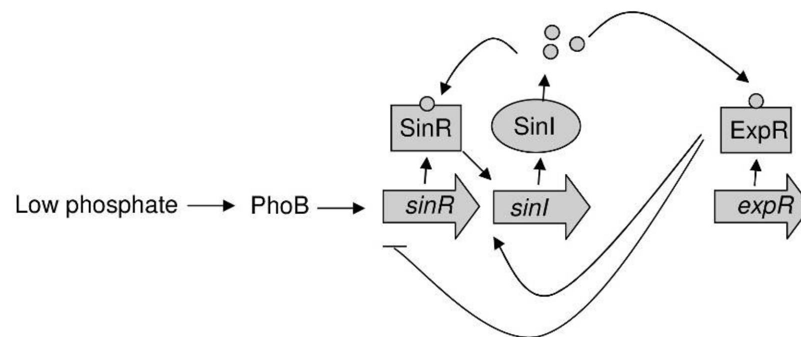


Figure 1.3: QS gene regulation in *S. meliloti.* SinR induces expression of *sinI,* even in the absence of SinI-made AHLs. SinI-made AHLs also activate ExpR, which induces the expression of *sinI* and represses the expression of *sinR.* PhoB induces the expression of *sinR* in response to low phosphate levels (see section 1.3.2).

In most studies, the *lasI/R* genes have been found to be hierarchically on top of the *rhlI/R* genes, but other studies have shown that this hierarchy is dependent on the environmental conditions (Duan and Surette 2007). Expression of *rhlR* is induced by LasR when it is activated by LasI-made AHLs (Latifi et al. 1996; Pesci et al. 1997). Further regulation of the *rhl* genes by the *las* genes is exerted post-translationally: at low population densities, the activation of RhlR by RhlI-made AHLs is inhibited by competitive binding of LasI-made AHLs to RhlR. Only at higher cell densities is the RhlI-produced AHL able to outcompete the LasI-made AHL (Pesci et al. 1997). Both mechanisms of control probably serve to ensure that the *las* and *rhl* genes are switched on in the right order, first the *las* genes and then the *rhl* genes. Recently, the repressor QteE was identified and it inhibits both the *lasI* and *rhlI* genes from being activated before the right population density is reached by reducing the stability of LasR and RhlR (Siehnel et al. 2010).

P. aeruginosa also possesses two other LuxR-type regulators and both of these affect the expression of the *rhlI* or *lasI* genes. QscR (quorum sensing control repressor) negatively affects the production of RhlI- and LasI-made AHLs and it does so in an AHL-independent manner, despite being able to bind AHLs (Chugani et al. 2001). QscR probably does not have a direct transcriptional effect on gene expression, but instead seems to function through the formation of inactive dimers with LasR and RhlR, by titering out AHLs and/or by competition for DNA binding sites at target genes for LasR and RhlR (Chugani et al. 2001; Ledgham et al. 2003b). VqsR (virulence and quorum sensing regulator) is another LuxR-type regulator, which has a key role in the *Pseudomonas* QS regulatory cascade. Microarray analysis showed that in a *vqsR* mutant the expression of *lasI* is greatly reduced (Juhas et al. 2004; Juhas et al. 2005). In addition, it was shown that the expression of *vqsR* itself is under the control of LasR (Li et al. 2007).

P. aeruginosa contains a third, AHL-independent PQS system, which seems to be in an intermediate position between *las* and *rhl* (Diggle et al. 2003). The PQS biosynthetic genes are induced by LasR at two levels. First, LasR controls the amount of PQS signal that is produced, by inducing the expression of *pqsH* (which catalyses the final step in PQS synthesis) (Deziel et al. 2004; Gallagher et al. 2002; Pesci et al. 1999). It has been shown however that under some circumstances, PQS synthesis can occur independently of LasR (Diggle et al. 2003). Second, the expression level of *pqsR* (also known as *mvfR*), which encodes a transcriptional regulator that is activated by PQS, is under direct control of activated LasR (Wade et al. 2005; Xiao et al.

2006b). Cross-regulation between the PQS and *rhl* QS systems has also been observed. Activated PqsR positively regulates *rhlI* expression (McKnight et al. 2000), while activated RhlR represses the expression of *pqsR* and *pqsABCDE* (Wade et al. 2005; Xiao et al. 2006b). Activation of the *pqs* biosynthetic genes also affects the production of RhlI-made AHLs by means of the response effector PqsE (Farrow et al. 2008). The exact mechanism by which this happens remains to be uncovered, but initial experiments point to a post-translational influence of PqsE on the activity of RhlR (Farrow et al. 2008; Yu et al. 2009). It has been shown that the PQS molecules only accumulate at late stationary phase, therefore it is not likely that PQS induces gene expression in the same population-density dependent way as AHLs (McKnight et al. 2000). It probably functions to link the induction of the *rhl* and *las* genes, providing an extra means of control in the hierarchical cascade to ensure that the *rhl* genes are only switched on after the *las* genes have been activated.

The three QS systems in *P. aeruginosa* are part of a complex regulatory network, and many other regulators that affect their expression and activity have been identified (summarised in Table 1.2): MvaT (Diggle et al. 2002), GidA (Gupta et al. 2009), the YebC-like protein PmpR (Liang et al. 2008a), AlgQ (Ledgham et al. 2003a), AlgR (Morici et al. 2007), VqsM (Dong et al. 2005), PA1196 (Liang et al. 2009), PpyR (Attila et al. 2008), PtxR (Carty et al. 2006), PPK1 (Fraley et al. 2007) and Lon protease (Bertani et al. 2007; Takaya et al. 2008).

In several other *Pseudomonas* species more than one QS system has been found, but their hierarchical organisation appears to be different from that seen in *P. aeruginosa* PAO1. In the plant-growth-promoting strain *P. aeruginosa* PUPa3, the *lasI/R* and *rhlI/R* genes are present, but their induction does not occur in a hierarchical fashion (Steindler et al. 2009). Likewise, in the plant pathogen *Pseudomonas aureofaciens*, the *phzI/R* and *csaI/R* genes are also not induced hierarchically. However, in this species the AHLs produced by PhzI cross-react with CsaR, and the AHLs produced by CsaI can interact with PhzR (Zhang and Pierson 2001).

***Vibrio* species**

QS has been studied extensively in *V. harveyi, V. cholerae* and *V. fischeri* and each of these species produces several different autoinducers.

a) *V. harveyi*

In the marine bacterium *V. harveyi* three autoinducers have been identified (Figure 1.5 A). These control the expression of bioluminescence, type-III secretion and metalloprotease production (Henke and Bassler 2004a). The use of three different autoinducers provides a way for the bacteria to decipher which species of bacteria are present in their occupied niche (Henke and Bassler 2004b; Waters and Bassler 2006).

HAI-1 (*harveyi* autoinducer-1, 3-OH-C_4-HSL) is produced by the LuxM AHL synthase (Bassler et al. 1993; Cao and Meighen 1989). HAI-1 is only produced by *V. harveyi* and its close relative *Vibrio parahaemolyticus,* and it is therefore proposed to be an intraspecies signal. The second autoinducer produced by *V. harveyi* is (*S*)-3-hydroxytridecan-4-one or CAI-1 (*cholerae* autoinducer-1), which is produced by CqsA (Higgins et al. 2007; Kelly et al. 2009; Miller et al. 2002). This molecule has been shown to be produced by many different *Vibrio* species, and therefore could act as an intragenus signal. The third autoinducer is AI-2, which is produced by LuxS in many bacterial species and could act as an interspecies signalling molecule (Bassler et al. 1997; Chen et al. 2002; Schauder et al. 2001). Each of these autoinducers is detected by its own two-component system sensor histidine kinase: HAI-1 by LuxN (Bassler et al. 1993; Freeman et al. 2000), CAI-1 by CqsS (Higgins et al. 2007; Miller et al. 2002) and AI-2 by the sensor histidine-kinase complex LuxPQ (Bassler et al. 1994; Neiditch et al. 2005). The three sensor histidine kinases transmit information through a phosporylation step into the same protein, LuxU, which subsequently relays the signal to LuxO (Freeman and Bassler 1999a; Freeman and Bassler 1999b). This mechanism allows three autoinducer signals, each of which is sensing a different aspect of the microbioal community, to be integrated into one response.

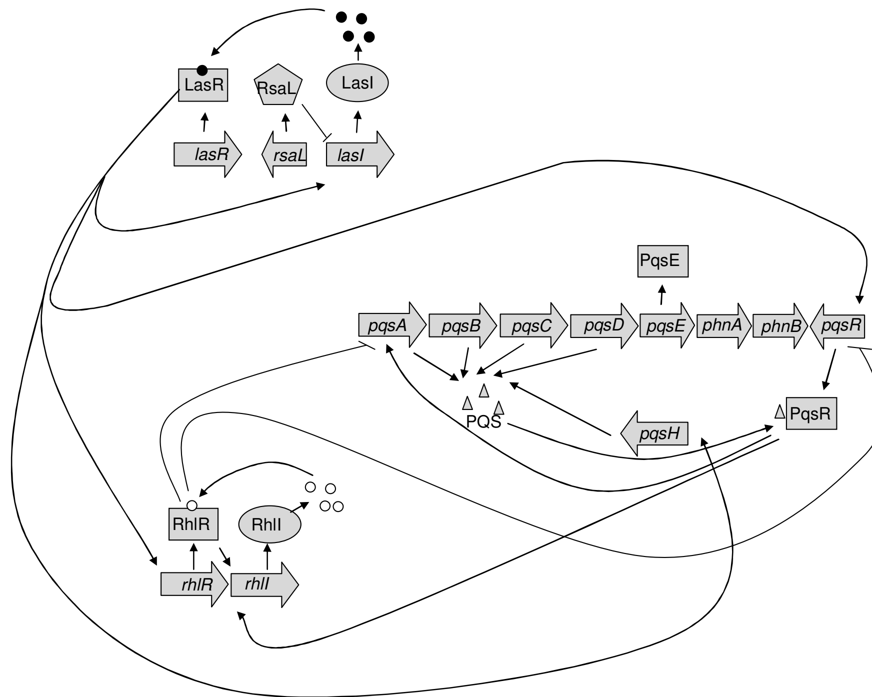


Figure 1.4: Hierarchical organisation of QS systems in *P. aeruginosa.* The *lasI* and *lasR* genes regulate gene expression of target genes and are on top of a hierarchical QS network. LasR activates *lasI* expression in response to LasI-made AHLs. RsaL, which is encoded between *lasI* and *lasR* represses the expression of *lasI.* Activated LasR also induces the expression of *rhlR* and *pqsR.* RhlR induces expression of target genes in response to RhlI-made AHLs, but represses the expression of the *pqsABCDE* operon and *pqsR.* PqsR induces gene expression in response to PQS molecules, which are synthesised by the proteins encoded by *pqsABCD.* Activated PqsR induces the expression of the *pqsABCDE* operon and *rhlI. pqsE* is cotranscribed with *pqsABCD* but its product is not involved in PQS biosynthesis and functions as a response effector.

At low population densities (when no autoinducers are present to activate the cascade) LuxN, CqsS and LuxQ function as kinases, phophorylating LuxU. LuxU-P relays the phosphate to LuxO, which causes this protein to be activated (Freeman and Bassler 1999a; Lilley and Bassler 2000). Activated LuxO-P then induces the expression of quorum regulatory RNAs (Qrr's) (Tu and Bassler 2007). The Qrr*'s* interact with the sRNA chaperone Hfq, and together they bind to the *luxR(vh)* mRNA, thus blocking translation of the QS 'master' regulator LuxR(vh) (Lenz et al. 2004; Showalter et al. 1990; Tu and Bassler 2007) (Figure 1.5 A). To avoid confusion in nomenclature with *V. fischeri* LuxR, *V. harveyi* LuxR is represented as LuxR(vh) while *V. fischeri* LuxR is represented as LuxR(vf) in this text. V. *harveyi* is capable of responding gradually to the presence of Qrr*'*s (Tu and Bassler 2007), which allows for the integration of the QS response with other environmental queues at the level of Qrr transcription. At high population densities, the presence of the autoinducer molecules switches the function of LuxN, CqsS and LuxQ to phophatases, ultimately leading to a dephosphorylation of LuxO-P, and thus repressing the expression of the Qrr*'*s. As a consequence, LuxR(vh) protein is produced and this regulatory protein is responsible for the activation or repression of QS responsive genes (Pompeani et al. 2008; Showalter et al. 1990; Swartzman et al. 1992).

b) *V. cholerae*

QS in the human pathogen *V. cholerae* is very similar to *V. harveyi*, but it only produces CAI-1 and AI-2 and not HAI-1 (Miller et al. 2002) (Figure 1.5 B). At high population densitities, *V. cholerae* QS represses biofilm formation and the expression of the virulence genes (Hammer and Bassler 2003; Higgins et al. 2007; Zhu et al. 2002). As in *V. harveyi,* the CAI-1 and AI-2 signals are transmitted to LuxU and LuxO to affect the expression level of the Qrr sRNA's. While the Qrr*'s* of *V. harveyi* function in an additive way, the Qrr*'s* of *V. cholerae* function redundantly (Lenz et al. 2004). This means that *V. cholerae* is extremely sensitive to the presence of autoinducers, and only one Qrr needs to be present for full repression of the QS regulator *hapR* .

Genetic evidence showed that even in the absence of LuxU the response regulator LuxO can control gene expression in a population dependent way (Miller et al. 2002). It was found that the small nucleoid protein Fis, which is highly expressed at low population densities (Ishihama 1999) is required for the expression of the *V. cholerae* Qrr sRNA's and this occurs probably due to direct binding of Fis to the promoter region of the Qrr sRNA's (Lenz and Bassler 2007). The Qrr's bind to and inactivate

the stability of *hapR* mRNA, which encodes the QS master regulator HapR (Kovacikova and Skorupski 2002; Zhu et al. 2002). HapR represses its own expression at two levels. At high population densities HapR binds directly to its own promoter (Lin et al. 2005), while at low population densities HapR activates the transcription of the Qrr sRNA's, thus indirectly destabilising *hapR* mRNA (Svenningsen et al. 2008). The latter is thought to speed up the inactivation of the QS response of *V. cholerae* cells when the population density reduces, for example upon invasion of a host. To be able to evade the host's immune response it is therefore important that the QS controlled virulence genes are inactivated as quickly as possible. A second target of the Qrr sRNA's was identified, *vca0939,* which encodes a GGDEF protein. Translation of *vca0939* is activated by the Qrr's in a HapR-independent manner (Hammer and Bassler 2007).

c) *V. fischeri*

In the squid symbiont *V. fischeri* the situation is slightly different from that in *V. harveyi* or *V. cholerae. V. fischeri* contains three QS systems, encoded by *ainS/R* (Gilson et al. 1995), *luxI/R* (Eberhard et al. 1981; Engebrecht and Silverman 1984) and *luxS/PQ* (Lupp and Ruby 2004) (Figure 1.5 C). These are responsible for regulating the expression of the luminescence genes and colonisation factors in the light organ of the squid and are organised in a hierarchical fashion (Lupp and Ruby 2005; Lupp et al. 2003).

The QS system encoded by the *luxI/R* genes functions like a traditional AHL QS system: LuxI synthesises 3-oxo-C_6-HSL, which activates the LuxR(vf) regulator (Engebrecht and Silverman 1984). Note that this LuxR(vf) regulator is not homologous to the one described previously for *V. harveyi* LuxR(vh). The *V. fisheri ainS/R* and *luxS/PQ* genes are similar to the *V. harveyi luxM/N* and *luxS/PQ* genes and they function in a similar fashion. AinS synthesises C_8-HSL, which is sensed by the sensor histidine kinase AinR (Gilson et al. 1995; Kuo et al. 1994), while LuxS-made AI-2 is sensed by the LuxPQ sensor histidine kinase complex (Lupp and Ruby 2004). As in *V. harveyi* and *V. cholerae,* high population density is sensed by AinR and LuxPQ to induce a phosphorelay via LuxO to relieve repression of the transcriptional regulator LitR (Miyashiro et al. 2010). LitR is the homologue of LuxR(vh) in *V. harveyi* and HapR in *V. cholerae* (Fidopiastis et al. 2002; Lupp and Ruby 2004; Miyamoto et al. 2003).

Induction of LuxS/PQ and AinS/R leads to the induction of the production of LuxI-made AHLs. This control is exerted at two levels. First, AinS-made AHLs are able to weakly activate LuxR(vf) (Lupp et al. 2003). It has been proposed that the AinS-made AHLs function as a competitor for the LuxI-made AHLs, which would ensure that higher population densities are reached before full activation of LuxR. Second, the LitR regulator induces the expression of *luxR(vf)* (Fidopiastis et al. 2002). In addition, LitR activates expression of *ainS,* thus establishing a positive feedback loop (Lupp and Ruby 2004).

Other examples of hierarchically organised QS systems

Yersinia pseudotuberculosis

The mammalian enteropathogen *Y. pseudotuberculosis* contains the *ypsI/R* and *ytbI/R* genes, both of which are involved in the regulation of cell aggregation and motility (Atkinson et al. 1999). YtbR induces the expression of *ytbI* in response to YtbI-made AHLs (C_6-HSL, 3-O-C_6-HSL, 3-O-C_7-HSL, 3-OH-C_8-HSL, 3-O-C_8-HSL, C_8-HSL and 3-O-C_{10}-HSL). YpsR represses the expression of y*psI* and y*psR* in response to YpsI-made AHLs (C_6-HSL, 3-O-C_6-HSL, 3-O-C_7-HSL). In addition it activates the expression of *ytbI* and *ytbR* (Atkinson et al. 2008). YtbR has got a positive effect on motility, while YpsR has got a negative effect (Atkinson et al. 2008).

Burkholderia cenocepacia (formerly *Pseudomonas cenocepacia*)

Burkholderia species are opportunistic pathogens in people with cystic fibrosis and they use AHLs for the regulation of virulence factors. In *B. cenocepacia,* the *cepI/R* (Lewenza et al. 1999) and *cciI/R* (Malott et al. 2005) genes are organised in a hierarchical fashion. CepI synthesises primarily C_8-HSL, and minor amounts of C_6-HSL (Lewenza et al. 1999; Lewenza and Sokol 2001). CciI synthesises primarily C_6-HSL and minor amounts of C_8-HSL (Malott et al. 2005). CepR is an inducer of gene expression, while CciR is primarily repressing gene expression (including autorepression of the *cciIR* operon). Several genes have been found to be regulated by both regulators reciprocally (O'Grady et al. 2009). CepR induces the transcription of the *cciIR* operon in response to C_8-HSL. A negative feedback loop is formed by two mechanisms: repression of *cepI* expression by CciR (Malott et al. 2005) and inactivation of CepR in the presence of high levels of C_6-HSL (Weingart et al. 2005). The closely related species *Burkholderia vietnamiensis* contains the *cepI/R* and *bviI/R* genes. Similar to the situation in *B. cenocepacia,* CepR is required for the expression of *bviI* (Malott and Sokol 2007).

A)

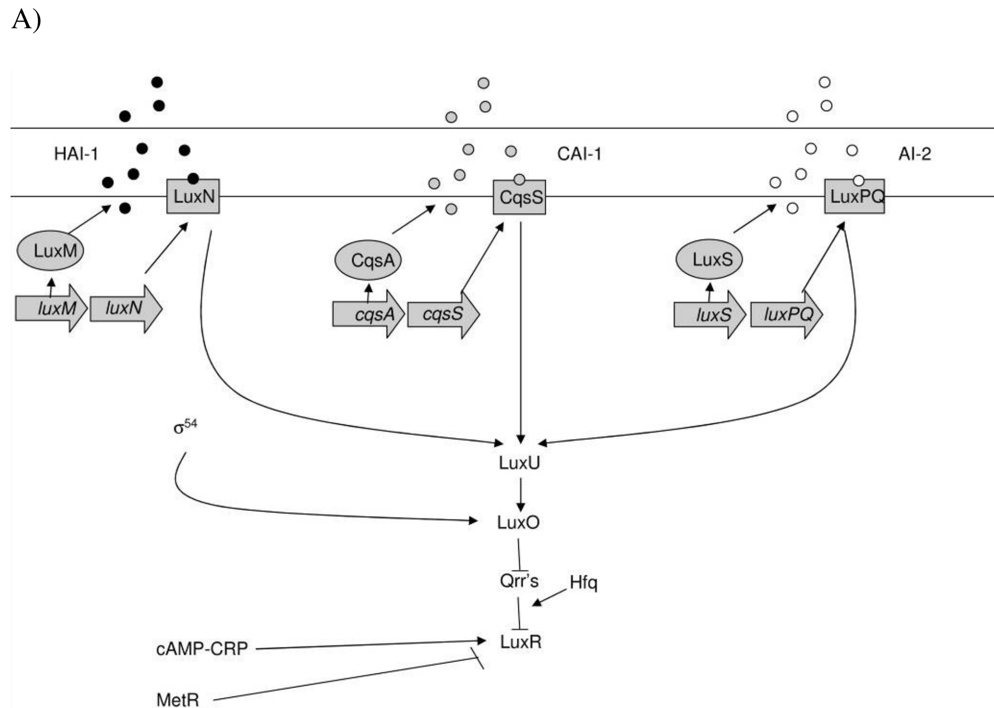


Figure 1.5: QS in *Vibrio* species. *V. harveyi, V. cholerae* and *V. fischeri* all produce more than one kind of autoinducer, and when these are perceived, the signals are integrated in one central signalling cascade which is similar in all three organisms. A: *V. harveyi* produces three autoinducer molecules: HAI-1 (produced by LuxM), CAI-1 (produced by CqsA) and AI-2 (produced by LuxS). These are perceived by the sensor histidine kinases LuxN (HAI-1), CqsS (CAI-1) and the LuxPQ complex (AI-2). At low population densities, LuxO-P is phosphorylated and induces the expression of the Qrr sRNA's, which repress translation of the QS regulator LuxR (vh) and interact with Hfq. At high population densities, the sensor kinases dephosphorylate LuxU. LuxU subsequently dephosphorylates LuxO, thus reducing the expression of the Qrr's and inducing the expression of LuxR(vh). Expression of LuxO is dependent on σ^{54}, while expression of *luxR(vh)* is regulated by cAMP-CRP and MetR (see section 1.3.2).

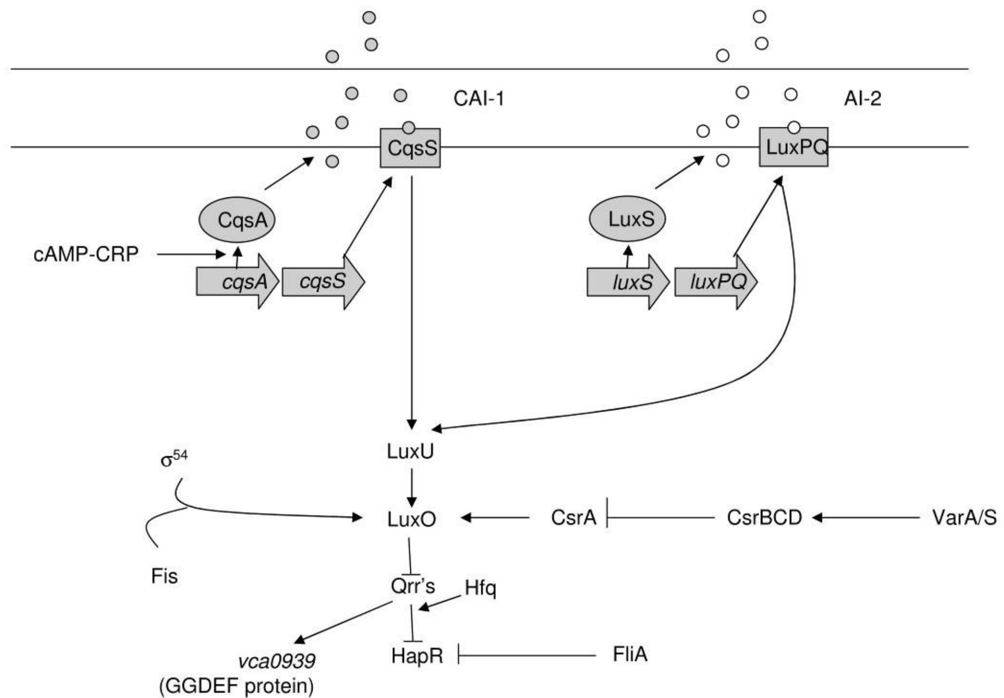


Figure 1.5: QS in *Vibrio* species.. B: *V. cholerae* produces two autoinducer molecules: CAI-1 (produced by CqsA) and AI-2 (produced by LuxS). These are perceived by the sensor histidine kinases CqsS (CAI-1) and the LuxPQ complex (AI-2). As in *V. harveyi,* expression of *luxO* requires s54. In addition, the small nucleoid protein Fis (which itself is expressed in a population density dependent manner) induces *luxO* expression. At low population densities, LuxO-P is phosphorylated and induces the expression of the Qrr sRNA's, which repress translation of the QS regulator HapR and interact with Hfq. At high population densities, the sensor kinases are activated, which leads to dephosphorylation of LuxU. LuxU subsequently dephosphorylates LuxO, thus reducing the expression of the Qrr's and inducing the expression of HapR. Induction of the Qrr's also activates the expression of the GGDEF protein *vca0939. V. cholerae* QS is also influenced by other signals as will be explained in section 1.3.2. cAMP-CRP modulates QS by a post-transcriptional effect on *cqsA* mRNA. VarA and VarS repress expression of LuxO via a regulatory cascade that involves the CsrBCD sRNA's and CsrA. The alternative sigma factor FliA induces expression of *hapR* upon arrival in the host environment.

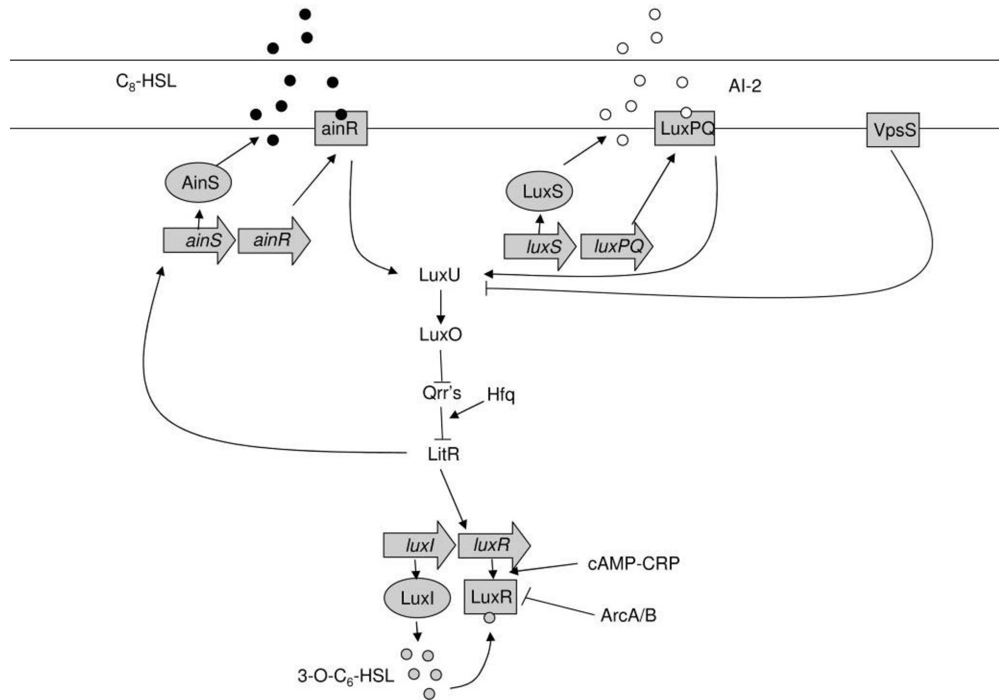


Figure 1.5: QS in *Vibrio* species. C: *V. fischeri* produces three autoinducer molecules: C_8-HSL (produced by AinS), AI-2 (produced by LuxS) and 3-oxo-C_6-HSL (produced by LuxI). C_8-HSL and AI-2 are perceived by the sensor histidine kinases AinR (C_8-HSL) and the LuxPQ complex (AI-2). At low population densities, LuxO-P is phosphorylated and induces the expression of the Qrr sRNA's, which repress translation of the QS regulator LitR and interact with Hfq. At high population densities, the sensor kinases are activated, which leads to dephosphorylation of LuxU. LuxU subsequently dephosphorylates LuxO, thus reducing the expression of the Qrr's and inducing the expression of LitR. LitR induces the expression of *ainS* and the *luxR*-type regulator *luxR(vf).* LuxR is activated by LuxI-made 3-oxo-C_6-HSL and induces expression of the bioluminescence genes. QS in *V. fischeri* is also influenced by environmental signals, as explained in section 1.3.2. Expression of LuxR is induced by cAMP-CRP. Under reducing conditions the response regulator ArcA can bind to the promoter of the *luxICDABEG* operon, thus inhibiting LuxR from binding. When the bacteria enter the light organ, oxidative conditions are met, which is sensed by ArcS. ArcS subsequently dephoshporylates ArcA, relieving it from the *luxICDABEG* promoter and allowing for LuxR(vf) binding. The hybrid sensor kinase VpsS phosphorylates LuxU, leading to phosphorylation of LuxO and induction of the Qrr's.

Ralstonia solanacearum

The plant pathogen *R. solanacearum,* which previously belonged to the *Pseudomonas* genus, produces two autoinducers: 3-OH PAME and SolI-made C_8- and C_9-HSL. The 3-OH PAME signal is detected by the PhcS histidine sensor kinase, which relays the information via PhcR to PhcA. PhcA induces *solR* expression and SolR induces gene expression in response to SolI-made AHLs (Clough et al. 1997a; Clough et al. 1997b; Flavier et al. 1997).

1.3.2 Environmental signals affecting QS gene regulation

In *P. aeruginosa,* adding AHLs exogenously to cultures did not always cause activation of QS (Diggle et al. 2002; Pearson 2002). Therefore, despite its name, quorum sensing is not just a matter of sensing quorum and population density is just one of the signals that bacteria use to determine which action to take in a certain environment.

Different environmental factors have been shown to influence QS signals. For example, transcriptome analysis of QS regulatory genes in different species has shown that factors like medium composition, temperature, oxygen availability, pH, glucose availability, osmolarity and redox state have a drastic impact on the expression of QS regulatory and QS regulated genes (Bazire et al. 2005; Bollinger et al. 2001; DeLisa et al. 2001; Duan and Surette 2007; Kim et al. 2005; McGowan et al. 2005; Sonck et al. 2009; Surette and Bassler 1999; Wagner et al. 2003). Understanding how the expression of QS genes is modified by environmental factors might give clues for new anti-virulence approaches that combat the activation of QS. In many cases the regulatory mechanisms behind these changes in expression are not clear and can probably be attributed to a general change in metabolic activity in the cell or lactonolysis of AHLs by pH or temperature. In most *E. carotovora* species, higher temperatures caused a reduction in production of AHLs (Hasegawa et al. 2005; McGowan et al. 2005). In *Y. pseudotuberculosis* increased temperatures caused degradation of AHLs, thus reducing the QS-dependent expression of the flagella genes (Yates et al. 2002). This allows the bacteria to swim until they are inside the host, but stop movement once they have arrived.

Nutrients

Although many effects of nutrient limitation on QS can probably partially be attributed to a change in metabolic state, there are specific regulatory mechanisms in place that couple nutrient sensing and QS gene regulation as well. Microorganisms must very often cope with low nutrient availability in their natural environment, and this can prevent the bacteria growing to high population densities. Therefore it is in some circumstances beneficial for the bacteria to elicit a QS response despite the fact that an appropriate quorum has not yet been reached. In addition, many pathogenic bacteria seem to be able to trigger QS in response to the low abundance of certain nutrients like Mg^{2+}, phosphate, etc. This low abundance might also serve as a signal for the bacteria, informing them that they have reached the right environment to switch on their virulence genes, which are often controlled by QS. Bacteria have different regulatory systems in place to sense and respond to nutrient conditions, for example the stringent response, catabolite repression and two-component systems.

a) The stringent response

When bacteria are confronted with low nutrient availability, they adapt by switching to a specific metabolic state, known as the 'stringent response' (for a review see Jain et al. 2006). This state is characterized by the inhibition of stable RNA (ribosomal and transfer RNA) synthesis, which is a result from the building up of high levels of the molecule guanosine 3', 5'-bidiphosphate (ppGpp) in the cell. When high concentrations of ppGpp are reached, it binds to the β-subunit of RNA polymerase and by doing so the promoter selectivity of the RNA polymerase is altered. In *E. coli* two proteins are involved in ppGpp accumulation: the ribosome-associated protein RelA functions as a ppGpp synthetase, while SpoT functions both as a ppGpp synthetase and a ppGpp hydrolase. It is thought that RelA mainly responds to amino acid starvation, while SpoT responds to other starvation conditions. The effect of the 'stringent response' is not limited to stopping stable RNA synthesis, but encompasses an inhibition of several other cellular processes. In several bacterial species the stringent response has been shown to be involved in modifying the expression of QS genes in a low nutrient environment.

One example of this is *P. aeruginosa,* where the 'stringent response' causes the premature activation of QS and virulence genes (van Delden et al. 2001). Some of the virulence genes encode tissue-degrading enzymes, which means that early activation of QS can enable the bacteria to access different nutrients during infection (Winstanley and Fothergill 2009). Induction of the 'stringent response' leads to

production of ppGpp by RelA and this leads to increased production of RhlI- and LasI-made AHLs via a transcriptional effect on the expression of *rhlR* and *lasR* (Erickson et al. 2004; van Delden et al. 2001). Apart from nutrient limitation, a change in the fluidity of the cell membrane under extreme environmental conditions can also trigger ppGpp synthesis and subsequent activation of the QS genes (Baysse et al. 2005). The protein DksA, originally identified as a repressor of *rhlI* (Branny et al. 2001), was shown to stabilise the interaction between ppGpp and RNA polymerase (Jude et al. 2003; Paul et al. 2004; Perron et al. 2005) (Table 1.2).

A role for the 'stringent response' in modulating QS was also found in *R. etli* and *A. tumefaciens.* In *R. etli* the stringent response causes an early activation of QS, as mutation of *relA* reduced the levels of both CinI- and RaiI-made AHL molecules (Moris et al. 2005). In contrast, in *A. tumefaciens* the stringent response has the opposite effect and is responsible for the activation of the lactonase AttM upon starvation. This subsequently leads to a decrease in the level of AHL molecules present and therefore inhibition of the QS-dependent conjugation of the Ti plasmid (Zhang et al. 2004).

b) Carbon catabolite repression

Catabolite repression is a global regulatory mechanism used by bacteria to regulate carbon catabolism. It was originally identified in *E. coli* and allows bacteria to adapt quickly to the presence of different carbon sources. In the presence of multiple carbon sources, bacteria can selectively use the one they prefer by inhibiting the expression of enzymes that catabolise carbon sources other than the preferred one (Bruckner and Titgemeyer 2002; Stulke and Hillen 1999). For example, in *E. coli* the preferred carbon source is glucose, which is taken up by the phosphoenolpyruvate phosphotransferase system. When the concentration of glucose inside the cell is high, adenylate cyclase (which converts ATP to cyclic adenosinemonophosphate or cAMP) is inhibited. Conversely, when glucose is absent, high levels of cAMP molecules build up. High glucose levels thus result in low levels of cAMP and low glucose levels result in high levels of cAMP (Deutscher et al. 2006). These cAMP molecules then bind to the cAMP receptor protein (Crp) and the activated cAMP-Crp complex is capable of binding to and induce the promoters of enzymes that catabolise less preferred carbon sources (Fic et al. 2009).

cAMP-Crp mediated carbon catabolite repression is a modulator of QS gene expression, causing an increase in AHL production when less of the preferred substrates are present. In *V. harveyi* cAMP-Crp functions as an activator of QS by direct binding of cAMP-Crp to the promoter of the master regulator *luxR(vh)* (Chatterjee et al. 2002) (Figure 1.5 A). In *V. cholerae* cAMP-Crp activates biosynthesis of CAI-1 autoinducers by an indirect post-transcriptional regulatory mechanism by influencing the stability of the *cqsA* mRNA, which encodes the CAI-1 synthase (Liang et al. 2007; Liang et al. 2008b) (Figure 1.5 B). In *V. fisheri* the cAMP-Crp complex is required for expression of *luxR(vf),* but it has not yet been shown at which level this regulation occurs (Dunlap 1999) (Figure 1.5 C).

In *E. coli,* cAMP-Crp influences QS gene regulation in two ways (Wang et al. 2005a). cAMP-Crp induces the expression of the Hfq-binding sRNA CyaR, which can bind to and destabilise *luxS* mRNA, causing less LuxS to be present for synthesis of AI-2 (De Lay and Gottesman 2009). In addition cAMP-Crp induces the expression of the AI-2 uptake system *lsr* by direct binding to its promoter (Xavier and Bassler 2005). AI-2 is thus being synthesised during early exponential growth (when glucose is present), but upon stationary phase it production ceases. Instead, AI-2 is being transported into the cells, possibly to be used as an alternative carbon source.

A role for cAMP-Crp in QS gene regulation was also identified in the phytopathogen *Erwinia chrysanthemi,* which contains the *expI/R* genes. ExpR activates the virulence genes in response to ExpI-made AHLs (Nasser et al. 1998; Reverchon et al. 1998). cAMP-Crp decreases *expI* expression, but increases *expR* expression. This could explain the observation that production of AHLs decreases after a quorum has reached and when the bacteria enter stationary phase (Reverchon et al. 1998)

Vfr, the homologue of Crp in *P. aeruginosa* was originally identified as a virulence factor regulator (West et al. 1994). The physiological role of this protein appears to be different from Crp in *E. coli* (Suh et al. 2002) and the main regulator of carbon metabolism and catabolite repression in *P. aeruginosa* is Crc (Wolff et al. 1991). The effect of Vfr on the expression of the virulence genes was due to induction of *lasR.* This effect was shown to be direct, as Vfr bound to the *lasR* promoter region in the presence of cAMP (Albus et al. 1997) (Table 1.2). In a *vfr* mutant, transcription of the transcriptional regulator of *rhlR* is reduced, but it has not yet been established whether this is due to a direct or indirect effect (Medina et al. 2003a). It has not yet been studied whether Crc influences the expression of the QS genes, although a recent

study showed that Crc modulates the expression of several QS-regulated virulence genes (Linares et al. 2010).

c) Nitrogen limitation

Sigma factors are subunits of RNA polymerase, and are required for gene transcription to occur. The expression of most genes in a bacterial cell is dependent on the expression of the 'housekeeping' sigma factor σ^{70}, but bacteria can express different sigma factors in response to different environmental conditions. These alternative sigma factors are involved in adaptation to specified niches, such as interactions with eukaryotic hosts. In many bacteria a link between one of these alternative sigma factors and QS gene regulation has been found. Under nitrogen starvation conditions the alternative sigma factor RpoN (σ^{54}) is activated and induces the expression of genes that are involved in nitrogen assimilation (Hendrickson et al. 2001).

In *V. cholerae* and *V. harveyi,* a link between RpoN and QS has been found, as the activity of the response regulator LuxO-P is dependent on the presence of RpoN, to promote the transcription of the Qrr sRNA's (Klose et al. 1998; Lenz et al. 2004; Lilley and Bassler 2000). Increased transcription of the *qrr* genes causes a destabilisation of the QS master regulator, and thus RpoN has a negative effect on the expression of QS-regulated genes (Figure 1.5 A+B).

Heurlier *et al.* (2003) found that RpoN reduced production of RhlI-made and LasI-made AHLs in *P. aeruginosa*. These effects were, at least partially, due to some indirect effects, as RpoN induced expression of *vfr* and repressed expression of *gacA.* In contrast with this are the observations from Thompson *et al.* (2003), who found that RpoN increased production of RhlI-made AHLs by induction of *rhlI* expression and Medina *et al.* (2003a)*,* who found that RpoN activated expression of *rhlR* (Table 1.2).

d) Iron limitation

A link between QS and iron deprivation has been observed in *P. aeruginosa* and *B. japonicum.* In *P. aeruginosa* expression of *lasI* and *lasR* is increased under iron-limited conditions (Bollinger et al. 2001; Jensen et al. 2006; Kim et al. 2005). Since invasion of the host is usually characterised by a shift to low-iron conditions, this can serve as a signal for early activation of the QS genes followed by the virulence genes. When host tissues become damaged as a consequence of the virulence factors, the resulting increase in iron concentrations should down-regulate the production of

virulence factor, which could favour host survival. Uptake of iron is controlled by a large set of genes, including siderophores, ferric uptake regulators, and sigma factors (Cornelis et al. 2009). The QS regulators PqsR (Deziel et al. 2005), VqsR (Cornelis and Aendekerk 2004; Juhas et al. 2004; Juhas et al. 2005), LasR and RhlR (Schuster et al. 2003) induce the expression of many iron responsive genes.

An effect of the iron concentration on the expression of the QS genes is exerted at different levels (Table 1.2). Expression of *pqsR* is increased in response to the iron starvation sigma factor PvdS (Ochsner et al. 2002). PqsR induces the expression of the *pqsABCDE* operon and the effector protein PqsE induces virulence. Under low iron conditions, the ferric uptake regulator (Fur) increases expression of two small regulatory RNAs encoded by *prrF1* and *prrF2*. The PrrF sRNAs destabilise the mRNA of the *antABC* genes that are responsible for the degradation of the PQS-precursor anthranilate, thus sparing anthranilate for PQS production and activating QS (Oglesby et al. 2008). The ability of PQS to trap iron is likely to reduce the amount of available iron in the cell (Bredenbruch et al. 2006).

Another example where iron deficiency causes an activation of QS gene regulation has been described in *B. japonicum,* as production of bradyoxetin was found to be maximal under low iron conditions (Loh et al. 2002a).

e) *phoB/R:* phosphate

Bacteria sense the presence of several nutrients by means of two-component systems. These typically consist of a response regulator and a sensor histidine kinase (Laub and Goulian 2007). The sensor histidine kinase senses a specific signal in the environment and upon doing so it phosphorylates itself and relays the signal to the corresponding response regulator. The activated response regulator then induces the expression of other genes. A link between two-component and QS gene regulation has been found in many species, although the signal recognised by the sensor kinase is not always known.

PhoR is a histidine sensor kinase that senses the amount of available inorganic phosphate in the environment. For this, it interacts with the ABC-type phosphate-specific transport system (Pst): at low phosphate concentrations PhoR is activated by autophosphorylation, after which the phosphoryl group is transferred to the response regulator PhoB. When there is sufficient phosphate in the environment, the Pst system is thought to form a repressing complex with PhoR, thus preventing activation of

PhoB (Lamarche et al. 2008). PhoB is not only activated by its partner histidine kinase PhoR, but also by other histidine kinases. For example, in *E. coli* the EnvZ sensor protein can activate PhoB in response to acetylphosphate in the absence of PhoR (Kim et al. 1996). Such cross-talk allows the integration of other environmental queues through PhoB.

PhoR homologues modulate QS gene regulation in several different bacterial species. In *S. meliloti* low phosphate conditions trigger an early activation of QS (Figure 1.3). This can be beneficial since in the soil phosphate levels are usually low. Phosphate uptake by the plant can actually create a zone of phosphate depletion in the rhizosphere, thus preventing the bacteria from reaching high population densities (Schachtman et al. 1998). Using microarray analysis the phosphate starvation response of *S. meliloti* was characterised and one of the effects was an increased expression of *sinR* (Krol and Becker 2004) (Figure 1.3). The mechanism by which PhoB regulates the expression of *sinR* has not yet been identified. A *pho* box was found in the upstream region of the *sinR* gene, but deletion of this sequence did not abolish regulation by PhoB, indicating that potentially the regulatory effect is mediated via an unidentified intermediate regulator (McIntosh et al. 2009).

In *Serratia* sp. ATCC39006 phosphorylated PhoB induced the expression of the AHL synthase *smaI* (Gristwood et al. 2009). In *P. aeruginosa* low phosphate conditions induced expression of *rhlR* and *pqsR* (Jensen et al. 2006; Zaborin et al. 2009).

f) *phoP/Q:* Mg^{2+}

The PhoP/Q two-component regulatory system mediates the adaptation of an organism to the Mg^{2+} concentrations, with PhoP serving as the response regulator and PhoQ as the histidine kinase sensor protein. It was first described in *Salmonella typhimurium* where it controls the expression of the virulence factors (Groisman 2001; Kier et al. 1979; Miller et al. 1989). PhoP induced the expression of the AHL synthase encoded by *pcoI in Pseudomonas fluorescens* 2P24 in response to low Mg^{2+} concentrations (Yan et al. 2009a). Under the same conditions, increased expression of PQS biosynthesis genes and *lasI* was observed in *P.aeruginosa*, but it has not yet been investigated whether the PhoP is responsible for this (Guina et al. 2003).

g) Amino acids

V. harveyi contains the LysR-type regulator MetR that monitors the amino acids in the environment. In response to homocysteine MetR causes a decrease in luminescence, and this was shown to be a direct effect as MetR binds to the *luxR(vh)* promoter (Chatterjee et al. 2002) (Figure 1.5 A).

Other environmental conditions

a) Oxygen

X. campestris QS gene regulation relies on the recognition of DSF by the sensor kinase RpfC (Figure 1.6). The signal is transmitted to the HD-GYP protein RpfG, causing hydrolysis of cyclic-di-GMP to cGMP. The altered levels of cGMP are sensed by the transcriptional regulator Clp, which induces target gene expression (Fouhy et al. 2006; He and Zhang 2008). Therefore the presence of other enzymes that modulate the levels of cGMP in the cell could alter the QS response.

Intracellular cyclic-di-GMP levels are typically modulated in two ways: proteins containing a GGDEF domain are responsible for the synthesis of cyclic-di-GMP, while proteins that contain an EAL or HD-GYP domain degrade cyclic-di-GMP (Ryan et al. 2006; Schirmer and Jenal 2009). In addition, some proteins contain both a GGDEF and an EAL domain. In *X. campestris* the proteins containing GGDEF, EAL or HD-GYP domains were all analysed by deletion mutagenesis studies. Only the deletion of *ravR* was capable of altering the DSF induced virulence response (He et al. 2009). RavR contains both a GGDEF and an EAL domain and is activated by the sensor kinase RavS (Figure 1.6). The RavR EAL domain was proven to be functional and degraded cyclic-di-GMP to cGMP, while the GGDEF domain (normally responsible for synthesising cyclic-di-GMP) was found to be not functional. RavS is very similar to the oxygen-sensing protein FixL from rhizobia and contains two domains with a conserved fold and key residues involved in haem binding (Gong et al. 1998; He et al. 2009; Key and Moffat 2005). Thus RavR increases the amount of intracellular cGMP in response to low-oxygen tension (Figure 1.6), and this is subsequently detected by the transcriptional regulator Clp, which can modulate the QS induced virulence response (Chin et al. 2010; He et al. 2009).

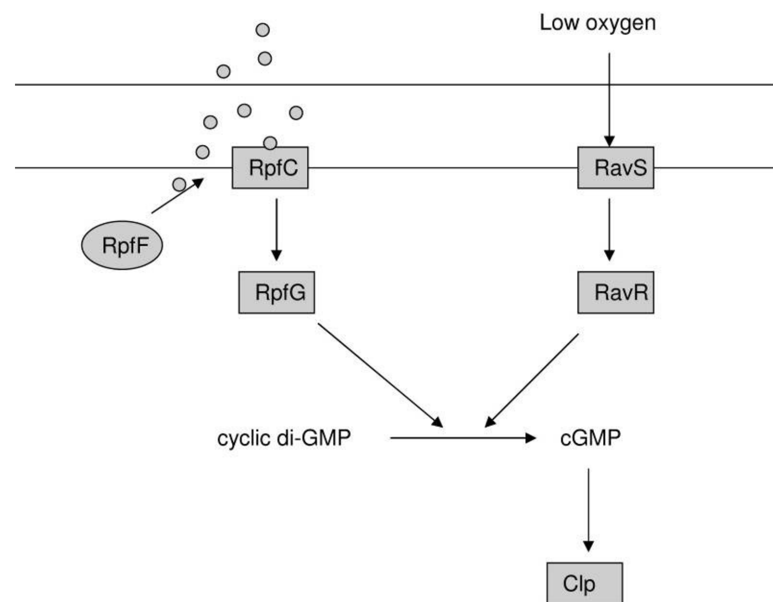


Figure 1.6: QS in *X. campestris.* DSF is produced by RpfF and is sensed by the two-component sensor kinase RpfC. RpfC transmits the signal to the HD-GYP protein RpfG. RpfG has a HD-GYP domain hydrolyses cyclic-di-GMP to cGMP. The regulator Clp senses cGMP and induces gene expression in response. cGMP levels are also modulated by the presence of the RavS sensor kinase, which senses low oxygen conditions.

Oxygen levels have also been shown to modulate the QS response in *P. aeruginosa.* The transcriptional regulator ANR belongs to the FNR (fumarate and nitrate reductase regulator) family and is activated under low-oxygen conditions (Spiro 1994). ANR is thought to function synergistically with LasR and RhlR (Pessi and Haas 2000) (Table 1.2). In addition expression of *lasR* was increased under oxygen stress (Kim et al. 2005).

Bioluminescence in *V. fischeri* is under QS control and light is generated by the products of the *luxICDABEG* genes. As the biochemistry of this reaction requires the use of oxygen and reducing power, one possible advantage of bioluminescence could be to protect the bacteria against intra- or extracellular oxidants upon colonisation of the light organ (Timmins et al. 2001; Visick et al. 2000). Hence it was predicted that the expression of the *lux* genes would be under redox control. Good candidate to mediate redox-dependent gene regulation were the ArcA and ArcB proteins. Homologues of *arcA* and *arcB* were originally identified in *E. coli* as part of a redox-sensitive two-component system (Georgellis et al. 1997). Mutation of *arcA* and *arcB* in *V. fischeri* strain ES114 affected the expression of the *lux* genes. In planktonic conditions (no oxidative stress) the phophorylated ArcA protein is activated by ArcB and binds to the *luxICDABEG* promoter, thus effectively blocking the binding of the AHL-binding transcriptional regulator LuxR(vf). Upon colonisation of the light organ, oxidative conditions are met, possibly due to host-generated reactive oxygen species. When this happens, ArcB dephosphorylates ArcA, which no longer binds to the *luxICDABEG* promoter. This allows LuxR(vf) to bind, thus inducing AHL-based QS (Bose et al. 2007) (Figure 1.5 C).

b) FliA: sensing arrival at colonisation site

The pathogen *V. cholerae* uses an alternative sigma factor FliA to sense that it has reached its site of colonisation in the small intestine. This depends on the fact that the flagella are broken from the cells during passage through the mucosal layer that covers the epithelial cells of the small intestine. Loss of the flagella leads to the release of the anti-sigma factor FlgM, which causes a derepression of FliA. FliA represses transcription of the QS regulator *hapR* and this causes a loss of HapR-mediated repression (hence activation) of the virulence genes that are under QS control (Liu et al. 2008; Tsou et al. 2008) (Figure 1.5 B).

c) *varA/S* (*gacA/S*)

The best studied two-component system with regards to its effect on QS gene regulation is the GacA/S system in *P. aeruginosa* (Reimmann et al. 1997), which is called VarA/S in *V. cholerae* (Lenz et al. 2005). The signal that activates GacS (VarS) has not yet been identified, but *Pseudomonas* and *Vibrio* species that grow to high population densities secrete GacA-activating signals, which are chemically unrelated to AHLs or AI-2. Because the signal accumulates at high population densities, the GacA/S two component system has been proposed to function as a QS system itself (Dubuis and Haas 2007).

In *V. cholerae* VarA and VarS control the transcription of three sRNA's (CsrB, CsrC and CsrD) that are homologous to the *E. coli* carbon storage regulator sRNA's CsrB and CsrC. These sRNA's bind to and inactivate the sRNA binding protein CsrA (Lenz et al. 2005). CsrA post-transcriptionally regulates the levels of LuxO and thus the expression of the Qrr sRNA's. At low cell densities VarS is not activated, and hence there is no transcription of the Csr sRNA's. This means CsrA is active and increases the amount of the response regulator LuxO-P, which leads to the induction of the Qrr sRNA's. The effect of CsrA on the amount of LuxO mRNA is probably not direct, but appears to be mediated by an as yet unidentified protein (Lenz et al. 2005) (Figure 1.5 B). The influence of the VarA on QS is not conserved in all *Vibrio* species, as in *V. fischeri* no effect on AHL production could be observed (Whistler and Ruby 2003).

In several *Pseudomonas* species GacA and GacS induce the production of AHLs (Chancey et al. 1999; Kay et al. 2006; Quinones et al. 2004; Reimmann et al. 1997) (table 1.2). In *P. aeruginosa* GacA induced the expression of the regulation of secondary metabolite sRNA's (RsmY and RsmZ) (Heurlier et al. 2004; Kay et al. 2006) that are capable of binding and inactivating a sRNA binding protein homologous to CsrA, RsmA. When active, RsmA reduces the expression of *rhlI* and *lasI* and the amount of RhlI- and LasI-made AHLs (Burrowes et al. 2005; Kay et al. 2006; Pessi and Haas 2001). This is likely to be a consequence of reduced expression of *rhlR* and *lasR* (Reimmann et al. 1997). A role for the global RNA chaperone Hfq in this regulatory mechanism has also been established, as Hfq binds to and stabilises RsmY (Sonnleitner et al. 2006). GacA and GacS also affect QS by inducing the expression of the *luxR*-type regulator *qscR* (Ledgham et al. 2003b). Two other sensor kinases-response regulator hybrids, LadS and RetS, control the expression of the sRNA RsmZ, affecting the activity of RsmA (Ventre et al. 2006). Thus LadS, RetS and GacS represent three different sensor kinases, which integrate different signals

into one central signalling cascade. GacA and GacS regulation of QS genes was also observed in other pseudomonads, like *P. aureofaciens* (Chancey et al. 1999; Zhang and Pierson 2001), *P. syringae* (Kitten et al. 1998; Quinones et al. 2004), *Pseudomonas* sp. M18 (Wang et al. 2008) and *P. fluorescens* (Yan et al. 2009b).

d) *bqsS/R* and *vpsS:* signal unknown

The BqsS/BqsR two component system in *P. aeruginosa* and the sensor kinase VpsS in *V. cholerae* affect QS. In both cases, the signal to which the sensor kinase responds is unknown. BqsR in *P. aeruginosa* was involved in biofilm decay, via a regulatory effect on the production of PQS and RhlI-made AHLs (Dong et al. 2008). As PqsR induces *rhlI* expression (Diggle et al. 2003), the effect of BqsR on the production of RhlI-made AHLs is likely indirect (Table 1.2). One possibility is that BqsR facilitates the conversion of anthranilate to PQS, as the transcription of the PQS biosynthetic genes *pqsA* and *phnA* was decreased in the mutant compared to wild type (Dong et al. 2008).

The *V. cholerae* hybrid sensor histidine kinase VpsS was identified based on its role in controlling biofilm formation by inducing the expression of the *vps* polysaccharide biosynthetic genes (Shikuma et al. 2009). Activated VpsS phosphorylates the phosphotransferase protein LuxU, which relays the phosphate to LuxO. Activated LuxO-P reduces the transcription of the global QS transcriptional regulator HapR, via induction of the Qrr's. VpsS can only affect HapR at low population densities, as at higher population densities the QS signals cause the dephosphorylation of LuxO-P (Figure 1.5 C). The observed effects of VpsS on biofilm formation are not strictly dependent on its effect on HapR though, as LuxO also activates two other transcriptional regulators that influence the expression of the *vps* genes, VpsT and VpsR (Shikuma et al. 2009).

e) RpoS: different stresses

The sigma factor RpoS (σ^{38}) is activated in stationary phase (Lange and Hengge-Aronis 1991) and in response to stresses like UV radiation, acid, temperature or osmotic shock, oxidative stress and nutrient deprivation (Durfee et al. 2008; Klauck et al. 2007).

The role of RpoS in *P. auruginosa* QS seems to be dependent on the experimental conditions used. Initial studies showed that RhlR activated transcription of *rpoS* (Latifi et al. 1996), which was later backed up by microarray analysis (Schuster et al. 2004; Schuster et al. 2003; Wagner et al. 2003). Other studies found that *rpoS* expression was not regulated by QS (Bertani et al. 2003; Whiteley et al. 2000), but instead RpoS repressed *rhlI* expression (Whiteley et al. 2000) (Table 1.2). Repression of QS by RpoS was also observed in *P. syringae* (Chatterjee et al. 2007) and *P. fluorescens* (Yan et al. 2009b) and it was found that, in stationary phase, more than 40% of the *P. aeruginosa* genes that were controlled by QS were also controlled by RpoS (Schuster et al. 2004). One report showed that RpoS repressed the expression of some QS-induced genes like *hcnABC* and *phzABC* (Whiteley et al. 2000), while an other report suggested that RpoS induced the expression of QS-controlled genes like *rhlAB* (Medina et al. 2003b). To explain these seemingly contradictory observations Schuster *et al.* (2004) proposed a model in which RpoS and QS regulated genes were divided into different categories, depending on whether the regulatory effects of both factors are direct or indirect.

In several other species, a role for RpoS modulating QS has been shown. In *Edwardsiella tarda* RpoS repressed the expression of the AI-2 synthase *luxS* (Xiao et al. 2009). In *R. solanacearum* RpoS induced expression of *solR* and *solI* (Flavier et al. 1998), In *E. coli* RpoS had a dual effect, namely the repression of *lsr* expression, resulting in reduced uptake of AI-2 (Wang et al. 2005a) and the induction of expression of the *luxS* homologue *ygaG* (Lelong et al. 2007). In *Vibrio anguillarum* RpoS induced the expression of the QS master regulator VanT (the homologue of the regulator LuxR in *V. harveyi* and HapR in *V. cholerae*). This effect was indirect and mediated through the repression of the expression of Hfq, destabilising the Qrr's and thus stabilising *vanT* mRNA (Weber et al. 2008). Other factors can modulate QS gene regulation through their effects on the sigma factor RpoS, sometimes in response to an environmental signal. For example, the type VI secretion system in *Vibrio anguillarum* induced the expression of *rpoS* (and thus *vanT*) by a currently unknown mechanism (Weber et al. 2009). PsrA in *P. syringae* and *P. chlororaphis* repressed AHL production by a transcriptional effect on *psyR* expression in response to fatty chain acids (Kang et al. 2009; Kojic and Venturi 2001). This repression of QS was shown to be an indirect effect, by induction of *rpoS* expression (Chatterjee et al. 2007; Girard et al. 2006).

1.3.3 Signals from other species

Microbial cross-communication

In natural conditions, bacteria usually occur as a mixture of species, and they have developed means of communicating with each other and to listen in on other conversations. Bacteria are thought to use AI-2 for interspecies communication and the variable chemical nature of AHLs allows intraspecies communcation. However various LuxR-type regulators can interact with non-cognate AHL molecules, and such interactions could lead to an unwanted activation or inhibition of QS (McClean et al. 1997; Schaefer et al. 1996a; Welch et al. 2000; Zhu et al. 1998). This could explain why other QS signals, like PQS in *Pseudomonas,* 3-OH PAME in *R. solanacearum,* bradyoxetin in *B. japonicum* and the DSF and DF in *Xanthomonas* species have been adopted, as a lack of cross-talk could provide a selective advantage. Several species have LuxR-type transcriptional regulators, although they do not produce any AHLs. *E. coli* and *S. typhimurium* contain the LuxR-type regulator SdiA, which can be activated by AHLs produced by other bacteria, possibly to indicate their arrival in the right environment to induce their virulence genes (Ahmer et al. 1998; Kanamaru et al. 2000; Michael et al. 2001).

Another mechanism to alter the bacterial QS response is by the production of autoinducer-degrading enzymes, which are found in many bacteria. Two kinds of AHL degrading enzymes have been identified, as described in section 1.2.2. (for a review, see Czajkowski and Jafra 2009; Dong and Zhang 2005; Uroz et al. 2009; Zhang 2003).

Communication with eukaryotes

Many bacteria use QS gene regulation for the regulation of factors that are involved in their relationship with the eukaryotic host. Examples include virulence gene expression in the human pathogens *P. aeruginosa* (Bjarnsholt and Givskov 2007), *V. cholerae* (Higgins et al. 2007), pathogenic *E. coli* (Sircili et al. 2004), *S. typhimurium* (Choi et al. 2007) and the plant plant pathogens *R. solanacearum* (Genin et al. 2005), *E, carotovora* (Barnard and Salmond 2007), *A. tumefaciens* (White and Winans 2007), *X. campestris* (He and Zhang 2008) and *S. marcescens* (Coulthurst et al. 2004), bioluminescence in *V. fischeri* (Fidopiastis et al. 2002) and *V. harveyi* (Bassler et al. 1993), and symbiotic nitrogen fixation in rhizobia (Downie and Gonzalez 2008). It is therefore not surprising that the eukaryotic hosts have developed mechanisms to

modulate bacterial QS. Eukaryotic signals have been identified that interfere with bacterial QS ('quorum quenching'), either by the production of AHL mimics or by the production of autoinducer-degrading enzymes, thus altering the level of autoinducers that are perceived by the bacteria rather then directly altering their level of production.

Perhaps the best known example of modulation of QS gene regulation by plant metabolites is found in the crown gall-inducing plant pathogen *A. tumefaciens* (Figure 1.7). This pathogen carries the tumour-inducing (Ti) plasmid, which contains oncogenic genes that are transferred to the plant cell nucleus. It also carries genes that can mediate the conjugation of the Ti plasmid to other *Agrobacterium* strains (Genetello et al. 1977). Conjugation is strongly stimulated by compounds that are produced by the tumour plant cells, namely opines or nopalines, which also serve as a source of food for the invading bacteria. Opines induce conjugation of the opine-Ti plasmid, while nopalines induce conjugation of the nopaline-Ti plasmid (Genetello et al. 1977; Kerr et al. 1977). In the opine-type Ti plasmid, the regulator OccR (octopine catabolism regulator) induces expression of both octopine catabolism and the plasmid transfer genes in response to opines (Habeeb et al. 1991). In nopaline-type Ti plasmids the regulator AccR (agrocinopine catabolism regulator) functions as a repressor that represses agrocinopine catabolism and the plasmid transfer genes in the absence of agrocinopines (Beck von Bodman et al. 1992). Regulation of Ti-plasmid conjugation by OccR and AccR is achieved by their induction of the LuxR-type regulator *traR* in response to opines and nopalines (Fuqua and Winans 1994; Piper et al. 1993). TraR then induces the expression of the plasmid transfer genes in response to TraI-made AHLs (Fuqua and Winans 1994).

Apart from opines and nopalines the plant tumour cells also produce mannopines and these inhibit the production of AHLs by TraI. Mannopines also serve as food source for the invading *Agrobacterium* but are less preferred then opines and nopalines. The mannopine-degrading cluster on the Ti-plasmid contains a LuxR-type regulator TrlR (also known as TraS) that is induced in the presence of mannopines (Chai et al. 2001; Oger et al. 1998; Zhu and Winans 1998). TrlR is very similar to TraR, but it has a frameshift mutation which results in a protein lacking the DNA binding domain. TrlR has retained the ability to interact with TraR, thus forming inactive heterodimers. Therefore, when mannopines are present TrlR is induced and this inhibits conjugation of the Ti-plasmid. In the presence of more appreciated food sources conjugation is induced. Apart from TrlR, *A. tumefaciens* also contains a gene coding for another anti-activator, *traM*, which is adjacent to TraR on the Ti-plasmid. Expression of *traM* is

under control of TraR and TraM binds to and inactivates TraR. This is thought to prevent TraR from reacting with AHLs that are produced in the cell itself (Hwang et al. 1995; Luo et al. 2000; Vannini et al. 2004) (Figure 1.7).

Expression of the virulence genes in the plant pathogen *Pantoea agglomerans* (also known as *Erwinia herbicola*) is induced by the *pag* QS genes. Upon formation of the plant tumour, the plant produces the plant hormones indole acetic acid (IAA) and cytokinin and these modulate the expression of *pagI* and *pagR* (Chalupowicz et al. 2009). In *Erwinia chrysanthemi* the transcriptional regulator PecS, which is thought to respond to the presence of plant phenolic compounds, repressed *expI,* encoding an AHL-synthase (Reverchon et al. 1998). Another interesting example of plant manipulation of QS has been found in *Rhodopseudomonas palustris,* which produces a new class of AHL molecules (coumaroyl-AHL), that require plant-produced coumaric acid rather than fatty acids as substrate (Schaefer et al. 2008). *Medicago sativa* produces L-canavanine, which has been shown to interfere with QS in *S. meliloti* (Keshavan et al. 2005). Many other examples of plant metabolites that affect bacterial QS have been found in plant essential oils and extracts, but it is often not known how they function (Al-Hussaini and Mahasneh 2009; Bodini et al. 2009; Feldman et al. 2009; Khan et al. 2009; Truchado et al. 2009).

In some cases a direct interaction of the compounds (AHL mimics) with the autoinducer receptor has been shown. The marine red alga *Delisea pulchra* inhibitis QS gene regulation by production of halogenated furanones that interact with bacterial AHL receptors, leading to degradation of the receptor (Givskov et al. 1996; Manefield et al. 2002). *Chlamydomonas* species produce a variety of AHL mimics, capable of activating some QS genes, while repressing others (Rajamani et al. 2008; Teplitski et al. 2004). Even higher plants, such as *Medicago truncatula,* pea, vetch, soybean, tomato and rice produce AHL mimics (Degrassi et al. 2007; Gao et al. 2003; Teplitski et al. 2000). In *Xanthomonas oryzae* and *Xanthomonas campestris* currently unidentified compounds in plant exudates were shown to activate the orphan LuxR-type regulators OryR and XccR (Ferluga and Venturi 2009).

Animal metabolites also modulate QS gene expression. For example, upon infection with *P. aeruginosa,* host stress is characterised by the release of the morphine-like chemical dynorphin, which can induce the PQS genes to induce virulence (Zaborina et al. 2007). In enterohemorrhagic *E. coli* (EHEC), the autoinducer AI-3 is thought to resemble the chemical structure of the mammalian hormones epinephrine and

norepinephrine (Sperandio et al. 2003). Therefore, when EHEC is present in the human colon, it can recognize these hormones and use them for the activation of the virulence genes (Clarke et al. 2006). The bacterivorous nematode *Caenorhabditis elegans* also produces compounds that inhibit *Pseudomonas* QS (Kaplan et al. 2009).

Eukaryotes can also affect autoinducer degradation. For example, in *A. tumefaciens* plant signals alter the expression levels of AHL lactonases that are encoded in the bacterial genome as a manner of defence against invasion (Chevrot et al. 2006; Haudecoeur et al. 2009a; Haudecoeur et al. 2009b). Other examples include the inactivation of *Pseudomonas* AHLs by human airway epithelia (Chun et al. 2004) and the degradation of AHLs by mammal paraoxonases (Draganov et al. 2005; Ozer et al. 2005; Teiber et al. 2008).

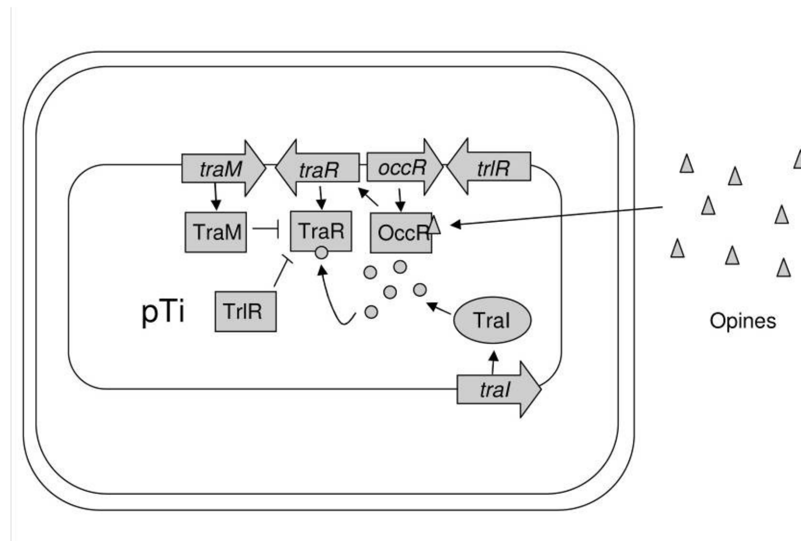


Figure 1.7: Induction of the *tra* QS system of *A. tumefaciens* by plant-made opines. The Ti plasmid carries the plasmid conjugation genes. Expression of these genes is induces by TraR in response to TraI-made AHLs. Expression of *traR* is induced by the transcriptional regulator OccR in response to plant-made opines. TraM and TrlR function as anti-activators of TraR.

Regulator	Function	Regulation of QS	Reference
RsaL	Transcriptional repressor	Repression of *lasI* transcription	(de Kievit *et al.*, 1999)
QscR	LuxR-type regulator	Formation of inactive dimers with RhlR and LasR	(Chugani *et al.*, 2001; Ledgham *et al.*, 2003b)
VqsR	LuxR-type regulator	Repression of *lasI* transcription	(Juhas *et al.*, 2004; Juhas *et al.*, 2005)
MvaT	HNS-like transcriptional regulator	Modulation of cell-density dependent gene expression	(Diggle *et al.*, 2002)
QteB	repressor	Regulation of quorum treshold	(Siehnel *et al.*, 2010)
GidA	Flavin adenine dinucleotide-binding protein	Post-transcirptional regulation of RhlR	(Gupta *et al.*, 2009)
PmpR	YebC-like protein	Repression of *pqsR* transcription	(Liang *et al.*, 2008)
AlgQ	Transcriptional regulator	Repression of *lasR* and *rhlR* transcription	(Ledgham *et al.*, 2003a)
AlgR	Transcriptional regulator	Repression of *rhlI* expression in biofilms	(Morici *et al.*, 2007)
VqsM	Transcriptional regulator	Induction of *rhl* and *las* QS systems, largely through effect on *vqsR*	(Dong *et al.*, 2005)
PA1196	Transcriptional regulator	Induction of rhl and PQS systems	(Liang *et al.*, 2009)
PpyR	Predicted membrane protein	Induction of PQS production	(Attila *et al.*, 2008)
Lon	Protease	Degradation LasI	(Bertani *et al.*, 2007; Takaya *et al.*, 2008)
Vfr	cAMP receptor	Induction of *lasR* expression	(Albus *et al.*, 1997)
RpoS	Stationary phase sigma factor	Repression of *rhlI* expression	(Whiteley *et al.*, 2000)
RpoN	Nitrogen starvation sigma factor	Repression of *rhl* and *las* QS systems, inductino of rhlI?	(Heurlier *et al.*, 2003; Medina *et al.*, 2003; Thompson *et al.*, 2003)

Regulator	Function	Regulation of QS	Reference
RsmA	Post-transcriptional regulator	Negatively controls *lasI* and *rhlI*	(Burrowes *et al.*, 2005; Kay *et al.*, 2006; Pessi *et al.*, 2001)
GacA	Response regulator	Induction of *lasR* and *rhlR* expression	(Reimmann *et al.*, 1997)
DksA	Repressor	Repression of *rhlI* by stabilisation of interaction between ppGpp and RNA polymerase	(Branny *et al.*, 2001; Jude *et al.*, 2003; Paul *et al.*, 2004; Perron *et al.*, 2005)
PhoB	Response regulator	Induction of *rhlR* and *pqsR* expression under phospate limited conditions	(Jensen *et al.*, 2006; Zaborin *et al.*, 2009)
PtxR	Transcriptional regulator	Repression of PQS biosynthesis and *rhlI* expression, induction of *lasI* expression	(Carty *et al.*, 2006)
PPK1	Polyphosphate kinase	Induction of AHL production	(Fraley *et al.*, 2007)
RelA	ppGpp synthase	Induction of *rhlR* and *lasR* expression	(Erickson *et al.*, 2004; van Delden *et al.*, 2001)
Fur	Ferric uptake regulator	Induction of PQS production	(Oglesby *et al.*, 2008)
PvdS	Iron starvation sigma factor	Induction of PQS production and expression of *pqsE*	(Ochsner *et al.*, 2002)
ANR	Transcriptional regulator	Functions synergistically with RhlR and LasR	(Kim *et al.*, 2005)
BqsS	Response regulator	Facilitating conversion of anthranilate to PQS	(Dong *et al.*, 2008)

Table 1.2: Overview of regulators that modulate QS in *P. aeruginosa*.

1.4 Aims of this project

In *R. leguminosarum* A34 induction of the *cin* genes leads to the induction of the *tra, rai* and *rhi* genes. Induction of the *rai* genes was shown to be mediated via the expression of a small gene *cinS* together with *expR* (Edwards et al. 2009). This project aimed to determine the molecular mechanism by which CinS regulates gene expression. This was addressed using the following approaches:

- purification and analysis of CinS protein
- identification of possible CinS interactors using EMSA, pull down and bacterial two hybrid analysis

In addition, the regulon of *cinS* was determined:

- using phenotypical studies
- using microarray analysis

Chapter 2: CinS, a novel regulator in the *cin* QS system

2.1 Introduction

The symbiosis between rhizobia and legumes is initiated by rhizobially made Nod-factors, which are synthesised in response to plant-made flavonoids and determine the ability of the bacteria to nodulate specific legume species (D'Haeze & Holsters, 2002). In addition, rhizobia produce surface EPS which are important in the initial stages of infection and contribute to the host-specificity of the symbiosis (Jones *et al.*, 2007; Skorupska *et al.*, 2006). For example, *Sinorhizobium meliloti* requires the production of at least one of two symbiotically active EPS, succinoglycan (EPSI) or galactoglucan (EPSII), for successful infection of alfalfa nodules and for evasion of the plant host defense response (Jones et al., 2008; Niehaus et al., 1993). It has been proposed that the low molecular weight fraction of these EPS is functioning as a symbiotic signal during infection and is required for biofilm formation (Gonzalez et al., 1996; Rinaudi & Gonzalez, 2009). In addition, other surface polysaccharides are also important for attachment and biofilm formation (Laus et al., 2005; Williams et al., 2008). In *R. leguminosarum* the acidic EPS is required for infection and root hair attachment. In addition the EPS glucomannan was shown to be important for lectin-mediated attachment to pea root hairs (Laus et al., 2006) and competitive nodule nodulation (Williams et al., 2008).

Many rhizobial species contain one or more AHL-based QS systems for intraspecies communication to optimise their interactions with the plant (Downie & Gonzalez, 2008; Sanchez-Contreras *et al.*, 2007). AHL-based QS systems are typically made up of two genes: one encodes a LuxI-type AHL-synthase that is responsible for the production of AHLs and the other encodes a LuxR-type transcriptional regulator that modulates gene expression in response to these AHLs. The AHL concentration is sensed by the bacteria to measure their population density, as well as the diffusional conditions that are encountered in the environment (Hense *et al.*, 2007; Williams,

2007). Different aspects of the *Rhizobium*-legume symbiosis have been shown to be regulated by QS (Downie & Gonzalez, 2008). EPS biosynthesis and processing is also found to be under the control of QS. In *S. meliloti* EPSI and II production is regulated by the QS regulator ExpR in conjunction with the *sinI/R* QS system (Glenn *et al.*, 2007; Gurich & Gonzalez, 2009; Marketon *et al.*, 2003; Pellock *et al.*, 2002). In *R. leguminosarum* the expression of the glycanase *plyB* is regulated by both ExpR and the *cin* QS system (Edwards *et al.*, 2009). This glycanase, which is secreted via a Type I secretion system (Finnie *et al.*, 1998) cleaves the nascent EPS chain and affects biofilm formation *in vitro,* although mutation of *plyB* had no effect on nodulation (Zorreguieta *et al.*, 2000).

In *R. l.* bv. *viciae* four different AHL-based QS systems have been described and these are made up of the *traI/R*, *raiI/R*, *rhiI/R* and *cinI/R* genes (Downie & Gonzalez, 2008; Sanchez-Contreras *et al.*, 2007). In addition, several LuxR-type regulators that are not directly linked to an AHL synthase (orphan LuxR-type regulators) have been found (Crossman *et al.*, 2008). Most research on QS gene regulation in *R. leguminosarum* has been carried out in strain A34, which contains all four QS systems (Lithgow *et al.*, 2000; Wisniewski-Dye *et al.*, 2002). The *cin* system is involved in stationary phase survival (Thorne & Williams, 1999) and is at the top of a regulatory cascade that affects the expression of the *rai, rhi* and *tra* QS systems (Lithgow *et al.*, 2000; Wisniewski-Dye *et al.*, 2002). The *tra* QS system regulates recipient-induced transfer of the symbiotic plasmid pRL1JI in response to CinI-made AHLs produced by potential recipient strains. This requires the presence of the orphan LuxR-type regulator BisR to induce *traR,* the product of which directly regulates plasmid transfer in a QS dependent manner in response to TraI-made AHLs (Danino *et al.*, 2003). The *raiI/R* QS genes are localised on a large, non-symbiotic plasmid; *raiI* and *raiR* have no detected biological role in *R. leguminosarum*, but in the closely related species *Rhizobium etli,* mutation of *raiI* increases the level of nodulation (Rosemeyer *et al.*, 1998). The *rhiI/R* QS genes on the symbiotic plasmid induce high levels of expression of the *rhiABC* operon in the rhizosphere, and affect nodulation in a strain that is already compromised for nodulation (Cubo *et al.*, 1992; Economou *et al.*, 1989; Rodelas *et al.*, 1999). *Mesorhizobium tianshanense* contains the *mrtI* and *mrtR* genes, which are highly similar to the *cinI* and *cinR* genes and affect root hair adherence and nodule formation (Zheng *et al.*, 2006) .

In recent work, the regulation of the *rai* system by the *cin* system was investigated in *R. leguminosarum* strain 8401. Strain 8401 is a derivative of A34, but it lacks the symbiotic plasmid, and therefore the *rhiI/R* and *traR/I* genes. In strain 8401, the *cin* system induces the expression of *raiR*, which results in increased *raiI* expression. This *cin*-dependent induction of *raiR* expression was shown not to be mediated by a transcriptional effect of the transcriptional regulator CinR in response to CinI-made AHLs (Edwards *et al.*, 2009). In AHL-based QS gene regulation, gene expression in an AHL synthase mutant can usually be induced by adding the AHLs to the medium. The AHLs diffuse into the cells, activate their cognate LuxR-type regulator and induce expression of the promoters regulated by the QS regulator. To examine the regulatory effect of the *cin* QS system on the expression of *raiR,* an attempt was made to induce *raiR* expression in a *cinI* mutant by adding CinI-made AHLs, but no induction of *raiR* was observed. This was not due to a lack of AHL perception by CinR in the *cinI* mutant, because the *cinI* promoter could be induced by adding CinI-made 3OH-$C_{14:1}$-HSL to a *cinI* mutant (Edwards *et al.*, 2009). This experiment clearly showed that CinR could be activated by exogenously added CinI-made AHLs, meaning that the *cinR/I* QS system must use a different, AHL-independent regulatory mechanism for the regulation of *raiR.* Further study of the regulation of *raiR* expression revealed that mutation of *cinI* had a polar effect on a downstream, previously unannotated gene *cinS*. Introduction of cloned *cinS* on a broad-host range plasmid was sufficient to induce *raiR* expression in a *cinI* mutant (Edwards *et al.*, 2009). In parallel work, the orphan *luxR*-type regulator *expR* was identified as another regulator of *raiR* expression. As both *expR* and *cinS* induced *raiR* expression, it was hypothesised that *cinS* and *expR* were functioning in the same regulatory pathway (Edwards *et al.*, 2009).

Apart from *raiR,* another regulatory target of *cinS* and *expR* was identified. Cloned *cinS* caused a ‘collapse’ of the colony morphology when strains were grown on TY agar. This collapse was characterised by a reduction in the mucoidy of colonies after 2-3 days of growth. As most of the mucoidy in *R.l.* bv. *viciae* is caused by the production of acidic exopolysaccharides (EPS), it was investigated whether the ‘collapse’ could be due to the premature degradation of EPS by an EPS-degrading enzyme. A likely candidate for this was the extracellular glycanase *plyB,* which had been identified in previous work in strain 8401 (Finnie *et al.*, 1998). It was then shown that expression of *plyB* required both *cinS* and *expR,* but not *raiR,* and that the increased expression levels of *plyB* in strains containing *cinS* on a plasmid were responsible for the ‘collapse’ phenotype (Edwards *et al.*, 2009).

In summary, it was shown that the *cinR, cinI* and *cinS* encoded QS system uses two systems of gene regulation: one that depends on the activation of the LuxR-type regulator CinR in response to CinI-made AHLs, and a novel mechanism, that depends on the regulatory protein CinS. The aim of this project was to uncover the molecular mechanism by which CinS and ExpR regulate gene expression. Further research was carried out in the closely related strain *R. l.* bv. *viciae* strain 3841, because this strain has been sequenced (Young *et al.*, 2006). *R. l.* bv. *viciae* 3841 contains the *cinR, cinI* and *cinS* genes and the orphan *luxR*-type regulator *expR* (RL4639), but lacks the *raiI* and *raiR* genes. It does contain the *rhiR* and *rhiI* genes (on the symbiotic plasmid pRL10JI). The *traR* and *traI* genes are not found in an operon on the symbiotic plasmid as in strain A34. Instead, genes homologous to *traI* and *traR* are encoded on different plasmids (pRL7JI and pRL8JI respectively).

In this chapter, *R. l.* bv. *viciae* 3841 *cinS* and *expR* mutants were generated and the phenotypes of these mutants were studied. Heterologously produced CinS protein was purified, and its regulatory function was examined by DNA- and RNA-binding studies.

2.2 Results

2.2.1 Bio-informatic analysis of CinS

In *R. l.* bv. *viciae* 3841 *cinS* is located downstream of *cinI,* as in strain A34 (Figure 2.1 A). Translation of *cinS* is predicted to be coupled to *cinI*, as the stop codon of *cinI* overlaps with the start codon of *cinS*. A tBLASTn database search using the CinS protein sequence identified (mostly unannotated) *cinS* homologues in several other rhizobial species (*R. l.* bv. *trifolii* WSM1325, *R. l.* bv. *trifolii* WSM2304, *M. tianshanense, R. etli* CNPAF512, *R. etli* CIAT652 and *R. etli* CFN42). In all of these, the *cinS* gene is located downstream of and apparently translationally coupled to a *cinI* homologue. In addition, the translated amino acid sequence of the *cinS* homologues is conserved (Figure 2.1 B), indicating that *cinS* is likely to encode a protein. To predict the biological role of CinS, different bio-informatical tools were used to search for conserved protein domains (Prosite, Uniprot, Interpro), but none were found. The secondary structure of CinS was analysed by using the Psipred prediction tool (McGuffin *et al.*, 2000), which predicted CinS to be mainly α-helical (Figure 2.1 C). The Fugue bio-informatical tool (Kwasigroch & Rooman, 2006) was used to identify proteins with a similar tertiary structure as CinS. Only one hit within the 95% confidence level was obtained, the *Mycobacterium tuberculosis* ArgP protein*,* which is a helix-turn-helix (HTH)-type transcriptional regulator. CinS was predicted to have a similar threedimensional structure as the DNA-binding N-terminal part of ArgP.

2.2.2 Characterisation of CinS and ExpR in *R. l.* bv. *viciae* 3841

***cinS* and *expR* mutants in *R.l.* bv. *viciae* 3841**

The *cinS* and *expR* (RL4639) genes were identified in *R. l.* bv. *viciae* 3841, but it still needed to be confirmed that these genes function as in strain 8401. Therefore, *cinS* (A1245) and *expR* (A1246) mutants were generated. No difference in growth of the mutants was observed when compared to WT 3841 (Figure 2.2 A+B). Nodulation of pea by the mutants was also normal, as both mutants formed similar numbers of big, pink nodules as induced by *R.l.* bv. *viciae* 3841 (results not shown).

A:

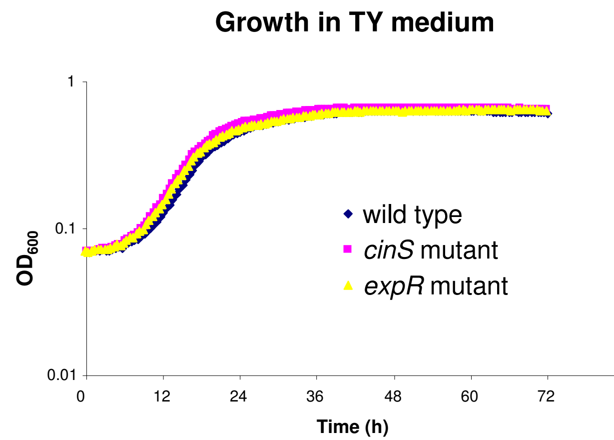
B)

```
a)1 MNRLAETATQTAIQKDIGALERHVLASRDIATQIGDPFLSYLLSMALFTIYEKKAHHENEALKSSFS  67
b)  ..............................................................R....
c)  ...........SM......................................................
d)  .......T...........................................................
e)  ........----.......................................................
f)  .......T..----...S......................................Q..........
g)  .......T..----...S......................................Q..........
h)  .K..V....P----...S......................................Q..........
```

C)

Figure 2.1: Bio-informatical analysis CinS sequence. A: The *cinR, cinI* and *cinS* gene region showing the translational coupling of CinI and CinS. B: Alignment of CinS in different rhizobia. The CinS sequence from *R. l.* bv. *viciae* 3841 was used for a tBLASTn analysis and CinS homologues were aligned using the BLAST algorithm. Residues that differ from *R. l.* bv. *viciae* 3841 CinS are indicated. Dashes represent residues that are deleted and dots represent amino acids that are identical. a) *R. l.* bv. *viciae* 3841, GenBank AM236080, b) *R. l.* bv. *trifolii* WSM1325, GenBank EDR73518, c*) R. leguminosarum* 8401, Genbank AF210630, d) *Mesorhizobium tianshanense*, GenBank DQ123807, e) *R. l.* bv. *trifolii* WSM2304, GenBank ACI55940, f) *R. etli* CNPAF512, GenBank AF393621, g) *R. etli* CIAT652, GenBank ACE92023, h) *R. etli* CFN 42, GenBank CP000133. C: The secondary structure of CinS was predicted using the Psipred prediction tool. C: coiled coil domain, H: α-helix

A)



B)

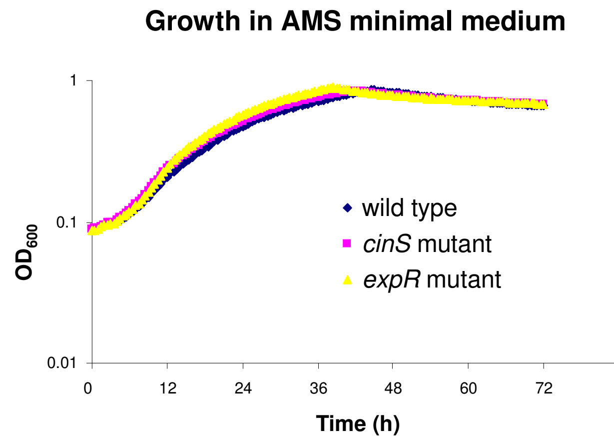


Figure 2.2: Growth of *cinS* (A1245) and *expR* (A1246) mutants. Strains were grown for 72 h in shaking microtiterplates and growth was monitored by measuring OD_{600}. A: TY medium. B: AMS minimal medium (30 mM pyruvate, 10 mM NH_4Cl).

***cinS* and *expR* have a similar role as in strain 8401**

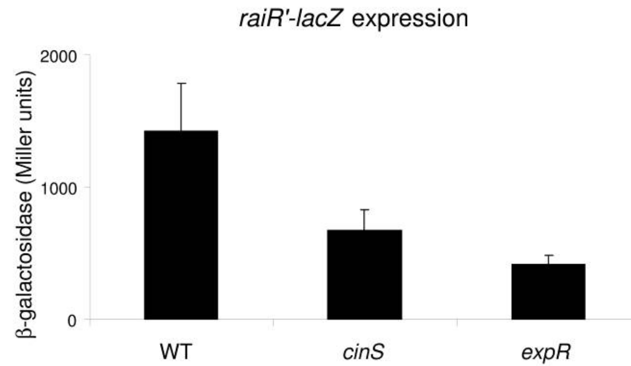
In strain 8401 the expression of *raiR* and *plyB* was decreased in the *cinS* and *expR* mutants (Edwards *et al.*, 2009). Therefore the expression of these genes (*raiR'-lacZ* on pIJ9272 and *plyB'-lacZ* on pIJ9252) was measured in strain 3841 and the *cinS* and *expR* mutants (Figure 2.3 A+B). As in strain 8401, the expression levels of *raiR* and *plyB* were decreased in the *cinS* and *expR* mutants. In addition, introduction of cloned *cinS* on a broad-host range plasmid (pIJ9692) caused a 'collapse' phenotype, similar to the one observed in strain 8401. A picture of this collapse is shown in Figure 2.3 C.

In strain 8401 the *cinS* and *expR* mutants were found to have an increased biofilm ring when grown in Y mannitol minimal medium for 5 days (Edwards *et al.*, 2009). The strain 3841 *cinS* and *expR* mutants had a similarly increased biofilm ring (Figure 2.4). Taken together, these data indicate that *cinS* and *expR* have a similar regulatory role in *R. l.* bv. *viciae* strains 3841 and 8401, justifying the further study of the regulatory roles of these genes in strain 3841.

***cinS* and *expR* influence the expression of the *rhiI/R* QS system**

Chromobacterium violaceum CV026 is commonly used as a biosensor strain that produces the purple pigment violacein in response to short-chain AHLs (McClean *et al.*, 1997). Strain 3841 induced a strong purple halo and this was predicted to be due to the production of RhiI-made AHLs. A library of Tn*5*-induced mutants of strain 3841 had been screened to identify mutants that no longer induced a halo (Maria Sanchez-Contreras). Mutations blocking AHL production were transduced into strain 3841 to demonstrate that the phenotypes co-transduced with the Tn*5*-transposon and the mutated genes were identified by sequencing the region adjacent to the Tn*5*. This way, mutations in *rhiI* (A850) and *rhiR* (A920) were identified (Figure 2.5 A), thus confirming that in strain 3841, the primary source of *C. violaceum* CV026-detectable AHLs is RhiI. Since mutation of *rhiR* also abolished the production of RhiI-made AHLs, expression of *rhiI* is dependent on RhiR, as has been described previously for the homologous genes in strain A34 (Rodelas *et al.*, 1999).

A:



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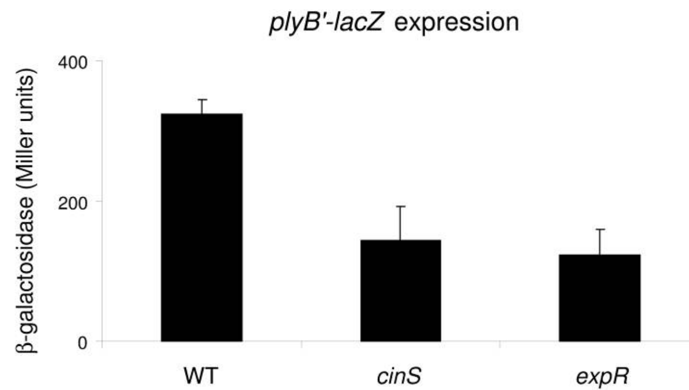


C:

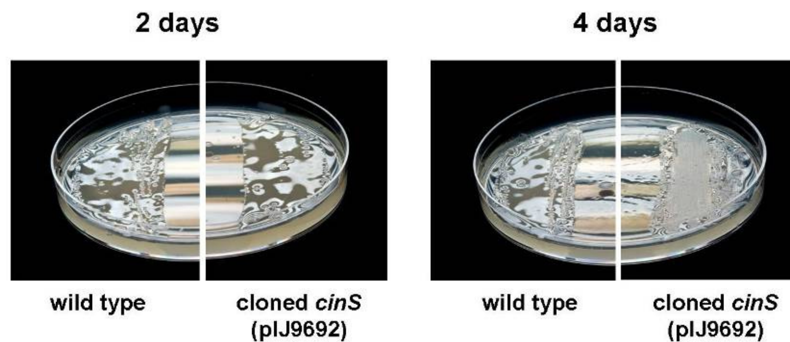


Figure 2.3: *raiR* and *plyB* expression in *cinS* and *expR* mutants. A: Expression of *raiR'-lacZ* (pIJ9272) and B: expression of *plyB'-lacZ* (pIJ9252) (measured by β-galactosidase activity) after three days of growth in Y mannitol minimal medium. Error bars indicate standard deviations. Strains used were WT, *cinS* (A1245) and *expR* (A1246) mutants. C: collapse phenotype. Strains were grown for three days on TY agar.

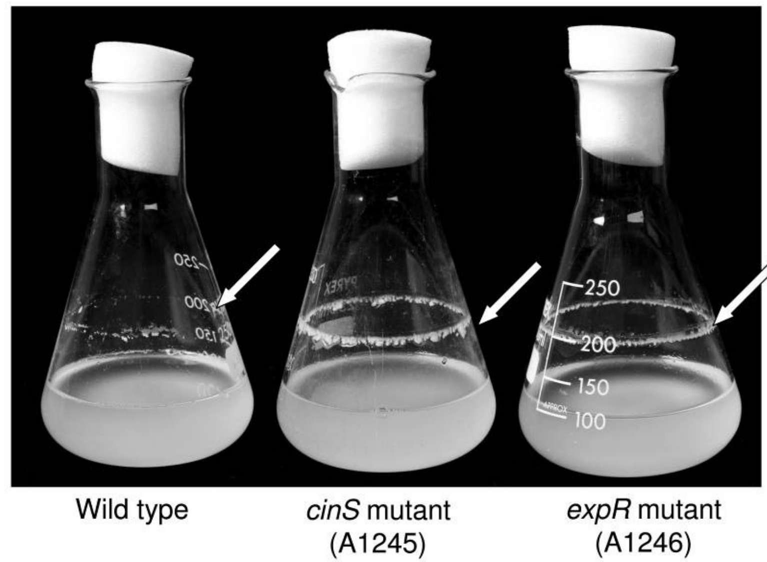
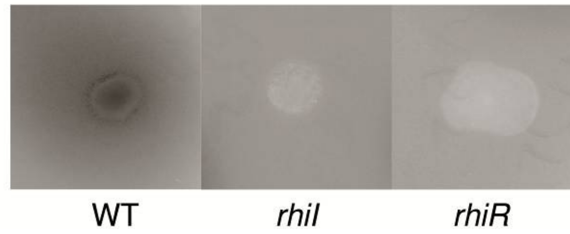
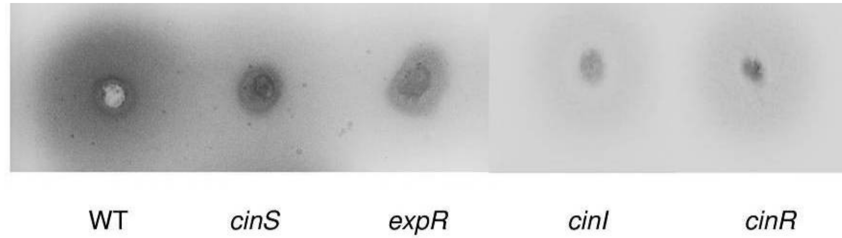


Figure 2.4 Biofilm rings formed by *cinS* (A1245) and *expR* (A1246) mutants. Strains were grown in Y mannitol minimal medium for 5 days. Arrows indicate the biofilm rings.

A)



B)



C)

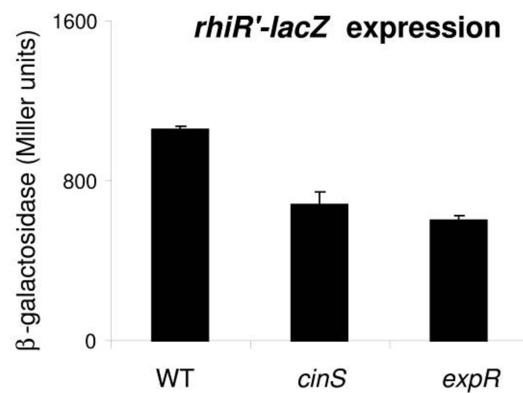


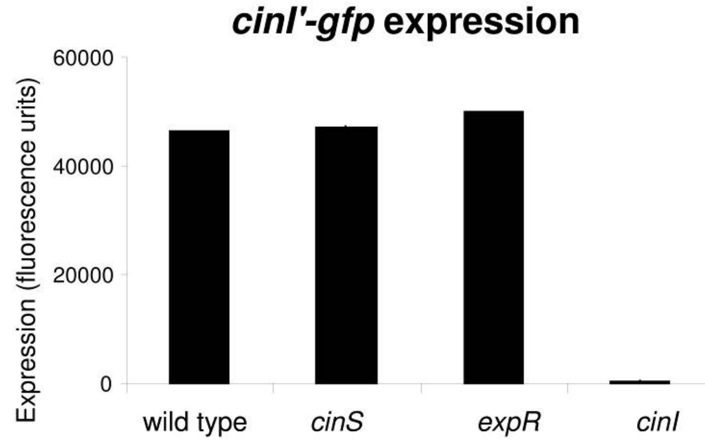
Figure 2.5: CinS and ExpR regulate the *rhi* QS system. RhiI-made AHLs were visualised by a purple halo on a lawn of *C. violaceum* CV026. A: Production of RhiI-made AHLs in WT 3841, *rhiI* mutant (A850) and *rhiR* mutant (A920). B: Production of RhiI-made AHLs in WT 3841, *cinS* mutant (A1245), *expR* mutant (A1246), *cinI* (A994) and *cinR* (A924). C: *rhiR'-lacZ* expression (pIJ9104) was measured after 2 days of growth in TY liquid medium. Error bars represent standard deviations.

Based on a *C. violaceum* CV026 bioassay it was found that mutations in *cinS*, *expR*, *cinI* (A994) and *cinR* (A924) significantly reduced the production of RhiI-made AHLs (Figure 2.5 B). CinS and ExpR thus positively regulate the expression of RhiI-made AHLs and it was tested whether this is due to a regulatory effect on the expression of the transcriptional regulator *rhiR* (Figure 2.5 C). There was a small but significant reduction in expression of *rhiR'-lacZ* in both the *cinS* and *expR* mutants and since RhiR induces *rhiI* expression, it is likely that the reduced expression of *rhiR* caused the reduced production of RhiI-made AHLs.

CinS and ExpR do not influence CinI activity

Expression of CinS is translationally coupled to expression of CinI. Often when the expression of two proteins is translationally coupled, this ensures that both proteins are present at equimolar concentrations. For example, the expression of sigma factors and anti-sigma factors is translationally coupled, because the anti-sigma factor has to be able to bind to and inactivate all the available sigma factor in the absence of its signal. This regulatory mechanism makes it essential that equal amounts of the sigma factor and anti-sigma factor are present in the cell (see review by Helmann, 2002). It was investigated whether CinS or ExpR were able to alter the expression level of *cinI* or the activity of CinI. Expression of *cinI* was measured by a *cinI'-gfp* plasmid (pIJ9611), which was conjugated into WT, the *cinS* and *expR* mutants, but no difference in expression between the mutants and WT was observed (Figure 2.6 A). The levels of CinI-made 3-OH-$C_{14:1}$-HSLs were examined by a bacteriocin-type assay in which 3-OH-$C_{14:1}$-HSL-induced growth inhibition of a pRL1JI-containing strain (A34) results in the formation of a halo. No difference in the halo size induced by the *cinS* or *expR* mutants compared to WT was observed (Figure 2.6 B).

A:



B:

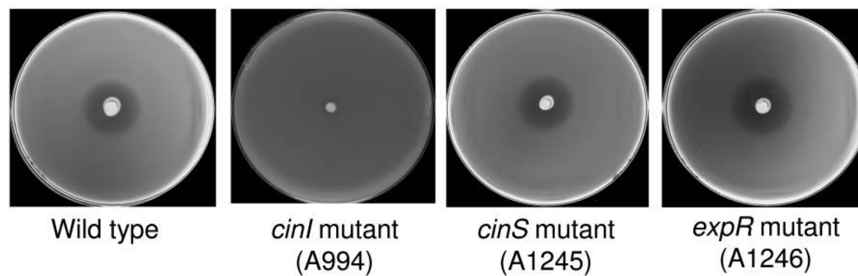


Figure 2.6: *cinS* and *expR* do not affect *cinI* expression or CinI activity. A: strains (containing *cinI'-gfp* on pIJ9611) were grown for 2 days in liquid TY medium after which fluorescence was measured. Strains used were wild type 3841, *cinS* (A1245), *expR* (A1246) and *cinI* (A994) mutants. Error bars represent standard deviations. B: production of CinI-made 3-OH-$C_{14:1}$-HSLs was assayed by measuring the halo size when grown on a strain carrying the symbiotic plasmid pRL1JI (A34).

2.2.3 *cinS* regulates swarming in *R. etli* CNPAF512

R. etli CNPAF512 is closely related to *R. leguminosarum* and contains orthologues of the *cinR, cinI* and *cinS* genes and the *raiI* and *raiR* genes. Mutation of *cinR* (FAJ4007) or *cinI* (FAJ4006) in *R. etli* CNPAF512 had previously been shown to abolish swarming behaviour (Daniels *et al.*, 2006). Addition of CinI-made AHLs to the swarming medium did not restore the full swarming phenotype, but it did induce a wrinkling in the edges of the colony. Introduction of cloned *cinI* did fully restore swarming in both the *cinI* and *cinR* mutants (Daniels *et al.*, 2006), leading the authors to conclude that the biosufactant properties of CinI-made AHLs were required for the swarming behaviour in *R. etli* CNPAF512.

On the basis of our results, it seemed likely that the *cinI* mutation in *R. etli* CNPAF512 was polar on *cinS*, which had not been identified in that work as a separate gene co-transcribed with *cinI*. Examination of the construct used for overexpression of *cinI* revealed that this construct contained the full *cinS* gene, indicating that the swarming phenotype might be caused by mutation of *cinS* and not *cinI*. Indeed, cloned *cinS* (from *R. leguminosarum* strain 8401, pIJ9692) fully restored swarming to the *R. etli cinI* mutant, but cloned *cinI* (without *cinS,* pIJ9655) did not (Figure 2.7). This indicates that the swarming is regulated by *cinS* and that the mutation of *cinI* is polar on *cinS* in *R. etli*. The *R. etli cinI* mutant carrying cloned *cinS* formed a swarm with a smooth edge, but the WT strain formed a swarm with a wrinkled edge. It is possible that the lack of wrinkling seen in this strain is caused by the absence of CinI-made AHLs. This would fit with the observations that CinI-made AHLs have got biosurfactant chemical properties (Daniels *et al.*, 2006). The swarming behaviour of *R. leguminosarum* strains 8401 and 3841 was tested under the conditions used for *R. etli* CNPAF512, but no *cinI*-dependent swarming was seen and so no difference between the control strains and the *cinI, cinR*, or *cinS* mutants could be detected.

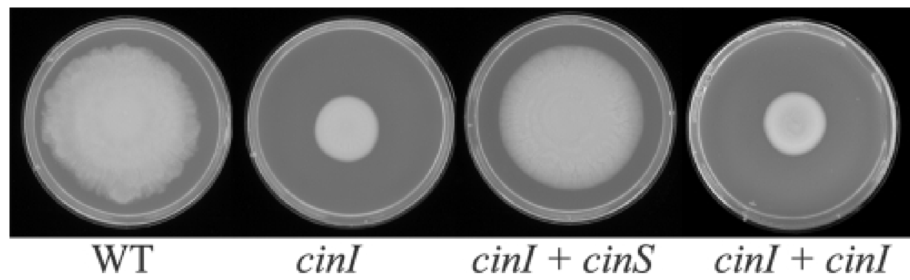


Figure 2.7: Swarming in *R. etli* CNPAF512. Strains were spot-inoculated on YEM medium for 7 days. Complementation of *cinI* mutant (FAJ4006) with *cinS* (pIJ9692) and *cinI* (pIJ9655).

2.2.4 Purification of CinS protein

C-terminally His_6-tagged CinS is functional

A C-terminally His_6-tagged CinS fusion protein was made by cloning the *R. l.* bv. *viciae* 3841 *cinS* gene in frame with a C-terminal His_6-tag into pET21a (forming pIJ11043). To test the functionality of the resulting fusion protein, the gene coding for His_6-tagged CinS was cloned into broad-host range vectors pBBR1-MC3 and pBBR1-MC5 (forming plasmids pIJ11051 and pIJ11052 respectively). These plasmids caused the characteristic collapse phenotype in strains 8401 and 3841 when grown on TY medium (Figure 2.8 A). In addition pIJ11052 complemented the strain 8401 *cinS* mutant (A1102) and the strain 8401 *cinR* mutant (A552) for production of RaiI-made AHLs (Figure 2.8 B). These results proved that CinS-His_6 was indeed functional.

Purification CinS-His_6 from *E. coli*

CinS-His_6 expressed in *E. coli* Bl21(DE3) cells carrying pIJ11043 was present in both the soluble and insoluble fraction. The soluble fraction was used to purify CinS-His_6 by fast protein liquid chromatography (FPLC) Ni^{2+}-chromatography using elution with an imidazol gradient. Fractions containing CinS-His_6 were identified by SDS-PAGE and pooled. Figure 2.9 A shows that the pooled sample of CinS-His_6 contained no significant contaminating proteins. The purified CinS-His_6 was analysed by quadrupole time-of-flight (Q-ToF) mass spectroscopy (Figure 2.9 B). This revealed that the purified protein was present in two forms: most of the protein was full-length CinS-His_6 (8557 Da), but an equal proportion of the purified protein represented a truncated form (8156 Da). This corresponded to a loss of 401 Da, equivalent to the lack of the first three amino acids Met, Asn and Arg.

Secondary structure of CinS-His_6

Purified CinS-His_6 was further examined by circular dichroism (CD) spectroscopy after dialysis against a buffer (20 mM phosphate buffer, pH 8) suitable for CD spectroscopy. Analysis of the CD-spectrum was done using the CDPro software, which uses three different algorithms (CDSSTR, ContinLL and SELCON3) to predict the secondary structure. Two training sets were used: a training set that only contained native proteins and a training set that contained both native and denatured proteins. All algorithms, predicted CinS-His_6 to be mainly α-helical (>30%) in secondary structure (Figure 2.10). This confirmed the prediction that CinS is mainly α-helical, although the Psipred analysis overestimated the degree of α-helicity (Figure 2.1).

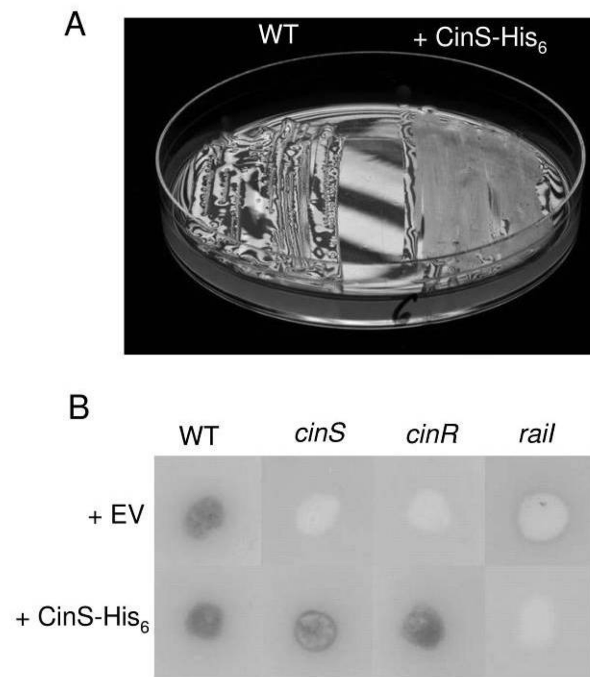
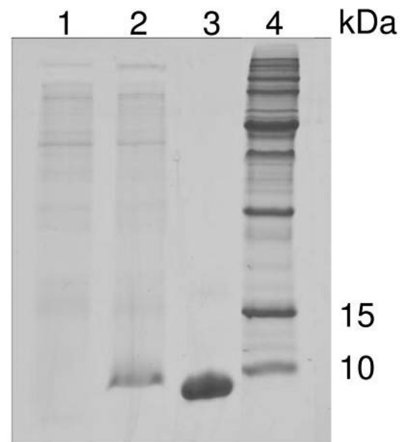


Figure 2.8: CinS-His$_6$ is functional. A: WT strain 3841 containing pIJ11052 (expressing CinS-His$_6$) showed a collapse of the exopolysaccharides after 3 days of growth on TY agar. B: *R. leguminosarum* strain 8401 and *cinS* (A1102), *cinR* (A552) and *raiI* (A789) mutant derivatives carrying an EV (pBBR1-MC5) or pIJ11052 (CinS-His$_6$) were grown for 3 days on TY agar and bioassayed on a lawn of *C. violaceum* CV026. The photographs show the accumulation of purple pigment by RaiI-made AHLs after one day of growth on *C. violaceum* CV026.

A)



B)

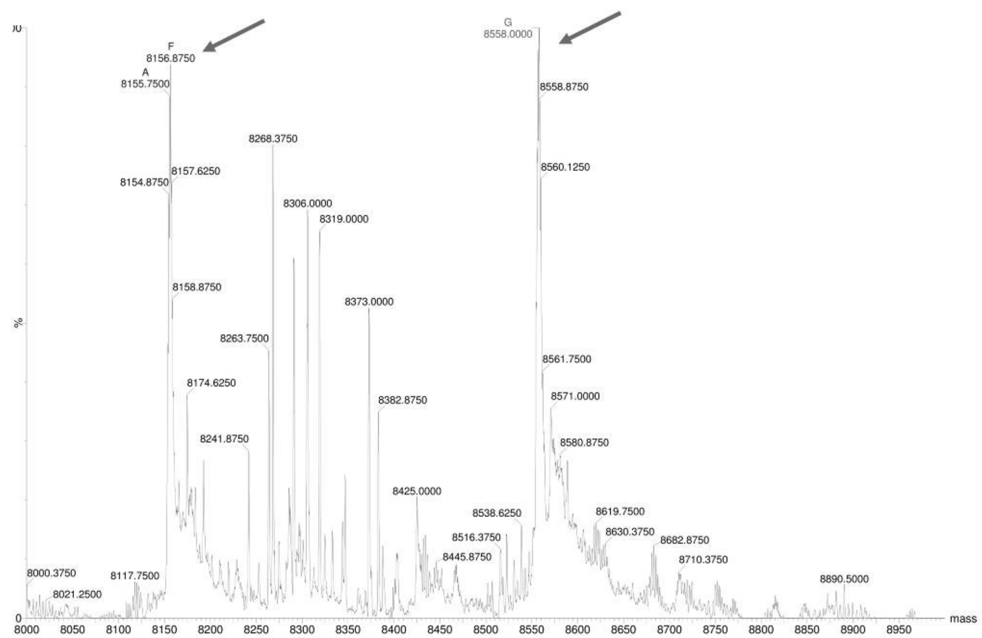
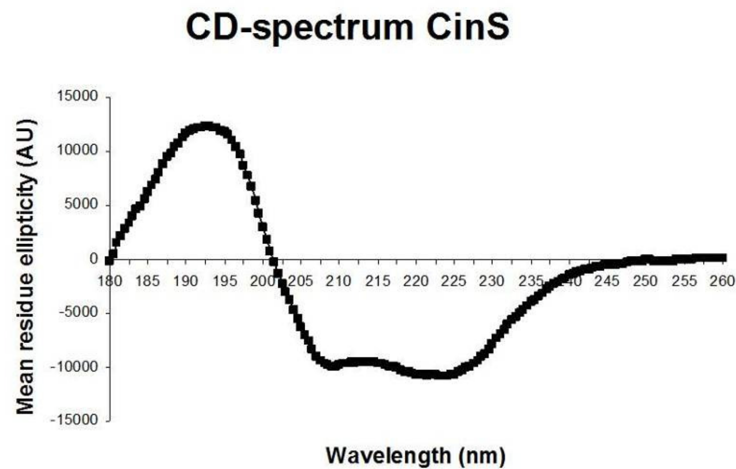


Figure 2.9: Purification CinS-His$_6$. A: SDS-PAGE analysis of purified CinS-His$_6$ protein. 1: uninduced cells, 2: induced cells, 3: pooled purified CinS-His$_6$ B: Q-ToF analysis of purified CinS-His$_6$ protein. Arrows indicate the molecular weight of the most abundant forms of CinS-His$_6$.

A)



B)

Algorithm	Training set	Ordered α-helices	Unordered α-helices	Ordered β-sheets	Unordered β-sheets	U-turns
CDSSTR	N	19,4 %	15,6 %	8,6 %	6,2 %	21,3 %
ContinLL	N	17,7 %	14,4 %	9,4 %	6,5 %	22,9 %
SELCON3	N	18,1 %	14,5 %	9,1 %	6,8 %	22,7 %
CDSSTR	N+D	19,1 %	13,8 %	7,9 %	5,8 %	18,0 %
ContinLL	N+D	17,9 %	14,4 %	9,3 %	6,0 %	22,0 %
SELCON3	N+D	18,1 %	14,2 %	8,9 %	6,6 %	22,1 %

Figure 2.10 : CD-spectrum analysis of CinS-His$_6$. A: CD-spectrum of CinS-His$_6$. AU: absorbance units B: Data were analysed using CDPro with three different algorithms: CDSSTR, ContinLL and SELCON3. N: native protein training set, D: denatured protein training set.

Quaternary structure of CinS-His$_6$

To determine the multimeric state of CinS-His$_6$, the dialysed purified protein was analysed with dynamic light scattering (DLS). This technique is used to estimate the size of particles in a solution based on the scattering of an incoming light beam. Most of the protein (>99% of the mass) was found to be present in a soluble multimeric state with a radius of 3.6 nm, predicting the molecular weight of the CinS-His$_6$ multimer to be 68 kDa, corresponding to a CinS-His$_6$ octamer (Figure 2.11). As more than 99% in mass of the protein was present in this state, the sample was monodisperse and only very little of the protein was aggregated.

Crystallisation trials of CinS-His$_6$

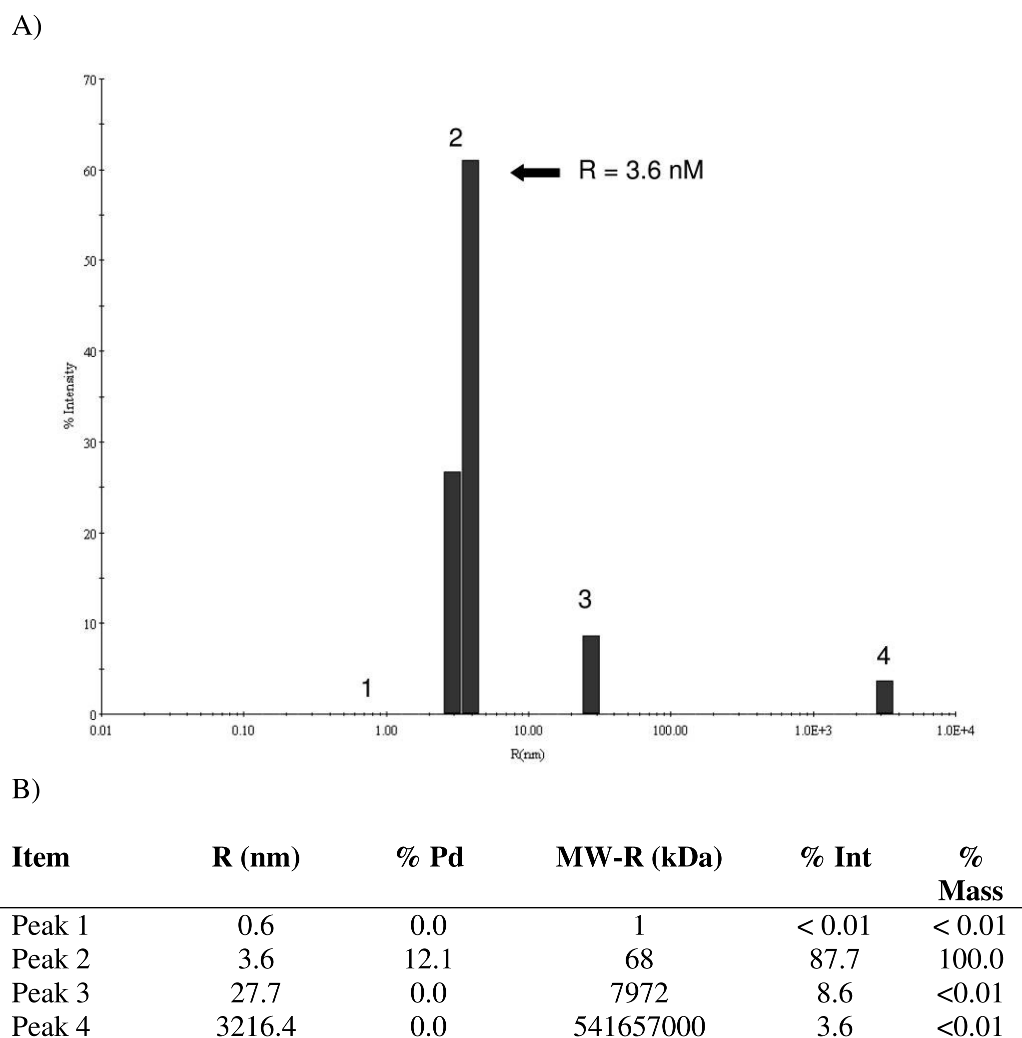
In collaboration with Clare Stevenson, crystallisation trials were set up for CinS-His$_6$. Different commercially available screens were used (Ammonium sulphate, PACT, Classics, PEG, Anion, Cation and JCSG screens). Unfortunately, no formation of crystals could be observed under any of the conditions tested. A possible reason for the unsuccessful crystallisation trials was the presence of two isomeric forms of CinS-His$_6$. To try to overcome this problem, an N-terminal His$_6$-tagged fusion protein His$_6$-CinS (pIJ11033) was generated. Purification of this protein using Ni^{2+}-chromatography was unsuccessful as the protein did not bind to the column resin. One explanation for this is that the N-terminal His$_6$-tag is buried in the threedimensional structure of the CinS protein. Alternatively, the full-length protein with the N-terminal His$_6$-tag is not soluble.

2.2.5 Regulatory role of CinS

CinS-His$_6$ does not bind to the *raiR* or *rhiR* promoter

Using the Fugue software, CinS was predicted to have a similar fold as the DNA-binding domain of a transcriptional regulator (see section 2.2.1). CinS-His$_6$ was tested for its abilities to bind DNA by using electrophoretic mobility shift assays (EMSA), using the *raiR* and *rhiR* promoters as targets. No retardation of the promoter fragment could be observed under any of the tested conditions (Figure 2.12).

A)

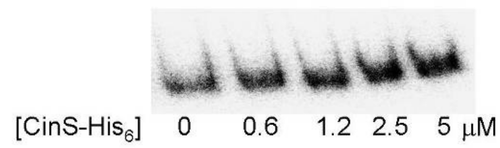


B)

Item	R (nm)	% Pd	MW-R (kDa)	% Int	% Mass
Peak 1	0.6	0.0	1	< 0.01	< 0.01
Peak 2	3.6	12.1	68	87.7	100.0
Peak 3	27.7	0.0	7972	8.6	<0.01
Peak 4	3216.4	0.0	541657000	3.6	<0.01

Figure 2.11: DLS analysis of CinS-His$_6$. A: DLS spectrum of CinS-His$_6$. B: Properties of peaks in DLS spectrum. R: hydrodynamic radius, % Pd: percentage polydispersity within a peak, should be less than 15%, MW-R: estimated molecular weight based on hydrodynamic radius, % Int: light scattering signal intensity of the specified peak divided by the total signal intensity of the measurement multiplied by 100, % Mass: estimated total mass of the particles in solution corresponding to the user-specified peak divided by the estimated total mass of all particles in solution.

A)



B)

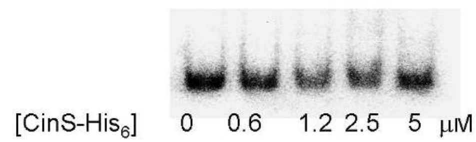


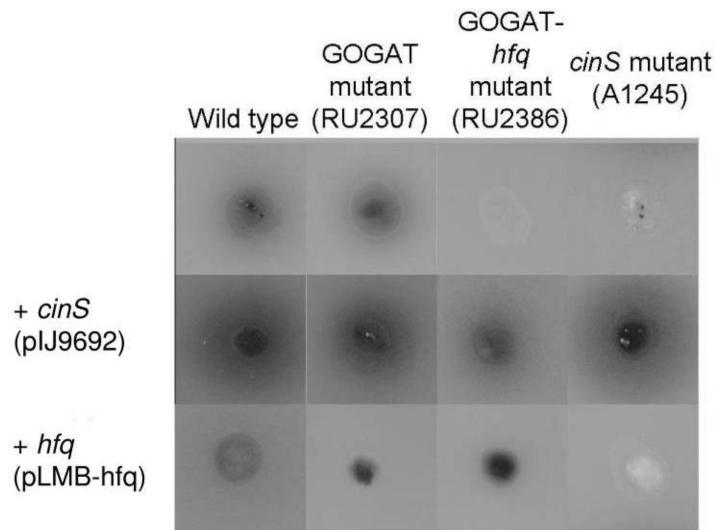
Figure 2.12: CinS-His$_6$ did not bind to the *raiR* or *rhiR* promoters. A: the *raiR* promoter and B: the *rhiR* promoter were incubated with increasing concentrations of CinS-His$_6$ (twofold dilution series).

A role for Hfq in CinS-dependent gene regulation?

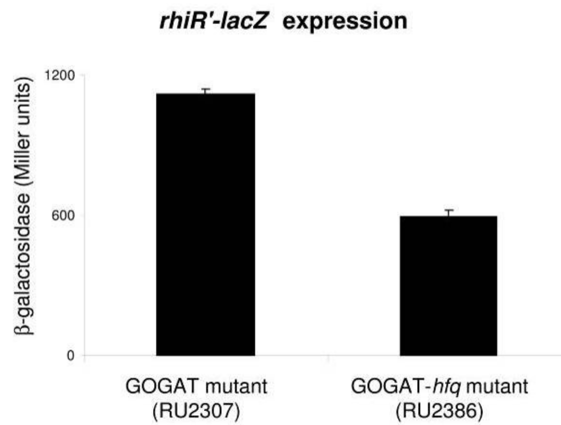
In Philip Poole's lab, an *R. l.* bv. *viciae* 3841 glutamate synthase (glutamine:2-oxoglutarate amidotransferase or GOGAT) mutant (RU2307) had been generated, which was not able to grow on glutamate as a nitrogen source. A spontaneous suppressor of the GOGAT mutation had been isolated (RU2386) and the suppressor mutation was identified using SOLEXA sequencing. It was found that in RU2386 there was a mutation in the *hfq* gene, causing a premature stop codon (Jay Mulley, unpublished results). *hfq* encodes the small RNA-binding protein Hfq, which plays an important role as a global regulator in many bacteria by binding to sRNA's, thereby influencing the degradation rate or the initation of translation of target mRNA's (Valentin-Hansen *et al.*, 2004). Microarray analysis of the *hfq*-GOGAT double mutant versus the GOGAT mutant revealed that *rhi* gene expression was decreased in the *hfq* mutant (Jay Mulley). This was confirmed using a *C. violaceum* CV026 bio-assay (Figure 2.13 A) and using the *rhiR'-lacZ* promoter fusion construct it was shown that the decrease of RhiI-made AHLs was probably due to decreased expression of the transcriptional regulator *rhiR* (Figure 2.13 B).

To examine whether Hfq played a role in CinS-dependent gene regulation, cloned *cinS* was introduced into the *hfq* mutant, and cloned *hfq* (pLMB-hfq, provided by Jay Mulley) was introduced into the *cinS* mutant. Both strains were tested for production of RhiI-made AHLs on a lawn of *C. violaceum* CV026 (Figure 2.14 A), showing that cloned *cinS* in the *hfq* mutant restored the levels of RhiI-made AHLs, while cloned *hfq* in the *cinS* mutant did not. This could indicate that Hfq regulates expression of the *cinIS* operon. The microarray analysis of the *hfq* mutant had not shown any effect on *cinIS* expression however (Jay Mulley, unpublished results). As the microarray analysis was done with strains grown until early exponential phase ($OD_{600} \approx 0.3$), this could be due to a growth phase effect and to eliminate this possibility, *cinIS* expression was analysed in stationary phase using the *cinI'-gfp* construct and a bacteriocin bioassay. No difference in expression was observed (Figure 2.13 C). These results show that the regulatory effects of Hfq on the expression of *rhiR* are not likely to be due to an indirect regulatory effect on the expression of CinS. Another possibility could be that CinS affects the regulatory mechanism by which Hfq regulates *rhiR* expression, for example by binding to sRNA's.

A)



B)



C)

Figure 2.13: Hfq regulates the *rhi* QS system. A: Production of RhiI-made AHLs was assayed by growing the indicated trains on a lawn of *C. violaceum* CV026. B: Expression of *rhiR* (*rhiR'-lacZ* on pIJ9104) was measured by β-galactosidase expression after 2 days of growth in liquid TY medium. Error bars represent standard deviations. C: bacteriocin test with GOGAT mutant (RU2307) and GOGAT-*hfq* mutant (RU2386) against A34 as a biosensor strain.

A collaboration with Prof. Guofan Hong was set up to test whether CinS functions by binding to sRNA's. In Prof. Guofan Hong's lab, a *R. leguminosarum* strain A34 sRNA library had been generated. These sRNA's were transcribed *in vitro,* and used in EMSA assays with the purified CinS-His_6 protein. To facilitate the screen, different sRNA's of the same length were pooled (up to 10 at the same time), and incubated with and without CinS-His_6 (Figure 2.14). No binding of CinS to any of the tested pools could be observed. As a positive control, Hfq protein (which was provided by Prof. Guofan Hong) was tested for binding to sRNA's in the same conditions. Unfortunately, no binding of Hfq to any of the sRNA's was found either (results not shown).

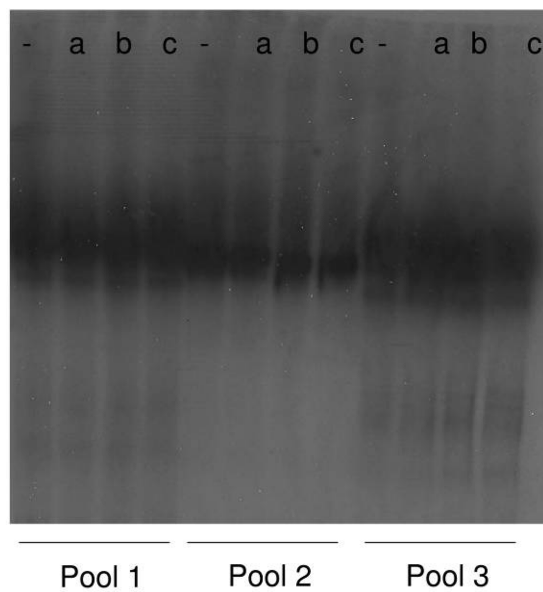


Figure 2.14: Screen of sRNA library by EMSA with CinS-His$_6$. Up to 10 sRNA's of similar size were pooled (examples shown are pools 1, 2 and3) and incubated with 1 μM CinS-His$_6$. -: no CinS-His$_6$, a: *sRNA* buffer A, b: sRNA buffer B, c: sRNA buffer C (recipes specified in Materials and Methods).

2.3 Discussion

Previous work in strain 8401 identified the presence of *cinS* downstream of *cinI* in the *cin* QS system. In this strain CinS was shown to regulate biofilm ring formation, EPS degradation and the expression of *raiR* (Edwards *et al.*, 2009). In this chapter the regulatory effects of CinS in *R.l.* bv. *viciae* 3841 were studied and as in strain 8401 and CinS regulated biofilm formation and EPS degradation. In addition, a regulatory effect on the expression of the *rhi* QS system was found and this was mediated by induction of the transcriptional regulator *rhiR*. Even relatively small changes in *rhiR* induction can be expected to have relatively large effects on promoters targeted by RhiR. This is due to the positive feedback on *rhiI* expression that occurs as a consequence of increased levels of RhiI-made AHLs (Rodelas *et al.*, 1999). Induction of the *rhi* QS system leads to increased expression of the *rhiABC* genes, which are highly expressed in the rhizosphere and have been shown to be involved in nodulation (Cubo *et al.*, 1992). CinS thus couples the induction of the *cin* QS system to the induction of the *rhi* and *rai* QS systems. Such hierarchical organisation of QS systems has been described in other species as well (see Chapter 1: Introduction). Despite the fact that CinS does not require AHLs for function, it does regulate gene expression in a population density dependent way, as expression of CinS is coupled to expression of *cinI.* CinS is predicted to be co-transtribed and translationally coupled to the expression of the AHL-synthase CinI. Although translational coupling of two proteins often indicates that they affect each others function, no influence of CinS on CinI activity could be found. The only promoter known to be be regulated by CinR is the *cinI* promoter itself. It is possible that the only function of CinR is the induction of expression of *cinS,* as all regulatory effects of the *cin* QS system that have been identified to date are mediated via CinS*,* and not via CinR. Further investigation will have to determine which genes are regulated by CinS (see Chapter 4).

CinS homologues were found in other rhizobia, namely *R. l.* bv, *trifolii* WSM1325, *R. l.* bv. *trifolii* WSM2304, *M. tianshanense, R. etli* CNPAF512, *R. etli* CIAT652 and *R. etli* CFN42. In these species, the *cinS* homologue is located downstream of and apparently translationally coupled to a *cinI* homologue, suggesting a similar role in QS gene regulation in these organisms. In *R. etli* CNPAF512 the *cin* system is involved in nitrogen fixation efficiency and swarming (Daniels *et al.*, 2002; Daniels *et al.*, 2006). In this work, it was shown that the *cinI* mutation in *R. etli* is probably polar on *cinS* because CinS was responsible for regulating swarming behaviour. One of the genes

that controls swarming in *R. etli* CNPAF512 is a gene with sequence similarity to *plyB* (Braeken *et al.*, 2008) and it is therefore likely that the effect of *cinS* mutation on swarming is caused by a reduction in expression of this *plyB*-like gene. The possible role of *cinS* in the efficiency of nitrogen fixation has not yet been studied.

The orphan LuxR-type regulator ExpR is conserved in several rhizobial species and *R. l.* bv. *viciae* ExpR is 58% identical to *S. meliloti* ExpR. Mutation of *expR* caused similar phenotypes as mutation of *cinS*, namely an increased biofilm ring and a reduction of production of RhiI-made AHLs. In *S. meliloti*, ExpR regulates EPSII production in reponse to SinI-made AHLs (Glenn *et al.*, 2007; Marketon *et al.*, 2003). This requirement for AHLs is not absolute, as ExpR also regulates gene expression in an AHL-independent manner (Gurich & Gonzalez, 2009). In *R. leguminosarum* strain 8401*,* ExpR seemed to function without a need for CinI-made AHL molecules (Edwards *et al.*, 2009). From this and previous work (Edwards *et al.*, 2009) it seems likely that CinS and ExpR function in the same regulatory pathway. The mechanism by which ExpR and CinS interact for gene regulation is not clear at this point.

A search of the protein databases did not identify any domains with homology to CinS although the tertiary structure of CinS was predicted to be similar to the DNA-binding domain of ArgP in *M. tuberculosis*. *In vitro* binding of CinS to the promoters of two of its regulatory targets could not be demonstrated. However, this does not mean that a role for CinS as a DNA-binding protein can be excluded; test conditions could have been sub-optimal, or the regulatory effect of CinS on the expression of *raiR* and *rhiR* could be indirect. Since no clear DNA-binding function for CinS could be found, other possibilities were investigated.

Hfq regulates many aspects of the rhizobial life, including QS (Barra-Bily *et al.*, 2010a; Barra-Bily *et al.*, 2010b; Gao *et al.*, 2010; Torres-Quesada *et al.*, 2010; Voss *et al.*, 2009). In the last few years, an important role for sRNA's and Hfq in QS gene regulation was found in *Vibrio* species (Lenz *et al.*, 2004). In *V. harveyi* and *V. cholerae,* AHLs function to activate the expression of Qrr sRNA's. These then bind to Hfq, and modulate the expression levels of the QS master regulators, LuxR and HapR respectively. A possible role of Hfq in CinS-mediated regulation was identified, as both Hfq and CinS induced the expression of *rhiR.* It was hypothesised that CinS might function as a sRNA-binding protein itself and to test this, a sRNA-library was screened for binding to CinS. As a control, the sRNA-library was screened with purified Hfq protein, but for both CinS and Hfq no interacting sRNA's were

identified. As Hfq is the major sRNA binding protein in most organisms (Valentin-Hansen *et al.*, 2004), it would be expected to interact with at least some of the sRNA's in the library. Therefore, it seems likely that if CinS would indeed function as a sRNA-binding protein, the experimental set-up used during these experiments was not suitable to detect this.

The regulatory mechanism by which CinS regulates gene expression remains unknown. Since no DNA- or sRNA-binding function could be demonstrated, these and other possibilities remain open. One other possibility is that CinS might function by interacting with other proteins. In *Bacillus subtilis*, a small QS-induced protein DegQ has been identified that stimulates phosphotransfer to a transcriptional regulator that affects motility and biofilm formation (Kobayashi, 2007). In the next chapter possible interactions of CinS with other proteins will be examined.

2.4 Summary

- The *cin* QS sensing system contains an unusual regulatory gene, *cinS*.
- The LuxR-type regulator ExpR is likely functioning in the same regulatory pathway as CinS.
- CinS and ExpR regulate the *rhi* and *rai* QS system by inducing the expression of the QS regulators *rhiR* and *raiR.*
- CinS did not require CinI-made AHLs for function.
- CinS and ExpR did not affect expression or activity of CinI.
- *cinS* is conserved in other rhizobia that contain a *cin* QS system. In *R. etli* CinS was required for swarming behaviour.
- CinS-His_6 was functional in *R. leguminosarum* and was purified from *E. coli.*
- No evidence could be found for CinS to bind to DNA or sRNA's.

Chapter 3: CinS is an antirepressor of PraR

3.1 Introduction

In the previous chapter *R.l.* bv. *viciae* 3841 *cinS* and *expR* mutants were analysed and CinS-His$_6$ was purified and characterised. It was examined whether CinS-His$_6$ could bind to the *raiR* and *rhiR* promoters and to rhizobial sRNA's but no evidence for binding could be found. In this chapter, the molecular mechanism by which CinS regulates gene expression was further studied, by using the purified protein for absorption of interacting proteins in *Rhizobium* cell lysate. This led to the identification of a transcriptional regulator PraR that interacts with CinS. Using *in vivo* transcriptional studies, bacterial-two-hybrid analysis and *in vitro* promoter binding studies it was shown that CinS acts as an inducer of gene expression by acting as an anti-repressor of the transcriptional repressor PraR.

3.2 Results

3.2.1 Identification of a protein interacting with CinS

To identify CinS-interacting proteins, purified CinS-His$_6$ protein was coupled covalently to cyanogen bromide-activated sepharose beads. These were then used to absorb interacting proteins from the soluble fraction of *R. l.* bv. *viciae* 3841 cell lysate. After several washes, bound proteins were released from the beads using a low pH buffer and quickly neutralised. Proteins in the different fractions were analysed by SDS-PAGE (Figure 3.1A). The fraction released at low pH contained three distinct bands, which were excised from the gel, digested with trypsin and analysed by Maldi-ToF mass spectroscopy. The two lowest migrating proteins (I and II) corresponded to CinS-His$_6$. The third protein (III) corresponded to a protein of about 16 kDa, which was identified using the Mascot software (within the 95% confidence level) as the product of the open reading frame RL0390 (Fig. 3.1B).

In *R. l.* bv. *viciae* 3841 RL0390 is located on the chromosome between a putative transmembrane acyltransferase (RL0391) and a putative *S*-adenosylmethionine synthetase *metK*. Homologues of RL0390 are widely conserved in the Alphaproteobacteria and orthologues have been described in *Sinorhizobium meliloti* WSM419 (*phrR*: pH regulated regulator) (Reeve *et al.*, 1998), and in *Azorhizobium caulinodans* (*praR, phrR*-like regulator conserved in the Alphaproteobacteria) (Akiba *et al.*, 2010). The genomic localisations in these strains are conserved and PraR from *R. l.* bv. *viciae* is 90% and 66% identical to PhrR and PraR respectively. In *S. meliloti* the expression of *phrR* was reported to be induced by acid pH (Reeve *et al.*, 1998; Tiwari *et al.*, 2004), but this was not found to be the case with the *A. caulinodans* gene (Akiba *et al.*, 2010). To test if expression of RL0390 in *R. l.* bv. *viciae* 3841 is induced in acid conditions, a *lacZ* promoter fusion (pIJ11112) was used to assay expression of RL0390 in exponentially growing strain 3841 after transfer from pH 7.0 to low pH 5.5 minimal AMS medium. No change in expression was observed (Figure 3.2). Since *phrR* was induced under similar conditions, but *praR* was not, the nomenclature used by Akiba *et al.* (2010) for *A. caulinodans* was followed and RL0390 was named *praR.*

3.2.2 A *praR* mutant has enhanced production of RhiI-made AHLs

In parallel work, a library of Tn*5*-induced mutants of *R. l.* bv. *viciae* strain 3841 had been screened to identify mutants producing altered levels of AHLs, as described in Chapter 2. One mutant (A963) produced increased levels of RhiI-made AHLs and this phenotype co-transduced with the Tn*5* transposon. Quantitative analysis of AHLs in TY liquid medium using *C. violaceum* CV026 revealed that the mutant produced about twice as much as wild type (WT) (43 ± 4 units compared to 21 ± 2 units). There was a parallel increase in *rhiI'-lacZ* expression (pIJ7794) from 7338 ± 114 to 28 052 ±1839 Miller units (Craig McAnulla). In addition, the mutant also formed an increased biofilm ring at the air-liquid interface when grown in Y mannitol minimal medium, similar to the *cinS* mutant (Figure 3.3). Sequencing from the end of the Tn*5* in A963 revealed that the transposon had inserted in the gene *praR*, identified above as encoding a protein that interacts with CinS. It had been observed previously that mutations of *cinI, cinR* and *cinS* (Chapter 2) reduced the levels of RhiI-made AHLs but it had not been established how *rhi* gene expression was coupled with *cinI/R*-mediated regulation. The observations that CinS interacted with PraR and that

mutation of *praR* caused increased levels of *rhiI* expression and associated AHLs suggested that the *cin*-dependent regulation of *rhiI* may be mediated via CinS and PraR.

3.2.3 CinS, ExpR and PraR regulate expression of *rhiR*

As described in Chapter 2, mutation of *cinS* caused a decrease in the production of RhiI-made AHLs (Figure 3.4B). This was likely due to the decreased level of *rhiR* transcription, as observed in assays of *rhiR'-lacZ* expression in the *cinS* and *cinI* (in which the *cinI* mutation is polar on *cinS*) mutants (Figure 3.4A). Conversely, the *praR* mutant showed increased levels of RhiI-made AHLs (see above and Figure 3.4A) and *rhiR'-lacZ* expression (Figure 3.4A). Introducing the *cinS* mutation into the *praR* mutant (making A1312) did not alter this increased expression of *rhiR'-lacZ* (Figure 3.4A). Cloned *praR* (pIJ11113) repressed RhiI-made AHL production by both the *praR* mutant and the WT. These results fit with a regulatory model in which PraR represses *rhiR* expression and CinS in some way relieves the repression.

Mutation of *expR* decreased both *rhiR'-lacZ* expression and production of RhiI-made AHLs (Chapter 2 and Figure 3.4 A+B). Introducing the *cinS* mutation into the *expR* mutant (making A1232) did not significantly change *rhiR'-lacZ* expression or levels of RhiI-made AHLs (Figure 3.4B). In contrast, introducing the *praR* mutation into the *expR* mutant (making A1313) increased production of RhiI-made AHLs and increased the level of *rhiR'-lacZ* expression to the same level as seen in the *praR* mutant (Figure 3.4A). These results suggest that like CinS, ExpR can also relieve the repression mediated by PraR.

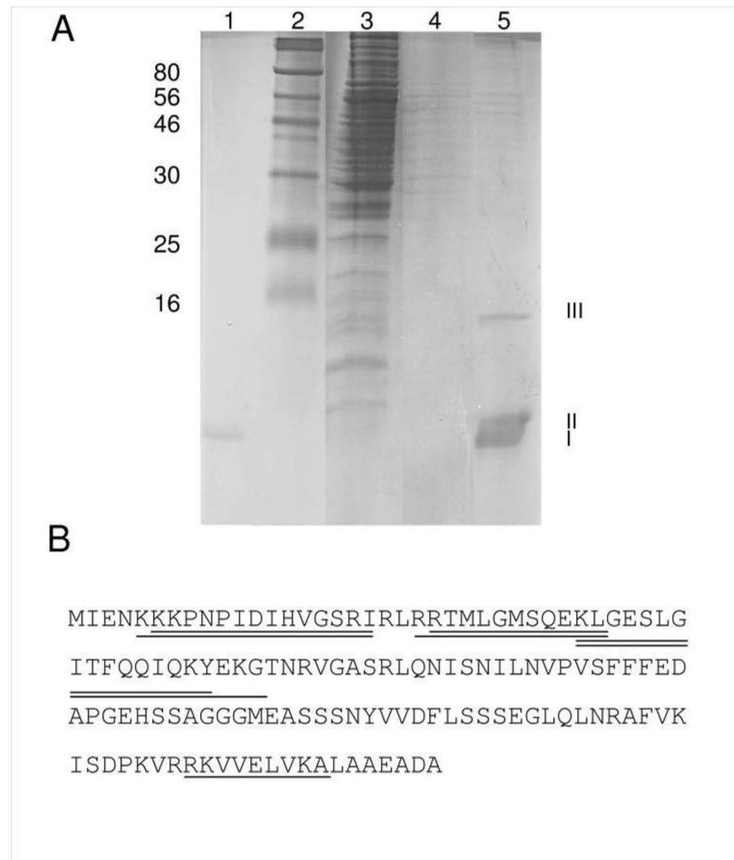


Figure 3.1: CinS interacts with the protein encoded by RL0390. A: Proteins interacting with CinS were isolated from *R. leguminosarum* cell lysate using CinS-His_6 covalently coupled to sepharose beads. The beads were washed ten times and then eluted with pH2.8 buffer. Lane 1, purified CinS-His_6; lane 2 molecular weight standards (sizes indicated); lane 3, first wash; lane 4, tenth wash; lane 5, proteins eluted at pH 2.8 and these were identified by MALDI-ToF as CinS-His_6 (I and II) and PraR (III). B: MALDI-ToF identification of the RL0390 protein. Detected protein fragments are underlined.

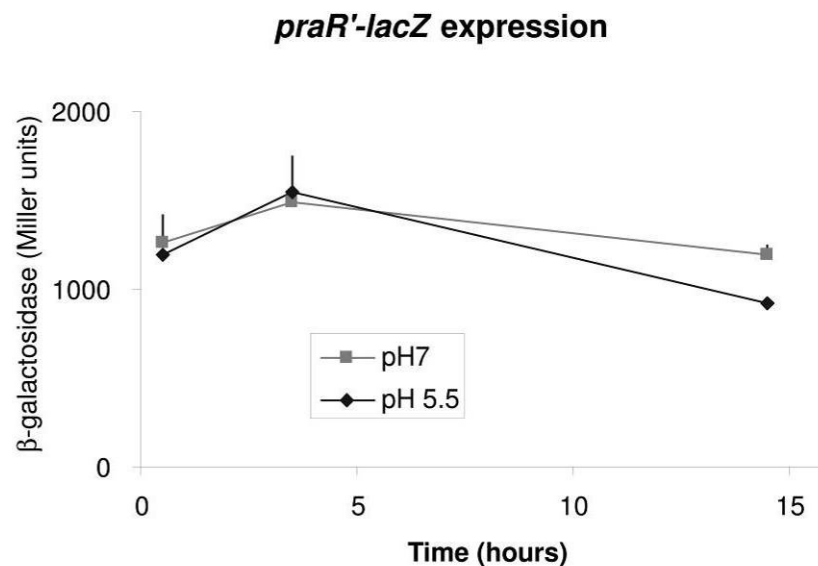


Figure 3.2: *praR* expression is not induced by acid shock in *R. l.* bv. *viciae* 3841. *R. l.* bv. *viciae* 3841 was pregrown in AMS (NH_4Cl, pyruvate, pH 7) until an OD_{600} of 0.4. Cultures were spun down and the cell pellet was transferred to fresh AMS (10 mM NH_4Cl, 30 mM pyruvate) cultures of either pH 7 or pH5.5. The expression of *praR* was measured (*praR'-lacZ* on pIJ11112) by measuring β-galactosidase activity. Error bars represent standard deviations.

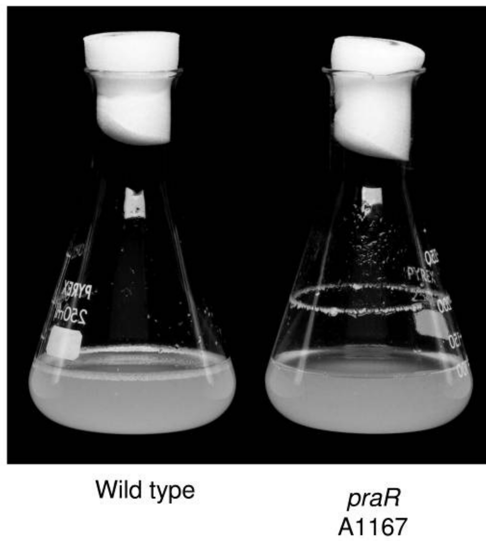


Figure 3.3: Biofilm ring formation of the praR mutant (A1167). Strains were grown in Y mannitol minimal medium for 5 days and the biofilm ring was examined.

Cloned *cinS* complemented the *cinS* and the *cinI* mutants for production of RhiI-made AHLs; this shows that CinS can induce production of RhiI made AHLs, even in the absence of *cinI* and CinI-made AHLs. Cloned *expR* complemented the *expR* mutant for production of RhiI-made AHLs, but also increased production of RhiI-made AHLs in the *cinS* and *cinI* mutants (Figure 3.4A). This indicated that ExpR did not require CinI-made AHLs for function. Based on the observations that mutations in *cinS* and *expR* decreased *rhiR* expression, it is probable that the restoration of RhiI-made AHLs by cloned *cinS* and *expR* are due to effects on *rhiR* transcription. However variability of *rhiR* expression during growth, coupled with the effects of two plasmids resulted in inconsistent measurements of *rhiR'-lacZ* expression.

These results, taken together with the observed interaction between PraR and CinS would be consistent with PraR repressing *rhiR* expression and CinS acting as a PraR antirepressor, thereby inducing *rhiR*. The role of ExpR is less clear; for example it could act directly by inducing *rhiR* or indirectly by repressing *praR* expression and thereby increasing *rhiR* expression.

3.2.4 PraR binds to the *rhiR* promoter and is displaced by CinS

PraR was isolated as an N-terminal protein fused to His_6-tagged maltose binding protein (MBP-PraR) using Ni^{2+} chromatography (Figure 3.5). The purified MBP-PraR protein was analysed by SDS-PAGE and migrated at an apparent Mw of ≈ 50 kDa, fitting with the calculated mass of MBP-PraR (58 kDa). The purified MBP-PraR protein was about 90 % pure, as estimated from the protein gel. This MBP-PraR was then tested for interaction with the *rhiR* promoter. Specific binding started at about 65 nM MBP-PraR and at 250 nM MBP-PraR the *rhiR* promoter fragment was fully shifted, with multiple supershifted bands (Figure 3.6 A). To test if the observed binding was specific, MBP-PraR was incubated with the *cinI* promoter, the expression of which was unaltered in *praR* or *cinS* mutants and no specific binding was observed up to 500 nM MBP-PraR (Figure 3.6 G). CinS-His_6 was tested for binding to the *rhiR* promoter, but no gel retardation could be observed (Figure 3.6 C). However 250 nM CinS-His_6 released MBP-PraR that was already bound to the *rhiR* promoter (Figure 3.6 B). CinS thus can function *in vitro* to displace bound PraR and this fits with its *in vivo* role as an inducer of *rhiR* gene expression by antirepression of PraR.

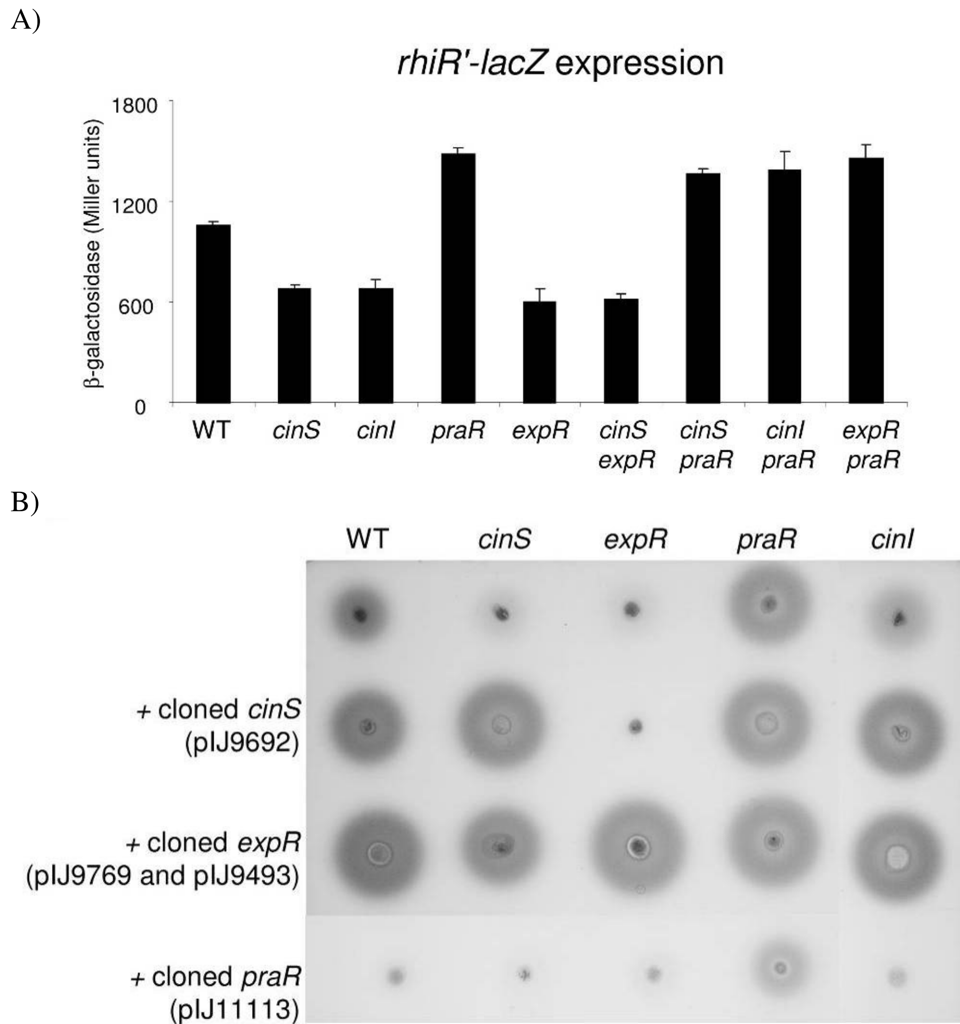


Figure 3.4: PraR, CinS and ExpR regulate *rhiR* expression. A: *rhiR'-lacZ* expression (pIJ9104) was measured by β-galactosidase activity (Miller units) after growth for 48h (OD600 ≈ 1.3) in TY medium. Strains: WT 3841 and the mutants: *cinS* (A1245), *praR* (A1167), *cinI* (A994) , *expR* (A1246), *cinS-expR* (A1232), *cinS-praR* (A1312), *cinI-praR* (A1314) and *expR-praR* (A1313). Error bars represent standard deviations. B: Effect of cloned *cinS* (pIJ9692), *expR* (pIJ9769 or pIJ9493) or *praR* (pIJ11113) on RhiI-made AHLs produced by the WT, *cinS, expR* and *praR* mutants bioassayed using *C. violaceum* CV026. The WT and A1246 (*expR*) contained pIJ9493; A1245 (*cinS*) and A1167 (*praR*) contained pIJ9697.

3.2.5 ExpR, CinS and PraR repress *praR* expression

To determine if ExpR acted on the promoter of *rhiR*, *praR* or *cinI,* it was attempted to purify ExpR, but no active ExpR protein could be obtained. Therefore a genetic approach was taken to further examine the role of ExpR. Mutation of *expR* did not affect the expression of the *cinIS* operon (pIJ9611) (see Chapter 2) but increased expression of *praR'-lacZ* (Figure 3.7A). The expression of *praR'-lacZ* was increased in the *cinS* and *praR* mutants (Figure 3.7A). To test this *in vitro,* MBP-PraR and CinS binding to the *praR* promoter was tested. MBP-PraR bound to the promoter and there were multiple retarded bands (Figure 3.6 D). CinS-His_6 did not bind to the promoter (Figure 3.6 E), but could dissociate bound MBP-PraR (Figure 3.6 F). The concentration of CinS-His_6 needed was lower than that needed for displacement of MPB-PraR from the *rhiR* promoter (65 nM was sufficient to start dissociation). The observation that CinS displaces PraR from the *praR* promoter seems to contradict the *in vivo* observation that CinS functions as a repressor. A possible explanation for this could be the absence of ExpR in the *in vitro* conditions, but because active ExpR could not be purified, this could not be tested *in vitro*. Instead the *in vivo* role of ExpR in the regulation of *praR'-lacZ* expression was further analysed.

To determine if the mutations in *cinS, expR* and *praR* had additive repressing effects on *praR*, its expression (*praR'-lacZ*) was measured in *cinS-praR*, *expR-cinS* and *expR-praR* double mutants. No additive effects were seen with the *cinS-praR* and *expR-cinS* double mutants, but the *expR-praR* double mutant showed a higher expression of *praR'-lacZ* than the *expR* or *praR* mutants (Figure 3.7A). This indicated that ExpR and PraR may function independently to decrease *praR* expression. If this was the case, *expR* should not require *cinS* or *praR* to be able to exert a regulatory effect. This hypothesis was tested by introducing cloned *expR* into the WT, the *cinS*, *expR* and *praR* mutants. As expected, cloned *expR* complemented the *expR* mutant for *praR'-lacZ* expression (and thus reduced the expression level). In addition, it reduced *praR'-lacZ* expression in the *cinS* and *praR* mutants (Figure 3.7B). This confirmed that ExpR can function independently of CinS and PraR to cause decreased expression of *praR*.

Cloned *cinS* was introduced into the different mutants to determine if ExpR and PraR are required for the repressing effect of CinS in the regulation of *praR'-lacZ* expression. Cloned *cinS* decreased *praR'lacZ* expression in WT and in the *cinS* mutant but no such decrease in expression was observed in the *praR* and *expR* mutants

(Figure 3.7B). This means that both ExpR and PraR are required for CinS to exert a repressing effect on the *praR* promoter. Taken together with the observation that mutation of *expR* does not affect *cinIS* expression, these results suggest that ExpR not only affects the expression of *praR'-lacZ,* but also may affect the activity of CinS.

3.2.6 ExpR interacts with PraR and CinS

Possible interactions between ExpR, PraR and CinS were examined by using a bacterial two hybrid system (Karimova *et al.*, 1998). This involves fusing the proteins to two parts of *E. coli* adenylate cyclase. If two proteins interact, the T18 and T25 parts are brought into proximity, reconstituting adenylate cyclase which leads to the induction of *lacZ,* which can be measured in *E. coli* by assaying β-galactosidase or by the development of a red colour on McConkey agar. As expected from the *in vitro* protein interactions (Figure 3.8), an interaction between PraR and CinS could be observed based on the increased levels of β-galactosidase when T25-CinS and PraR-T18 were expressed in *E. coli* (Table 3.1). In the opposite orientation (T25-PraR and CinS-T18) no increased activity was observed, but such negative results can occur due to steric effects. Based on the increased levels of activity seen with CinS-T18 and T25-CinS, and with PraR-T18 and T25-PraR, it is clear that both CinS and PraR can form homo-multimers (Table 3.1).

The same strategy was used to determine whether ExpR interacted with CinS and PraR. ExpR-T18 together with either T25-CinS or T25-PraR induced β-galactosidase activity (Table 3.1 and Figure 3.8) indicating that ExpR interacts with both CinS and PraR. T25-ExpR was found to be auto-active, and this construct could therefore not be used for further analysis. These results show that PraR, ExpR and CinS can all interact with each other. PraR had already been identified as a CinS interactor as it was isolated from total *R. leguminosarum* cell lysate. ExpR was not identified in that experiment, which may mean that the interaction between CinS and ExpR is weaker than that between CinS and PraR. The observations on expression of *praR* in the *cinS, expR* and *praR* mutants together with the observation that CinS, ExpR and PraR all interact with each other fits with a model in which a) PraR represses *praR* expression, b) ExpR can repress *praR* expression independently of PraR and c) the presence of ExpR and PraR together on the *praR* promoter prevents CinS from acting as a PraR-antirepressor.

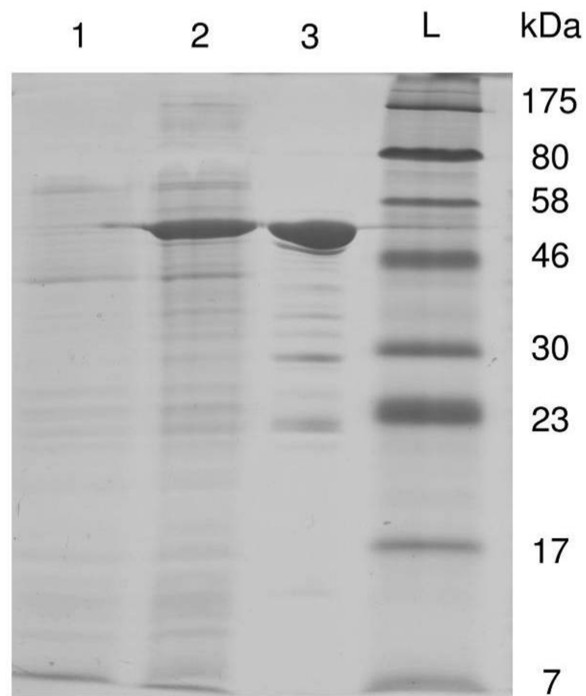


Figure 3.5: Purification of MBP-PraR. SDS-PAGE analysis of purified MBP-PraR protein. 1: uninduced cells, 2: induced cells, 3: pooled purified MBP-PraR. L: ladder

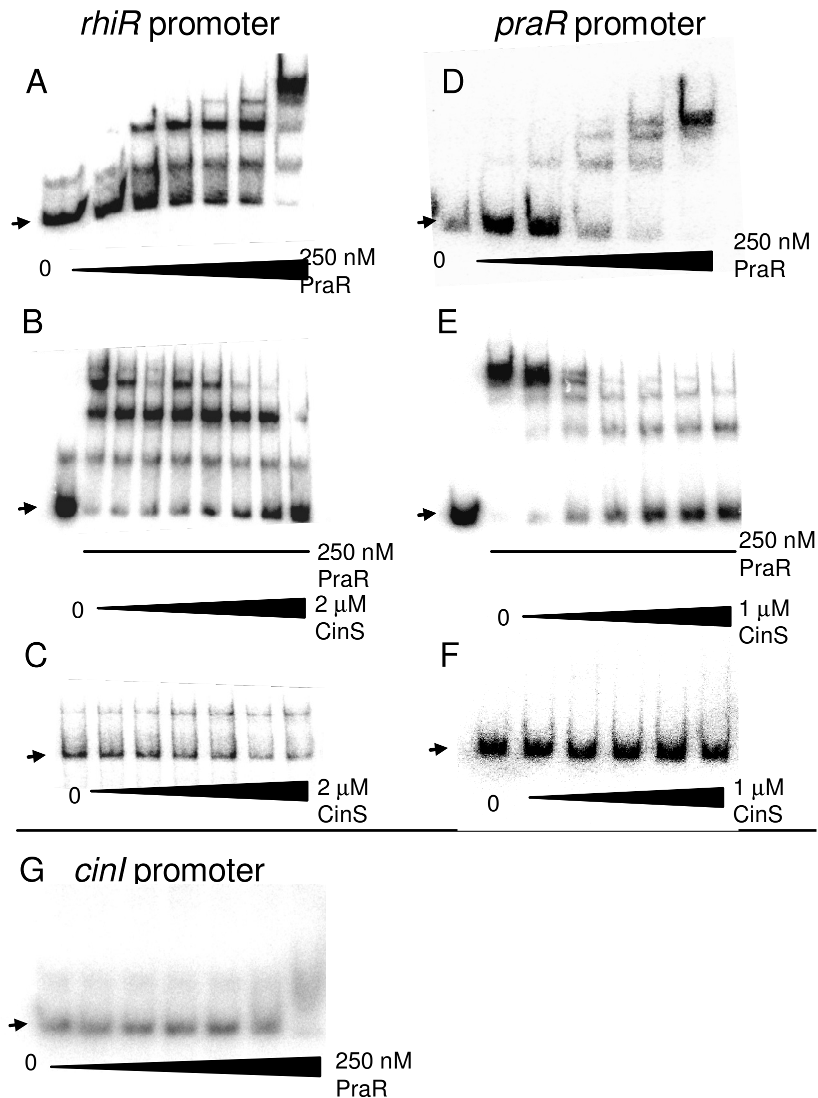


Figure 3.6: *In vitro* analysis of MBP-PraR and CinS-His$_6$ binding to *praR, rhiR* and *cinI* promoters. Radioactively labelled promoters were incubated with purified proteins, after which the reactions were analysed by non-denaturing gel electrophoresis. A-C *rhiR* promoter: A, with increasing levels of MBP-PraR B, with 250 nM MBP-PraR and increasing levels of CinS-His$_6$; C, with increasing levels of CinS-His$_6$. D-F: *praR* promoter: D, with increasing levels of MBP-PraR; E, with: 250 nM MBP-PraR and increasing levels of CinS-His$_6$; F, with increasing levels of CinS-His$_6$. G: *cinI* promoter incubated with increasing levels of MBP-PraR Protein concentrations were a twofold dilution series. Arrows indicate unshifted fragments.

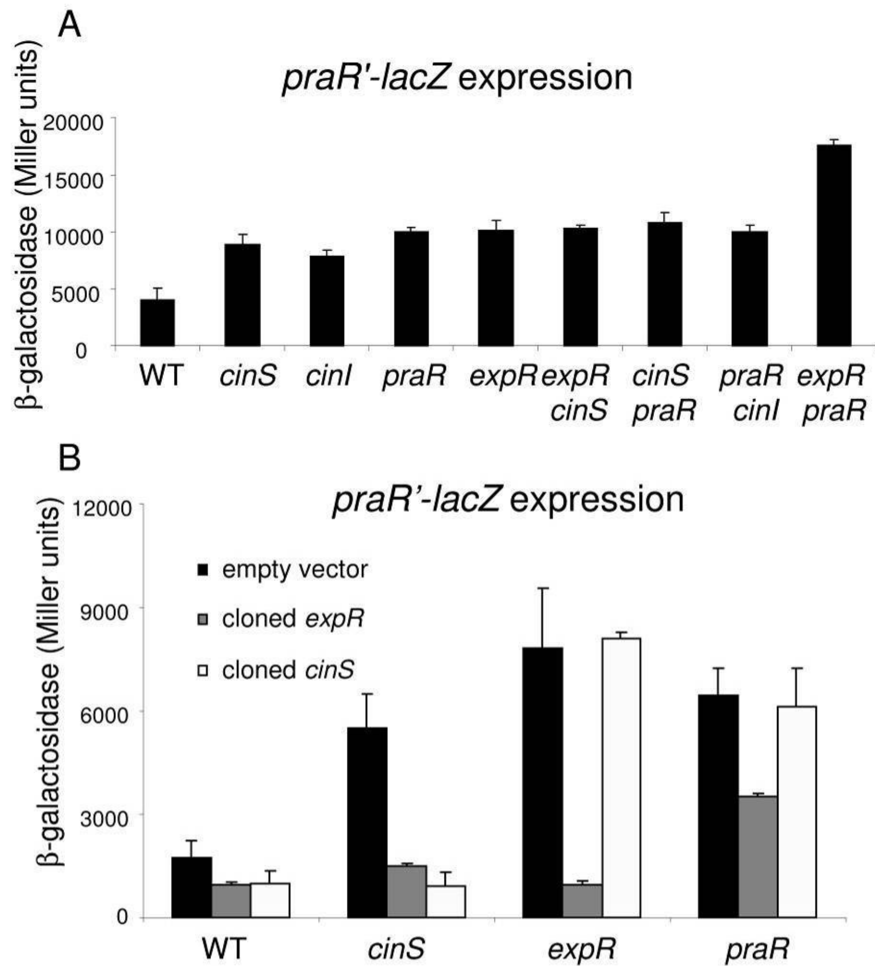


Figure 3.7: PraR, CinS and ExpR repress transcription of *praR*. A and B *praR'-lacZ* expression (pIJ11112) was measured by β-galactosidase activity (Miller units) after growth for 48h (OD600 ≈ 1.3) in TY medium. A): expression was measued in WT 3841 and the mutants: *cinS* (A1245), *cinI* (A994), *praR* (A1167), *expR* (A1246), *cinS-expR* (A1232), *cinS-praR* (A1312), *cinI-praR* (A1314) and *expR-praR* (A1313). B) expression was measured in WT (3841) and the *cinS* (A1245), *expR* (A1246) and *praR* (A1167) mutants containing cloned *cinS, expR* or the empty vectors. WT, A1245 (*cinS*) and A1167 (*praR*) contained pIJ11051, pIJ9769 or pBBR1-MC2. A1246 (*expR*) contained pIJ11052, pIJ9493 or pBBR1-MC5.

3.2.7 Identification of PraR consensus binding site

To identify the PraR binding consensus sequence, systematic evolution of ligands by exponential enrichment was used (Oliphant *et al.*, 1989). After ten rounds of enrichment, the resulting fragments were cloned and sequenced (Figure 3.9A). Alignment of these fragments identified the consensus palindrome sequence CAACnnnnnGTTG to which PraR is predicted to bind. One such fragment (tctttaCAACccaggGTTGt) was shown to interact with MBP-PraR (Figure 3.9B), while no interaction was seen with the mix of random oligonucleotides (Figure 3.9C). Interestingly, with this fragment multiple supershifted bands occurred. This means that the cause of these supershifted bands must be due to the formation of higher order multimeric states of the protein, as only one binding site is present in this fragment (Figure 3.9B). The consensus PraR binding sequence was also found in the *praR* and *rhiR* promoter regions (CAACgtggcGTTT and CAACataccGTTG respectively – one mismatch was present for the *praR* promoter) and in front of *plyB* (CACCtttcgGTCG) and *raiR* (CAAGctgtatGTTG) (two mismatches compared to consensus).

3.2.8 Expression of *rhiR,* but not *praR* is growth dependent

Since CinS is expressed in a population dependent way, it was examined whether the expression of *rhiR* and *praR* were dependent on growth. Expression of *rhiR'-lacZ* increased very strongly during exponential growth (Figure 3.10 A) as would be expected from a QS induced gene. No significant change in the expression of *praR'-lacZ* was seen during growth (Figure 3.10 B). This was surprising, as earlier results showed that CinS acts as a repressor of *praR'-lacZ.* Nevertheless these results do show that CinS is not acting as an inducer of *praR* expression.

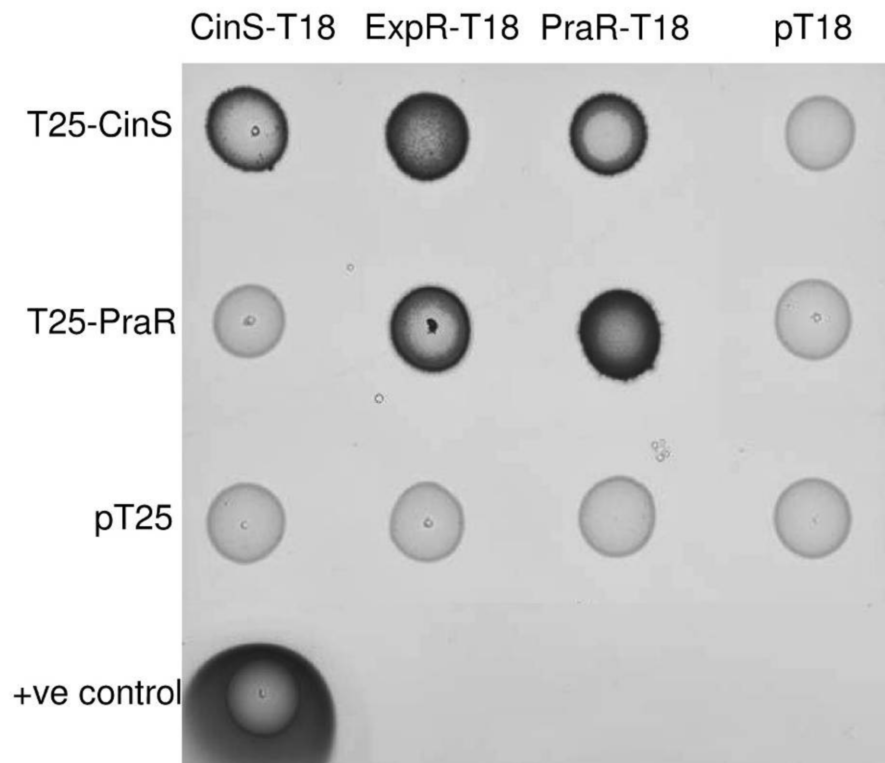


Figure 3.8: Bacterial two hybrid analysis of interactions between CinS, ExpR and PraR. Interactions between CinS-T18 (pIJ11159), ExpR-T18 (pIJ9716), PraR-T18 (pIJ11132), pT18, T25-CinS (pIJ9717), T25-PraR (pIJ11133) and pT25 were examined by bacterial two hybrid analysis. Positive interactions result in the formation of a red pigment on McConkey agar. As a positive control pT25-zip and pT18-zip were analysed.

Interactions between CinS, Csi and ExpR (β-galactosidase assay, Miller units)				
	CinS-T18	**Csi-T18**	**ExpR-T18**	**Empty pT18**
T25-CinS	583 ± 30	613 ± 125	1682 ± 62	153 ± 24
T25-PraR	296 ± 36	5666 ± 159	1062 ± 34	242 ± 5
T25-ExpR	n.d.	n.d.	n.d.	Auto-active
Empty pT25	120 ± 40	175 ± 12	231 ± 24	135 ± 15

Table 3.1: Bacterial two hybrid analysis of interactions between CinS, ExpR and PraR. Interactions between CinS-T18 (pIJ11159), ExpR-T18 (pIJ9716), PraR-T18 (pIJ11132), pT18, T25-CinS (pIJ9717), T25-PraR (pIJ11133) and pT25 were examined by bacterial two hybrid analysis. Positive interactions result in the activation of β-galactosidase activity. n.d.: not determined

3.2.9 CinS is not stable in a *praR* mutant

Antiserum against the CinS-His$_6$ protein was raised and used to study CinS in *R. leguminosarum* by Western blotting (Figure 3.11A). CinS was detected in WT *R. l.* bv. *viciae* 3841 and the lack of a signal in the *cinS* or *cinI* mutants confirmed the specificity of the antiserum. CinS was not detected in the *praR* mutant. This was very unexpected, as mutation of *praR* had no effect on transcription of the *cinIS* operon (measured by *cinI-gfp*) (Figure 3.11B). Production of CinI-made AHLs was normal in the *praR* mutant, as shown by a bio-assay (Figure 3.11C) that assesses the levels of CinI-made AHLs, confirming that the *cinIS* operon is transcribed normally in the *praR* mutant. Therefore the reduced level of CinS must be due to a post-transcriptional effect. One possibility is that the PraR-CinS interaction protects CinS from degradation. Alternatively, PraR could affect the translation initiation of CinS independent of the translation initiation of CinI. This seems unlikely however, as CinS is thought to be translationally coupled to CinI (Edwards *et al.*, 2009).

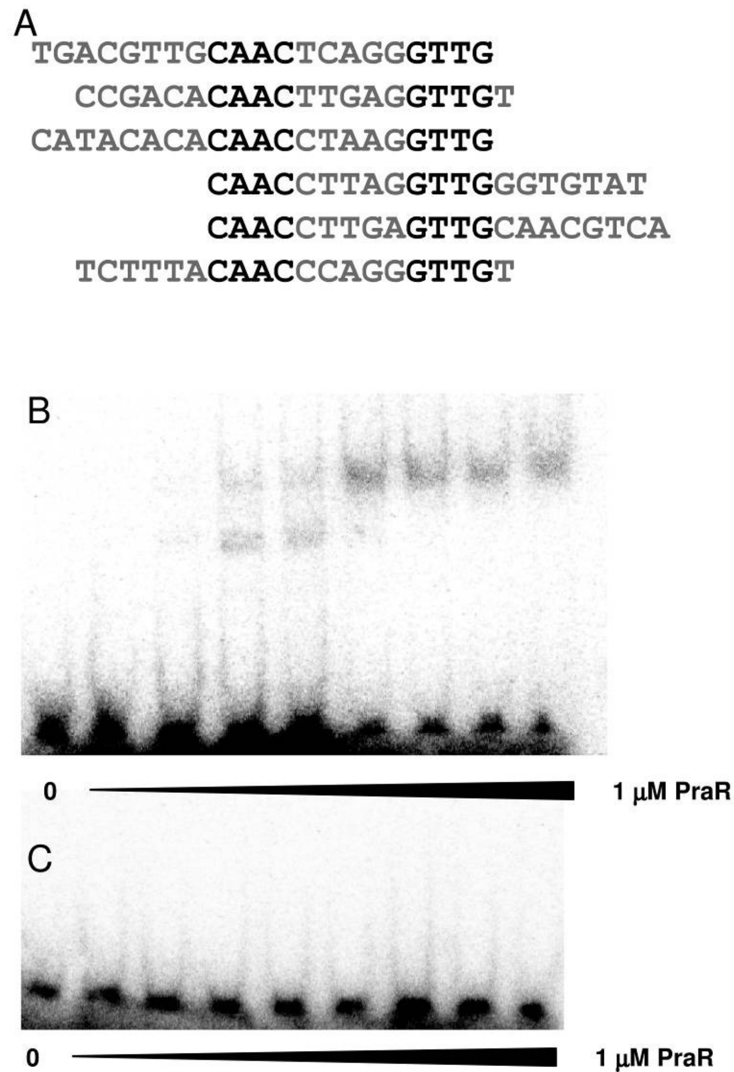
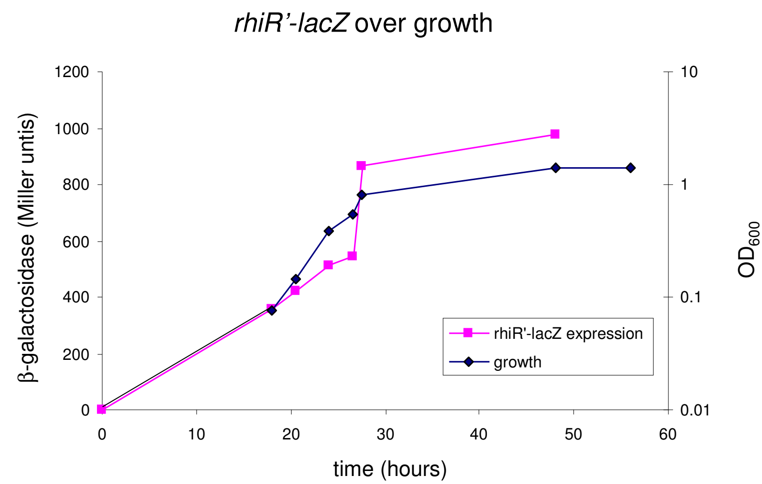


Figure 3.9: PraR binds to a palindrome sequence in the *praR* and *rhiR* promoters. A: a library of random oligonucleotides was enriched for fragments binding to MBP-PraR. The enriched fragments were cloned in pGEM T-easy, sequenced and aligned. B+C: Radioactively labelled fragments were incubated with purified proteins, after which the reactions were analysed by non-denatureing gel electrophoresis. B: EMSA analysis of tctttaCAACccaggGTTGt oligonucleotide with increasing increasing levels of MBP-PraR. C: library of random oligonucleotides with increasing levels of MBP-PraR. Protein concentrations were a twofold dilution series.

A)



B)

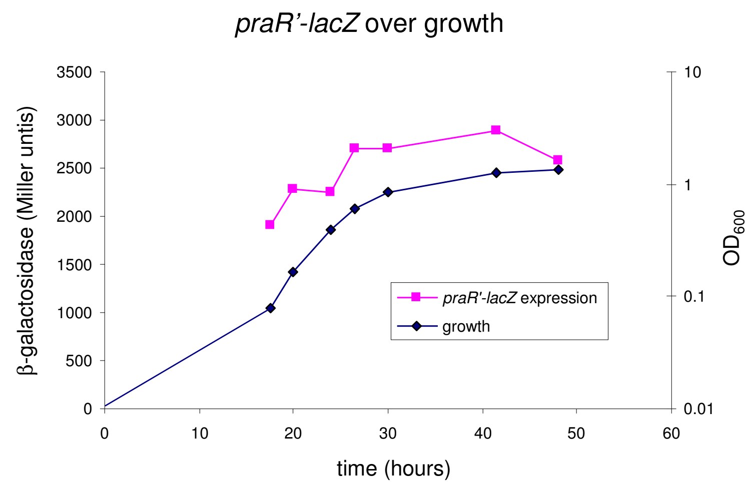


Figure 3.10: Expression of *rhiR,* but not *praR* is growth phase dependent. Cultures were grown in 50 mL TY shaken flasks. *rhiR'-lacZ* (pIJ9104) and *praR'-lacZ* (pIJ11112) expression was measured by β-galactosidase activities at different time points over growth.

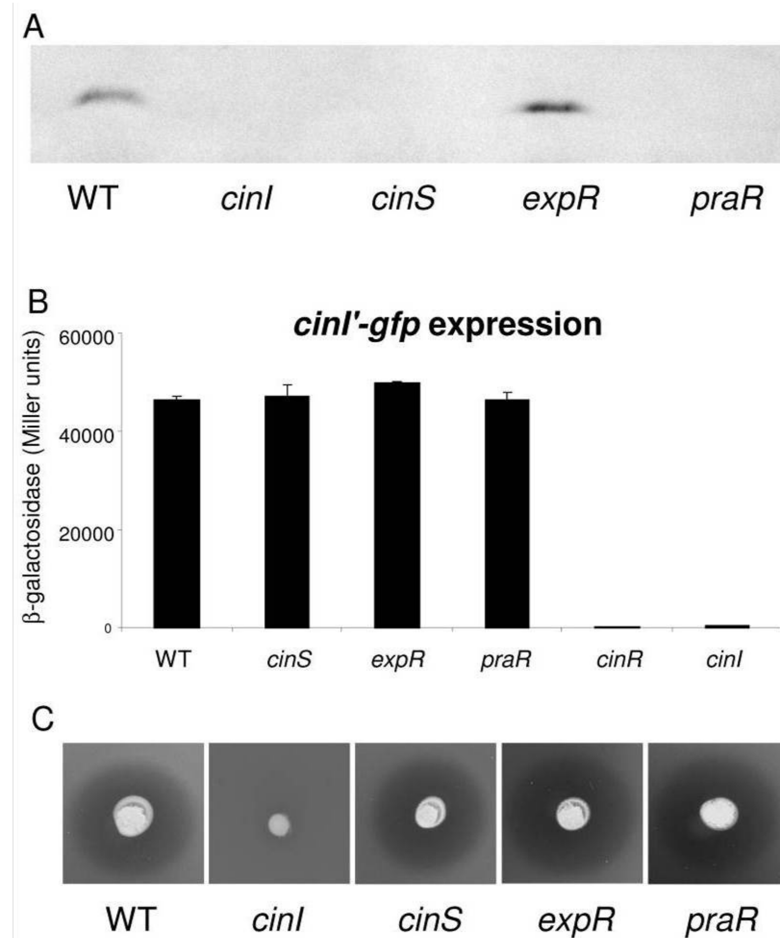


Figure 3.11: CinS is not stable in a *praR* mutant. A: Western blot detecting CinS in different mutants. B: Expression of *cinI* in different mutants was measured by a *cinI'-gfp* promoter fusion (pIJ). C: Assay of CinI-made AHLs by the bacteriocin activity against the biosensor strain A34. Strains used were WT, *cinS* (A1245), *expR* (A1246), *praR* (A1167), *cinI* (A994) and *cinR* (A924) mutants.

3.3 Discussion

It was shown that QS gene regulation can be mediated by the induction of *cinS* and that CinS acts by attenuating repression by the PraR regulator. Although *cinS* is induced in a population density-dependent manner under *cinR* and *cinI* control, once it is expressed it can act independently of both CinR and the CinI-made AHLs, even though *cinS* and *cinI* appear to be translationally coupled. As CinS levels rise during population growth it can increasingly displace PraR from target promoters, thereby inducing their expression in a QS manner (Fig. 3.12). This regulatory mechanism differs from other QS induction systems.

Anti-activation has been found in QS systems as a mechanism to modulate the QS response. For example, in *Agrobacterium tumefaciens* the antiactivators TraM (Chen *et al.*, 2007) and TrlR (Chai *et al.*, 2001) inactivate TraR, which normally induces plasmid transfer genes. In *Pseudomonas aeruginosa* the orphan LuxR-type regulator QscR modulates the expression of the *las* and *rhl* systems by forming inactive dimers with LasR and RhlR (Chugani *et al.*, 2001; Ledgham *et al.*, 2003). In addition, a repressor QteE was recently identified that inhibits early activation of both systems by reducing the stability of LasR and RhlR (Siehnel *et al.*, 2010). Thus, modulation of QS in both these species occurs by a direct interaction with the LuxR-type regulators. In contrast, CinS attenuates repression by targeting an AHL-independent repressor PraR.

Regulation of *praR* expression is important for CinS-dependent regulation. No significant change in *praR* expression throughout growth was observed. It had been shown that the LuxR-type regulator ExpR increased *raiR* expression independently of AHLs, which means ExpR is likely to function without a need for AHLs (Edwards *et al.*, 2009). Orphan LuxR-type regulators that respond to signals other than AHLs have been previously reported. For example, in *Xanthomonas* species unidentified compounds in plant exudates have been shown to activate the orphan LuxR-type regulators OryR and XccR (Ferluga & Venturi, 2009). The mechanism by which ExpR is relieved from repressing *praR* expression is not known. Additional to ExpR-mediated repression, PraR functions as an autorepressor. Bacterial two hybrid analysis indicated that PraR and ExpR interact. Both PraR and ExpR can independently attenuate expression of *praR*, because a *praR*-*expR* double mutant showed higher expression at the *praR* promoter than either single mutant. The regulation of *praR* is complex, because although CinS can displace PraR from the *praR* promoter,

paradoxically, mutation of *cinS* increased *praR* expression (Fig. 3.12). The basis for this is not known; it could e.g. be due to a change in expression of another regulator or possibly due to some change in stability of the interactions of PraR and ExpR at the *praR* promoter. However the net effect would be that CinS could attenuate both *praR* expression and PraR repression.

PraR is highly conserved in the Alphaproteobacteria (Akiba *et al.*, 2010) but *cinS* is only present in the few rhizobia that contain *cinI* and *cinR* (Edwards *et al.*, 2009). This suggests that if the *praR*-like genes in other species encode repressors, a different mechanism must be involved in relief of repression. The roles of *praR*-like genes seem to be different even among different rhizobia. In *A. caulinodans,* mutation of *praR* caused a loss of symbiotic nitrogen fixation, probably due to the induction of the *reb* genes, which are absent from most rhizobia (Akiba *et al.*, 2010). Mutations of *praR* in *R.l.* bv. *viciae* and the closely-related gene *phrR* in *S. meliloti* (both lacking the *reb* genes) do not affect symbiotic nitrogen fixation (Reeve *et al.*, 1998). In *S. meliloti* the *phrR* gene has been reported to be acid inducible and to respond to other stresses (Reeve *et al.*, 1998), but no such acid induction of *praR* was observed in *R.l.* bv. *viciae*.

The only known target of CinR is the *cinI* promoter and we propose that the primary function of CinR and CinI could be to induce the expression of *cinS* in a population-dependent manner, but it can not be excluded that CinR has a CinS-independent regulatory function. It is unlikely that the *rhiR* and *praR* promoters are the only targets of PraR. Given the similar dependence of *raiR* and *plyB* expression on ExpR and CinS (Edwards *et al.*, 2009) and the predicted PraR binding sites in their promoters, it seems likely that they are also repressed by PraR. It is possible that acquisition of the *cinR*, *cinI* and *cinS* genes by horizontal gene transfer could be a mechanism of modulating the activity of PraR, thereby putting PraR-regulated promoters under QS control. Such regulation could be quite subtle and could positively influence the adaptation of the *R.l.* bv. *viciae* to specific lifestyle switches. Since mutations in *plyB, rhiR* and *raiR* affect biofilm formation, rhizosphere growth and symbiotic interactions (Cubo *et al.*, 1992; Edwards *et al.*, 2009; Russo *et al.*, 2006), QS regulation via CinS, ExpR and PraR could play a role in optimising interactions between the symbiotic partners.

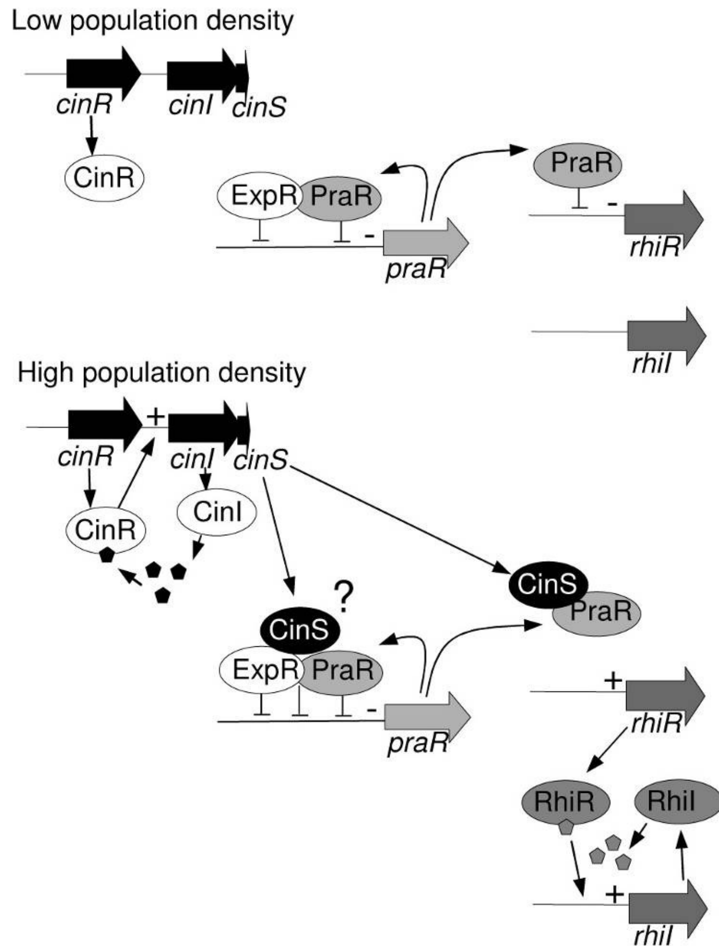


Figure 3.12: Model for CinS, ExpR and PraR function. At low population density, the *cinIS* operon is expressed at a low level, so there is little CinS present. PraR is expressed at a level that represses the expression of *rhiR*. At high population density, the *cinIS* operon is strongly induced, leading to the production of CinS which displaces PraR from the *rhiR* promoter. Increased production of RhiR will induce *rhiI* and positive feedback by RhiI-made AHLs on RhiR will increase expression of rhiI and other RhiR-regulated promoters. The regulation of the *praR* promoter is complex and involves ExpR, PraR and CinS. CinS might bind to ExpR and PraR and lower *praR* expression.

3.4 Summary

- CinS interacted with the transcriptional repressor PraR
- PraR is a repressor of *rhiR* and *praR* expression.
- CinS is an inducer of *rhiR* expression, but a repressor of *praR* expression.
- PraR bound directly to the *rhiR* and *praR* promoters and CinS was capable of displacing PraR. A binding box for PraR was identified.
- ExpR interacted with both CinS and PraR.
- ExpR is an inducer of *rhiR* expression, and an independent repressor of *praR* expression.
- In the proposed model ExpR prevents CinS from acting as a PraR antirepressor on the *praR* promoter.
- ExpR did not require CinI-made AHLs for function.
- CinS needed PraR for stability in *R. leguminosarum*

Chapter 4: PraR regulates root hair attachment and competitive nodulation

4.1 Introduction

Inoculation of legume seeds is an efficient way of introducing effective rhizobia to the soil. A common problem with this method is that the inoculated rhizobia may not be able to compete with the indigenous soil rhizobia. Therefore many studies have tried to determine which factors influence nodulation competitiveness. These can be divided into three different categories: an increased ability to attach to the plant roots, an increased survival in the soil or infection thread and the ability to respond appropriately to plant signals.

Rhizobial attachment to plant roots can contribute to efficient nodulation, as higher numbers of attached bacteria give them a higher chance to be entrapped by the curling root hair and thus to infect the nodule. Attachment of *R. l.* bv. *viciae* to root hairs occurs in two stages. The first loose attachment is mediated via Ca^{2+}-dependent adhesion proteins like rhicadhesin (Smit *et al.*, 1989b) and RapA1 (Mongiardini *et al.*, 2009). Polysaccharides are also important during this first stage of attachment. Different rhizobial species produce different kinds of surface polysaccharides and a role for polysaccharides in nodulation competitiveness has been shown in many species (Bittinger *et al.*, 1997; Janczarek *et al.*, 2009; Milner *et al.*, 1992; Pobigaylo *et al.*, 2008; Williams *et al.*, 2008). *R. l.* bv. *viciae* strain 3841 produces three polysaccharides important for attachment: acidic EPS, glucomannan and cellulose (Williams *et al.*, 2008). Acidic EPS was involved in *in vitro* biofilm formation and root hair attachment and a mutant unable to produce acidic EPS was defective for nodule infection (Russo *et al.*, 2006; Williams *et al.*, 2008). Glucomannan was important for attachment to root hairs in acidic conditions due to its interaction with a plant lectin and was required for competitive nodule infection (Laus *et al.*, 2006; Williams *et al.*, 2008). Cellulose was essential for the formation of a biofilm cap,

although a mutant unable to produce cellulose nodulated normally (Laus *et al.*, 2005; Smit *et al.*, 1987; Williams *et al.*, 2008).

The ability of the rhizobia to survive in the soil or in infection threads is also very important and this is often coupled with metabolic changes. Rhizobia can promote survival in the soil by producing antibacterial compounds. For example, *R. l.* bv. *trifolii* T24 and *Bradyrhizobium elkanii* produce anti-rhizobial compounds (trifolitoxin and rhizobitoxine respectively) to compete against sensitive strains (Robleto *et al.*, 1998; Yuhashi *et al.*, 2000). Nodulation competitiveness can also be affected by the ability to carry out specific metabolic processes, such as catabolism of rhamnose by *R. l.* bv. *trifolii* (Oresnik, 1998) and catabolism of *myo*-inositol and rhizobially synthesised rhizopines (which are derivatives of *myo*-inositol) by *R. l.* bv. *viciae, S. meliloti* and *Sinorhizobium fredii* (Fry *et al.*, 2001; Gordon *et al.*, 1996; Jiang *et al.*, 2001). Some rhizobia contain nodulation formation efficiency (*nfe*) genes (Soto *et al.*, 1993; Soto *et al.*, 1994). Although the function of the *nfe* genes is not clear, it has been hypothesised that they are responsible for the degradation of an unidentified rhizopine (Garcia-Rodriguez & Toro, 2000). Other phenotypes affecting rhizosphere growth include proline catabolism (Jimenez-Zurdo *et al.*, 1995) and production and degradation of intracellular poly-3-hydroxybutyrate in *S. meliloti* (Aneja *et al.*, 2005). Survival in infection threads is enhanced when the rhizobia can adapt better to host-induced osmotic stresses, and they can do this by production of trehalose (Ampomah *et al.*, 2008; Dominguez-Ferreras *et al.*, 2009) or catabolism of choline and glycine (Boncompagni *et al.*, 1999).

Some rhizobia are more able to respond appropriately to signals from the plant. The ability of some *R. leguminosarum* species to respond better to plant factors like flavonoids increased nodulation competitiveness (Maj *et al.*, 2008). In addition, rhizobia are able to move towards plant-made chemoattractants, which may guide them to the plant root hairs (Armitage *et al.*, 1988; Caetano-Anolles *et al.*, 1988a; Dharmatilake & Bauer, 1992) and increase nodulation efficiency (Gulash *et al.*, 1984; Miller *et al.*, 2007; Yost *et al.*, 1998).

The aims of this chapter were to investigate the phenotypes associated with mutation of *cinS, praR* or *expR.* It was found that a *praR* mutant attached very strongly to root hairs compared to WT, and that this led to increased nodulation competitiveness.

4.2 Results

4.2.1 Nodulation by the *cinS, praR* and *expR* mutants

The *cinS, expR* and *praR* mutants formed equivalent numbers of pink nodules, indistinguishable to those formed by WT. To test whether any of the mutants showed a difference in nodulation competitiveness when co-inoculated with WT, the *cinS, expR* and *praR* mutations were transduced into strain 300, a non-streptomycin resistant derivative of *R. l.* bv. *viciae* 3841, yielding strains A1325, A1326 and A1345 respectively. All of these mutants retained the phenotypes of the original mutants (formation of an increased biofilm ring and changes in production of RhiI-made AHLs). Nodulation competitiveness was tested by co-inoculating each of these strains with equal amounts of (streptomycin resistant) WT 3841 on peas. After three to four weeks, bacteria were isolated from the nodules and identified based on antibiotic resistances. Typically, 20% or less of nodules contained both WT and mutant strains and these were omitted from the analysis. The *cinS* and *expR* mutants did not show any difference with WT in the efficiency by which they infected nodules, as each strain occupied about 50% of the nodules (Figure 4.1). In contrast, the *praR* mutant (A1345) was more efficient than WT, as it occupied about 85% of the nodules (Figure 4.1).

4.2.2 The *praR* mutant attaches more strongly than WT

It had previously been observed that a *praR* mutant formed an increased biofilm ring at the air-liquid interface when grown in Y mannitol minimal medium (see Chapter 3). Therefore the increased biofilm formation of the *praR* mutant could affect root hair attachment. The attachment properties of the *praR* mutant to biotic and abiotic surfaces were studied by Anna Swiderska. *In vitro* biofilm formation by the *praR* mutant was assayed by staining the surface-attached bacteria with crystal violet after growth in microtiter plates (Figure 4.2 A). Attachment to plant root hairs was examined by quantification of the root hair-attached bacteria. This showed the *praR* mutant attached twice as efficiently to pea roots than WT (Figure 4.2 C) and indicated that the increased biofilm formation by a *praR* mutant could be the cause of the increased nodulation competitiveness.

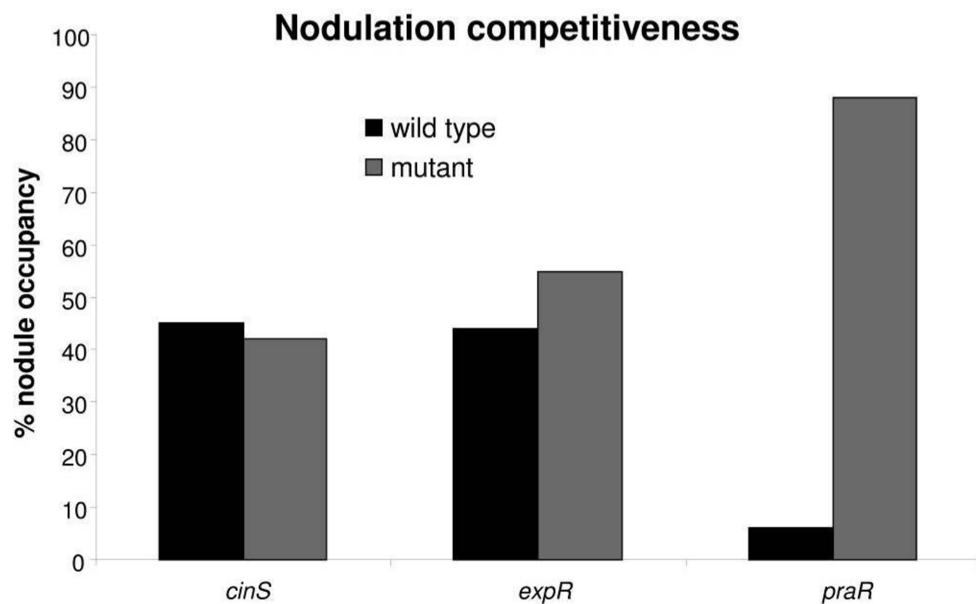
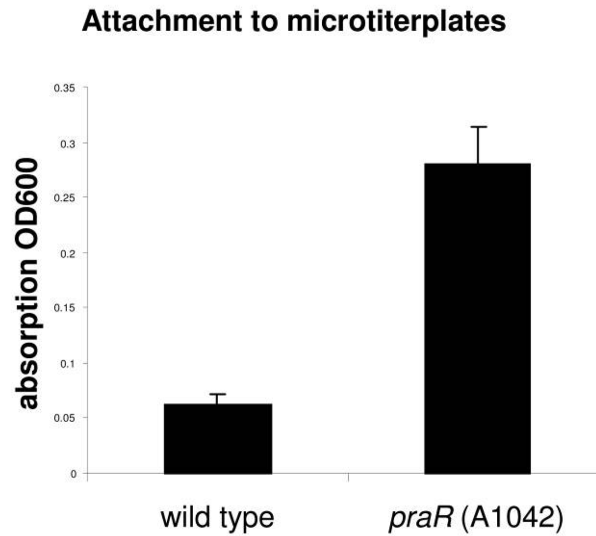


Figure 4.1: Nodulation competitiveness of the *cinS* (A1325), *expR* (A1326) and *praR* (A1345) mutants. Plants were co-inoculated with a 1:1 mixture of WT and mutant. After three weeks, the bacteria were isolated from the nodules and identified based on their antibiotic resistance. Typically about 20% of the nodules contained both kinds of bacteria, and these were omitted from the analysis

A)



B)

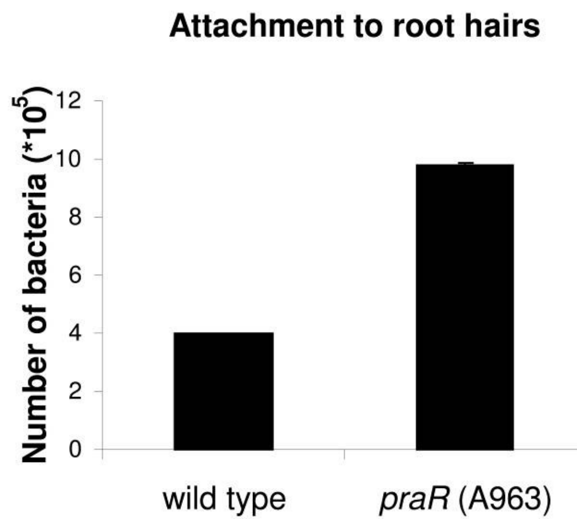


Figure 4.2: *In vivo* and *in vitro* attachment assays of the *praR* mutant. A: Absorption of surface-attached, crystalviolet-stained bacteria. B: *In vivo* attachment of bacteria to plant root hairs. Error bars represent standard deviations. Data from Anna Swiderska.

4.2.3 Role of the *rhiI/R* QS system in competitiveness

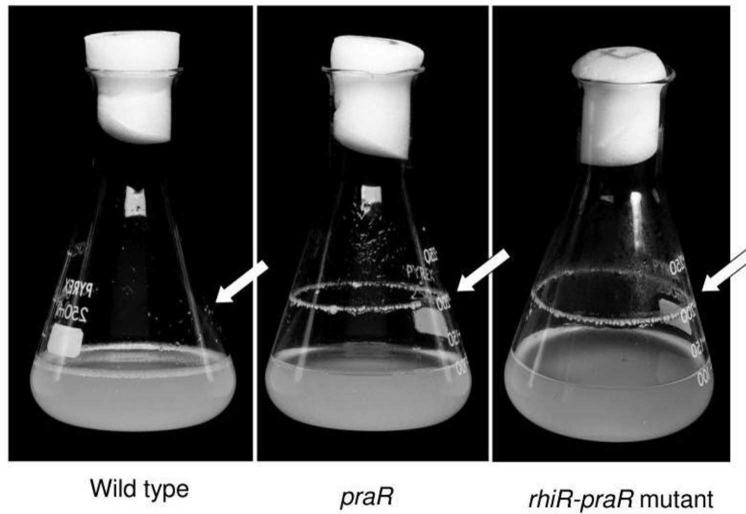
As described in Chapter 3, one gene known to be regulated by PraR is *rhiR* and increased expression of *rhiR* leads to induction of *rhiI* and the *rhiABC* genes, which are very highly expressed in the rhizosphere (Cubo *et al.*, 1992). RhiA and RhiB are cytoplasmic proteins, while RhiC is predicted to be a periplasmic protein. Based on these localisations, the RhiABC proteins are unlikely to be involved in attachment or biofilm formation. To test this, the *rhiR* mutation was transduced into the *praR* mutant and the resulting *rhiR-praR* double mutant (A1370) was examined for biofilm ring formation. The biofilm rings formed by the *rhiR-praR* double mutant were no different from those formed by the *praR* mutant (Figure 4.3 A).

It has previously been hypothesised that the RhiABC proteins are involved in metabolism of certain plant-produced metabolites (Cubo *et al.*, 1992) and this ability could give them a competitive advantage. Therefore it was tested whether the *rhiR-praR* double mutant behaved differently from the *praR* mutant in competition experiments (Figure 4.3 B). This was not the case, as the *rhiR-praR* mutant occupied 85 % of the nodules. Other PraR-regulated genes must therefore be involved in enhanced attachment and/or competitiveness and so I looked for PraR-regulated genes.

4.2.4 Identification of genes regulated by CinS, ExpR and PraR

The transcription profiles of the *cinS*, *expR* and *praR* mutants were analysed using microarrays in collaboration with Ramakrishnan Karunakaran and Philip Poole. RNA was isolated from strains that were grown in liquid AMS medium until an OD_{600} of about 0.7 (late-exponential phase). At this OD_{600} the bacteria had grown sufficiently high to induce expression of the *cinIS* operon (Figure 4.4) while sufficiently high yields of RNA could be obtained. At later stages of growth, only very low yields of RNA could be obtained, probably due to the presence of EPS in the cultures. The RNA of the mutants and WT was converted to cDNA, differentially labelled and hybridised onto two-channel microarray slides. Three independent microarray experiments of each mutant were done and after analysis in Genespring, genes with altered expression levels were selected (Supplementary tables 1-4 and Table 4.1).

A)



B)

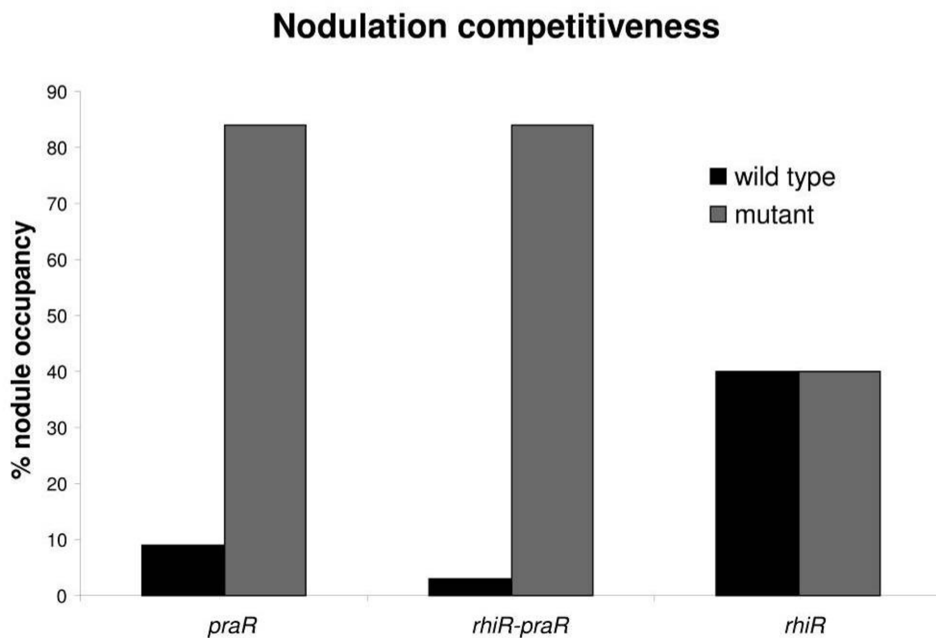


Figure 4.3: The *rhi* genes are not responsible for the increased attachment or nodulation competitiveness phenotype of a *praR* mutant. A: biofilm ring formation of WT, the *praR* mutant (A1167) and the *rhiR-praR* double mutant (A1370). Arrow indicates biofilm ring. B: competition experiments of the *praR* mutant (A1345), the *rhiR-praR* double mutant (A1370) and the *rhiR* mutant (A920) versus WT. Plants were co-inoculated with a 1:1 mixture of WT and mutant. After three weeks, the bacteria were isolated from the nodules and identified based on their antibiotic resistance. Typically about 20% of the nodules contained both kinds of bacteria, and these were omitted from the analysis.

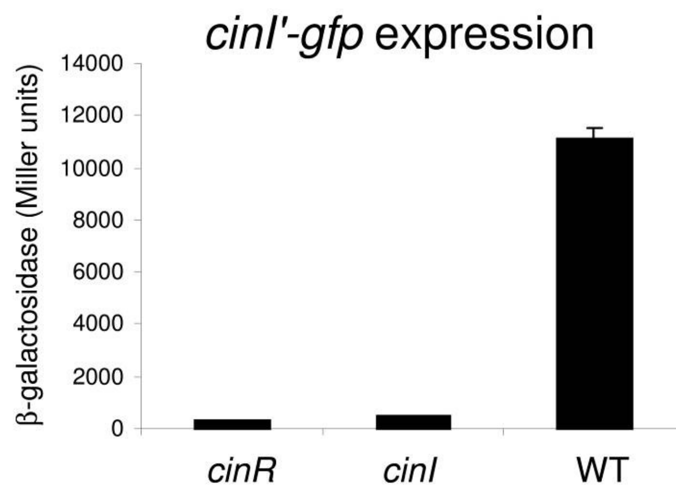


Figure 4.4: Expression of *cinI'-gfp* (pIJ9611) in microarray conditions. Strains were grown in AMS minimal medium (10 mM NH_4Cl, 30 mM glucose) until an OD_{600} of ≈ 0.7 and the expression from the *cinI* promoter was measured by the fluorescence units. Error bars represent standard deviations.

First all genes with low expression (cut-off arbitrarily chosen as an expression level of 500) and a low significance (cut-off arbitrarily chosen at the 90% confidence level) were removed from the analysis. It was clear that the mutants had only very small changes in expression when compared to WT, indicating that the conditions used for growth of the mutants were probably not ideal. Therefore the cut-off values for the fold change were chosen to be 1.5-fold for genes that were upregulated and 0.66-fold for genes that were downregulated in the mutants (Supplementary tables 1-4 and Table 4.1). Subsequently the data were cross-referenced between the different microarrays, leading to the inclusion of some data that didn't make the cut-offs in the first round: this made it possible to observe some trends in gene expression that would otherwise have been lost (these data are shaded in Supplementary tables 1-4). As this second round of analysis was probably a bit subjective, the expression of a subset of the selected genes was confirmed by promoter-*lacZ* studies.

The *cinS* and *expR* mutant microarrays showed a nearly identical pattern of gene expression and most differentially expressed genes were downregulated. The *praR* mutant microarray showed that most differentially expressed genes were upregulated (as would be expected for a repressor). The complete expression data (including raw values and p-test scores) are represented in Supplementary tables 1-4. The *lacZ* expression data for a subset of these genes are represented in Supplementary table 5. Table 4.1 summarises the results for the most interesting genes from Supplementary tables 1-5.

Four groups of genes could be distinguished:

- Group A: reduced expression in the *cinS* and *expR* mutants, with increased expression in the *praR* mutant
- Group B: increased expression in *cinS, expR* and *praR* mutants
- Group C: increased expression in the *cinS* and *expR* mutants, with reduced expression in the *praR* mutant
- Group D: altered expression in the *praR* mutant, but no observed difference in the *cinS* and *expR* mutants

Group A: reduced expression in the *cinS* and *expR* mutant, with increased expression in the *praR* mutant

Most genes that were differentially expressed in the *cinS* and *expR* mutants were downregulated and did not show increased expression in the *praR* mutant. However, when a subset of the genes in Group A were analysed with promoter-*lacZ* fusion constructs, all the tested genes showed increased expression levels in the *praR* mutant (Supplementary table 1 and Table 4.1). A possible explanation for the absence of increased expression levels of these genes in the *praR* mutant microarrays is the different time point used (late-exponential phase for microarray analysis versus stationary phase for promoter-*lacZ* studies). The observed expression changes were small, even at this later time-point, but they were reproducible and statistically significant. Based on their expression pattern, it is likely that the genes from Group A are regulated by the same mechanism as the one described for *rhiR* in Chapter 3.

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As expected from the previous results (Chapter 3), *rhiI* and the *rhiAB* genes were found in Group A. No effect on *rhiC* expression was observed, possibly due to its relatively low expression level. *rhiR* (the product of which regulates the expression of the *rhiABC* genes in response to RhiI-made AHLs) was eliminated from the microarray analysis because of its low expression level (below cut-off). It is however clear from the results in the previous chapter that *cinS, expR* and *praR* regulate the expression of *rhiR* and it is therefore likely that the changed levels of RhiR caused the changes in expression of *rhiI* and *rhiAB* (Rodelas *et al.*, 1999).

In Group A, RL3074 encodes a predicted Rap (*Rhizobium* adhesion protein) and RL3073 encodes a conserved hypothetical protein which is probably co-transcribed with RL3074. PraR bound to the RL3074 promoter (Figure 4.5 A). Other genes in Group A encode three chemotaxis proteins in the *che2* chemotaxis cluster (RL4031, RL4032 and RL4037) and an aquaporin (RL3302). This aquaporin Z is a member of the major intrinsic protein family of transporters and is predicted to be involved in the transport of glycerol (http://www.membranetransport.org/), suggesting that it could function in osmoregulation. The other genes in Group A do not have a clear function and are mostly conserved hypothetical proteins.

Group B: increased expression in the *cinS, expR* and *praR* mutants

Two genes upregulated in all three microarrays were *praR* itself and pRL100451 (Supplementary table 2 and Table 4.1). The repression of *praR* expression by PraR, CinS and ExpR has been described in Chapter 3. pRL100451 encodes a *Rhizobium*

adhesion protein similar to RL3074 (Group A). PraR bound to the pRL100451 promoter and CinS could displace PraR from the promoter (Figure 4.5 B+C), so it is likely that pRL100451 is regulated in the same way as *praR*.

Group C: increased expression in the *cinS* and *expR* mutants, with reduced expression in the *praR* mutant

Seven genes had increased expression in the *cinS* and *expR* microarrays and these had mostly unaltered expression in the *praR* mutant (Supplementary table 3). The promoter of one of them (RL4371, which is upstream of and probably cotranscribed with RL4370) was fused to *lacZ,* confirming that its expression was increased in the *cinS* and *expR* mutants (Supplementary table 5 and Table 4.1). In addition, its expression was decreased in the *praR* mutant in stationary phase. The expression pattern of RL4371 was thus the inverse of the expression pattern of the genes in Group A. The expression of RL1065, encoding a predicted chemotaxis protein, followed a similar pattern (Supplementary table 3). Possibly one of the transcriptional regulators in Group A represses the genes from Group C. Alternatively PraR could also function as an inducer on some promoters.

The pRL120625-pRL120627 operon and pRL100465 were in Group C, but could also belong to Group B. To confirm in which group they belong, their expression would require promoter-*lacZ* fusion assays in stationary phase.

Group D: altered expression in the *praR* mutant, but no observed difference in the *cinS* or *expR* mutants

Group D genes can be subdivided into these which were upregulated (Group D1) or downregulated in the *praR* mutant (Group D2). None of them showed a change in expression level in both the *cinS* and *expR* mutant, although RL3634 was upregulated in the *cinS* mutant (Supplementary table 4). One of the genes in Group D1, RL0149, encodes a transcriptional regulator that is very similar to PraR (Table 4.1). The role of this regulator will be discussed later in this chapter. None of the other genes in Group D1 and Group D2 were analysed with promoter-*lacZ* constructs. It is therefore possible that further analysis would place these genes into Groups A, B or C.

	cinS		*expR*		*praR*		
	microarray	**promoter-*lacZ***	**microarray**	**promoter-*lacZ***	**microarray**	**promoter-*lacZ***	
Group A							
pRL110060	0.55	0.73	0.69	0.73	1.36	1.35	putative transmembrane component of ABC transporter
pRL110096	0.72	0.44	0.47	0.28	1.19	1.49	conserved hypothetical protein
pRL110097	0.34	0.40	0.42	0.43	1.40	2.22	conserved hypothetical protein
RL1940	0.61	0.58	0.53	0.11	1.04	1.72	hypothetical protein
RL2169	0.59	0.70	0.60	0.72	1.31	1.36	hypothetical exported protein
RL2331	0.65	0.69	0.59	0.69	1.20	2.31	lipase/esterase
RL2423	0.62	0.66	0.46	0.63	1.05	1.49	probable peptidase/protease
RL3074	0.65	0.65	0.48	0.63	1.92	1.49	*rapC, Rhizobium* adhesion protein
RL3302	0.46	0.55	0.50	0.53	1.38	1.60	putative aquaporin Z
RL4031	0.58	n.d.	0.62	n.d.	1.08	n.d.	putative sensory transducer methyl-accepting chemotaxis protein
RL4032	0.75	n.d.	0.62	n.d.	1.65	n.d.	putative sensory transducer methyl-accepting chemotaxis protein
RL4037	0.46	n.d.	0.51	n.d.	1.21	n.d.	conserved hypothetical protein
RL4665	0.41	0.28	0.40	0.26	1.95	1.89	conserved hypothetical protein
Group B							
RL0390	1.88	2.20	2.77	2.52	4.34	2.49	*praR,* HTH transcriptional regulator
pRL100451	1.93	n.d.	1.90	n.d.	3.40	n.d.	*rapA2, Rhizobium* adhesion protein
Group C							
RL4371	1.95	1.51	2.67	1.57	0.75	0.50	conserved hypothetical protein
Group D							
RL0149	1.10	2.19	0.99	0.67	3.67	6.92	HTH transcriptional regulator

Table 4.1: Summary of microarray and promoter-*lacZ* expression data in supplementary tables 1-5. Values represent the fold change that was observed in the different assays. n.d.: not determined. For further details, raw values, p-values and standard deviations, see supplementary tables 1-5. For the microarray analysis, RNA was isolated from cultures grown in AMS minimal medium until an OD600 of ≈0.7. β-galactosidase assays were done with cultures grown for three days in AMS minimal medium.

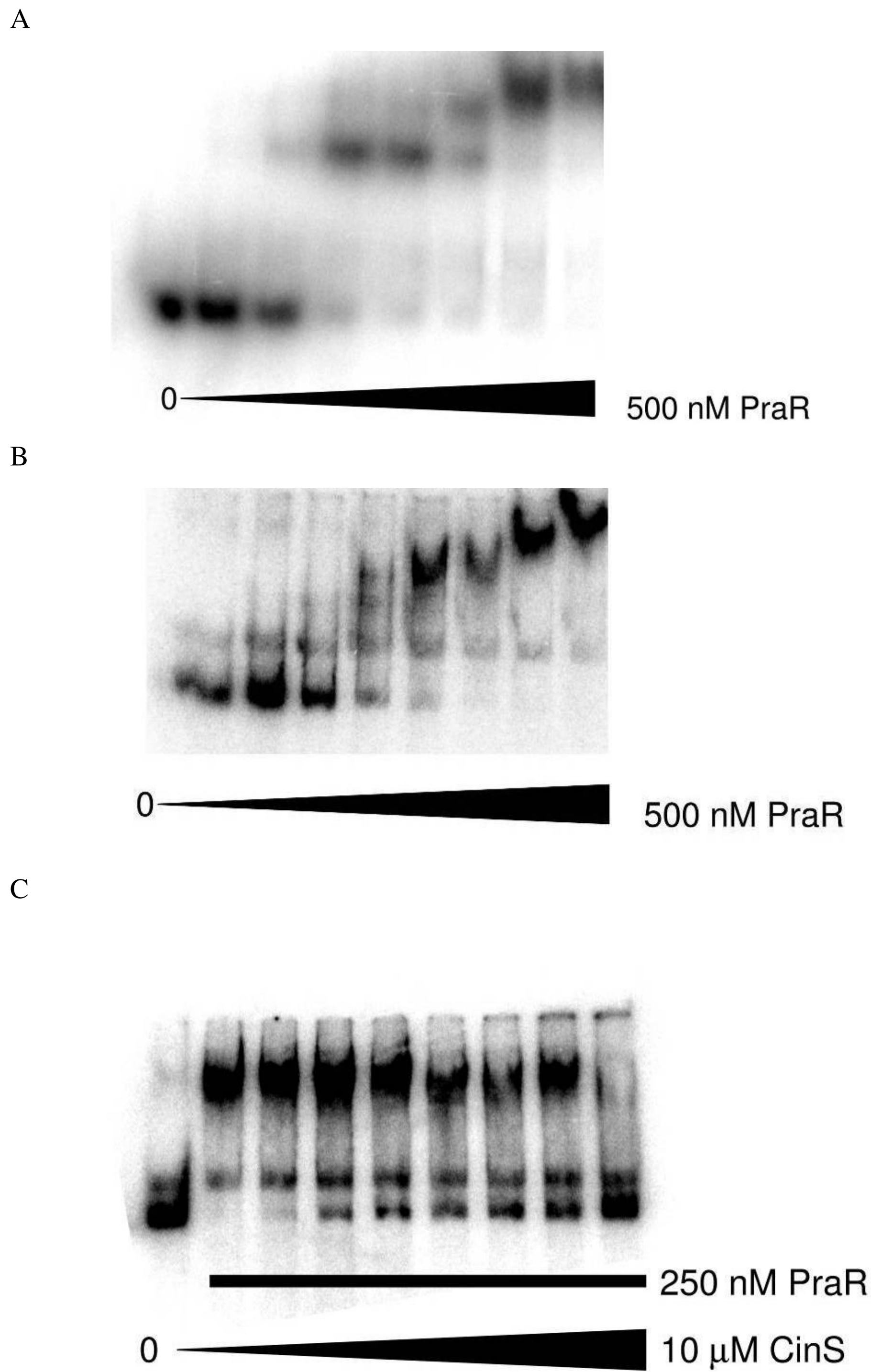


Figure 4.5: *In vitro* EMSA analysis of MBP-PraR binding to the RL3074 and pRL100451 promoters. A) The RL3074 promoter and B) the pRL100451 promoter were incubated with increasing MBP-PraR concentrations (twofold dilution series).C) the pRL100451 promoter incubated with 250 nM MBP-PraR and increasing CinS-His$_6$ concentrations (twofold dilution series).

4.2.5 Role of Rap proteins and chemotaxis in competitiveness

Role of the *Rhizobium* adhesion genes RL3074 and pRL100451

Two genes encoding *Rhizobium* adhesion proteins (Rap proteins) were identified during the microarray analysis. The expression of RL3074 (Group A) was increased in the *praR* mutant and decreased in the *cinS* and *expR* mutants, while the expression of pRL100451 (Group B) was increased in the *praR, cinS* and *expR* mutants. The increased expression of the Rap proteins could be responsible for the increased attachment properties of the *praR* mutant. Rap proteins were first identified in *R. l.* bv. *trifolii,* where RapA1 was isolated using a phage display approach as a protein that interacted with a polysaccharide on the surface of *R. l.* bv. *trifolii,* thus promoting rhizobial autoaggregation (Ausmees *et al.*, 2001). An additional role for RapA1 in the interaction with plant roots was found more recently, as overexpression of RapA1 increased attachment to root hairs (Mongiardini *et al.*, 2008) and nodulation competitiveness (Mongiardini *et al.*, 2009).

RL3074 had been annotated in the *R. l.* bv. *viciae* 3841 genome as *rap1A,* but a BLAST analysis showed that it has most similarity with the *R. l.* bv. *trifolii* RapC protein and RL3074 was therefore renamed *rapC.* pRL100451 was annotated as *rapA2* in the genome and showed most homology to *R. l.* bv. *trifolii rapA1*. In this species *rapA1* was found to be located near a cluster of genes involved in the synthesis of EPS. In *R. l.* bv. *viciae,* a gene homologous to *rapA1* was found in a similar location (RL3660), but this gene was not functional due to a frame-shift mutation. Because pRL100451 was located elsewhere in the genome, the name *rapA2* for pRL100451 was kept despite its homology to *rapA1.* In addition to *rapC* (RL3074) and *rapA2* (pRL100451), there is another gene encoding a Rap protein, RL3911, which has been annotated as *rapB*. A closer examination of the expression level of *rapB* (RL3911) in the microarrays showed no differential expression in any of the mutants and therefore this gene was not analysed further.

The role of *rapC* and *rapA2* in biofilm ring formation and nodulation competitiveness was studied by generating *rapC* and *rapA2* mutants (A1362 and A1206 respectively). The mutations were transduced into a *praR* mutant (A1345), yielding *rapC-praR* and *rapA2-praR* double mutants (A1374 and A1328). These double mutants did not show an alteration in biofilm ring formation (results not shown) or nodulation competitiveness (Figure 4.6) compared to the *praR* mutant. It would be interesting to examine the effect of mutating both *rapC* and *rapA2* in the *praR* mutant, but due to time constraints this mutant has not yet been made.

Role of motility and chemotaxis

The expression of the chemotaxis genes RL4031, RL4032 and RL4037 was reduced in the *cinS* and *expR* mutants and the expression of RL4032 and RL4031 was increased in the *praR* mutant. In several bacterial species the flagella have a structural role in biofilm formation (Barken *et al.*, 2008; Fujishige *et al.*, 2006; Kim *et al.*, 2008; Lemon *et al.*, 2007; Merritt *et al.*, 2007). In *R. leguminosarum* the flagella were not involved in root hair attachment (Smit *et al.*, 1989a) but mutations in some flagella biosynthesis genes delayed biofilm formation (Fang Xie, unpublished results). In all tested rhizobia, flagella play an important role in competitive nodulation (Ames & Bergman, 1981; Caetano-Anolles *et al.*, 1988b; Liu *et al.*, 1989), suggesting that although flagella may not be necessary for attachment, swimming towards plant-made chemoattractants is. In *R. l.* VF35SM, the chemosensory proteins *mcpB* and *mcpC* were implicated in nodulation competitiveness (Yost *et al.*, 1998). The production of flagella has to be tightly regulated however, as overproduction of flagella reduced the efficiency of nodule invasion (Gurich & Gonzalez, 2009).

To test whether flagella and motility are important for competitive nodulation, mutations were made in the flagellar hook gene *flgE* (RL0728) and the motility gene *motA* (RL0703) (Fang Xie and Michael Hynes) and transduced to *R. l.* bv. *viciae* 300 (strains A1344 and A1378 respectively). Co-inoculation experiments with these mutants and WT showed that as in other rhizobia motility is essential for nodulation competitiveness (Figure 4.7 A+B). The *flgE-praR* and *motA-praR* double mutants had a reduced nodulation competitiveness phenotype (strains A1369 and A1377), as shown in Figure 4.7 C+D.

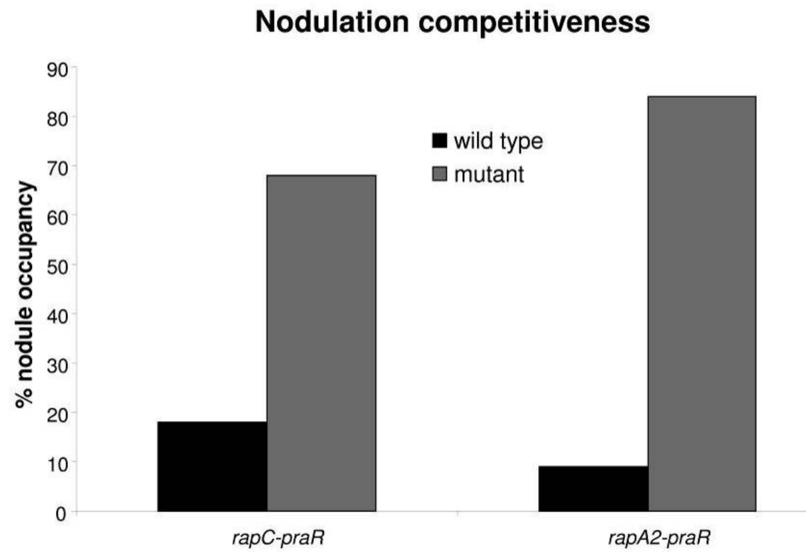


Figure 4.6: Nodulation competitiveness of the *rapC-praR* (A1374) and *rapA2-praR* (A1328) mutants. Plants were co-inoculated with a 1:1 mixture of WT and mutant. After three weeks, the bacteria were isolated from the nodules and identified based on their antibiotic resistance. Typically about 20% of the nodules contained both kinds of bacteria, and these were omitted from the analysis

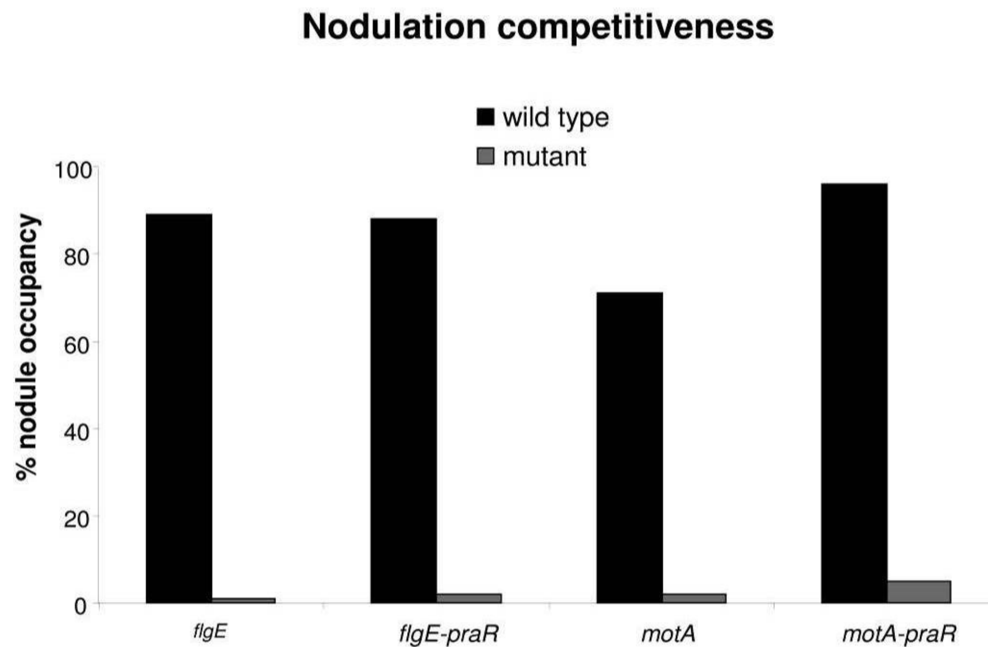


Figure 4.7: Nodulation competitiveness of the *flgE* (A1344), *motA* (A1378), *flgE-praR* (A1369) *and motA-praR* (A1377) mutants. Plants were co-inoculated with a 1:1 mixture of WT and mutant. After three weeks, the bacteria were isolated from the nodule and identified based on their antibiotic resistance. Typically about 20% of the nodules contained both kinds of bacteria, and these were omitted from the analysis

One possibility was that the increased expression of the *che2* chemotaxis genes in the *praR* mutant caused increased nodulation competitiveness, by increasing the ability of the rhizobia to move towards the plant roots. The role of the two major chemotaxis clusters *che1* and *che2* in *R. l.* bv. *viciae* 3841 had already been studied (Miller *et al.*, 2007). Both clusters are apparently transcribed in an operon (Figure 4.8). The *che1* cluster (RL0685-RL0695) was centrally located in the cell, while the *che2* cluster (RL4037-RL4028) was located at the cell pole, embedded in the cell membrane (Miller *et al.*, 2007). These localisations suggest that the *che1* cluster senses the general nutrient status of the cell, while the *che2* cluster is involved in specific movement towards chemoattractants (for example plant-made metabolites). The chemotaxis response of *che1* and *che2* mutants to different molecules as chemoattractants was tested (Miller *et al.*, 2007), but surprisingly for all tested molecules the *che1* and not the *che2* cluster was responsible for chemotaxis. Both mutants were tested for their role in competitive nodulation but only the *che1* mutant had an effect on nodulation competitiveness (Miller *et al.*, 2007). Considering these results, it was decided that further investigations of the role of the *che2* cluster and RL4031, RL4032 and RL4037 were not worth pursuing.

4.2.6 Analysis of proteins secreted by the *praR* mutant

Analysis of protein secretion in the *praR* mutant

As the microarray analysis was unlikely to identify all PraR-regulated genes, other approaches were taken. Proteins secreted by a *praR* mutant (A1167) were compared with proteins secreted by WT (Figure 4.9, by Anna Swiderska) and two proteins were identified that were more abundant in the *praR* mutant than in WT. These proteins were identified using Maldi-ToF as the cadherin-like proteins encoded by RL2961 (*cadA*) and pRL100309 (*cadB*), which had been shown to be secreted by the Type 1-secretion system PrsDE (Krehenbrink & Downie, 2008). Differential expression of RL2961 and pRL100309 had not been found in the microarray analysis, but based on these results the microarray expression data of RL2961 and pRL100309 were re-examined. There was a twofold induction of pRL100309 but because of a high p-value (p=0.12) this gene had been eliminated from the analysis. No change in expression of this gene was seen in the *cinS* and *expR* microarrays. RL2961 did not show any differential expression in the *praR, expR* and *cinS* mutants.

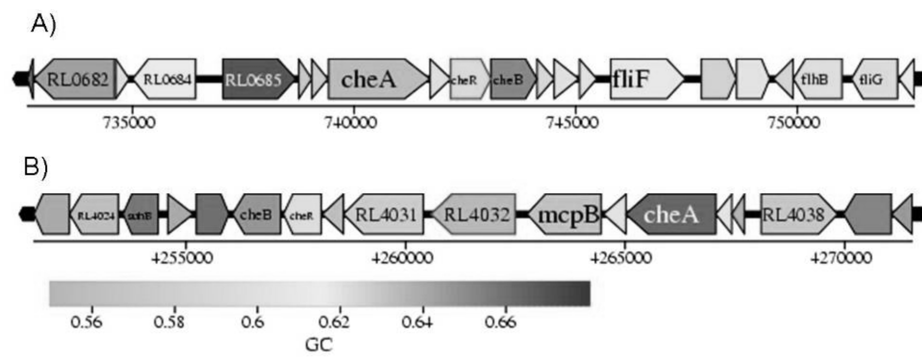
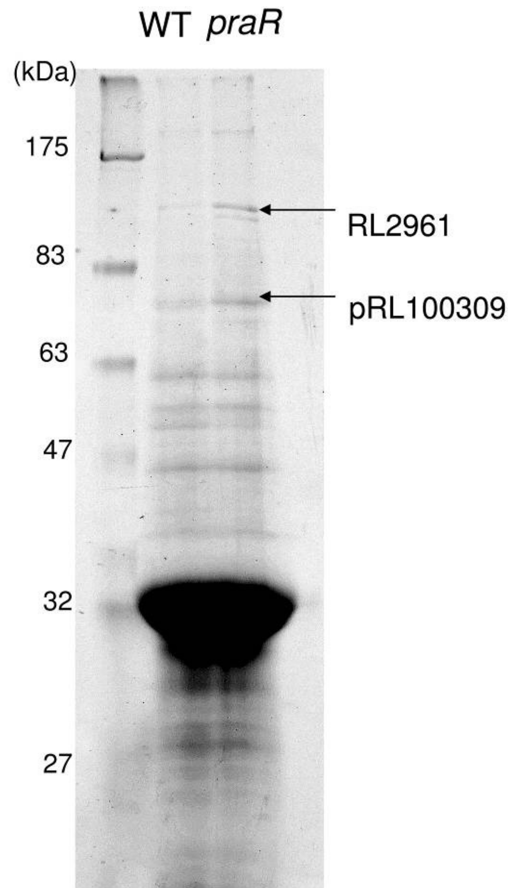


Figure 4.8 : Chemotaxis clusters in *R. l.* bv. *viciae* strain 3841. Genomic localisations of A) the *che1* cluster and B) the *che2* cluster.



	Apparent Mw (kDa)	Theoretical Mw (kDa)
RL2961 (CadA)	100	95.0
pRL100309 (CadB)	75	60.7

Figure 4.9: Analysis of the secreted proteins of the *praR* mutant. Proteins were identified by MALDI-ToF. Data from Anna Swiderska.

Role of the cadherin-like proteins CadA (RL2961) and CadB (pRL100309)
Cadherins are cell-adhesion proteins and have mainly been studied in multicellular eukaryotes (Ivanov *et al.*, 2001), although they have also been found in unicellular organisms (Abedin & King, 2008; Fraiberg *et al.*). They typically contain extracellular Ca^{2+}-binding domains that adhere to each other, causing autoaggregation. CadA and CadB both contain two nearly identical C-terminal cadherin domains. A BLAST analysis showed that proteins similar to CadA and CadB can be found in different biovars of *R. leguminosarum* and in *R. etli* species, but their role has not been studied. Because of their role in adherence in eukaryotic cells, it seemed possible that CadA and CadB were involved in rhizobial attachment to root hairs.

cadA and *cadB* mutants were made (A1263 and A1254), and the mutations were transduced into a *praR* mutant. To make sure that CadA and CadB were not functioning redundantly, a *cadA*-*cadB*-*praR* triple mutant was generated as well (A1383). This mutant showed no difference in biofilm ring formation compared to the *praR* mutant (results not shown). The nodulation competitiveness of the triple mutant was also examined and no difference with the *praR* mutant was found (Figure 4.10).

4.2.7 Exopolysaccharide production by the *praR* mutant is altered

An important factor in bacterial attachment to root hairs is the presence of polysaccharides and these have been shown to be involved in nodulation competitiveness in several species (see 4.1 Introduction). In *R. l.* bv. *viciae* 3841 the acidic EPS is required for attachment to root hairs, biofilm formation and normal nodulation, while glucomannan is involved in polar attachment to root hairs and competitive nodulation (Laus *et al.*, 2005; Williams *et al.*, 2008). A third polysaccharide, cellulose, is involved in the formation of a biofilm cap on the root hairs (Laus *et al.*, 2005; Williams *et al.*, 2008). The aim of this section was to determine if the *praR* mutant was affected in polysaccharide production or processing.

Role of cellulose

Cellulose production by the *praR, cinS* and *expR* mutants was analysed by growing the bacteria on Congo-Red-containing medium, which stains the cellulose fibrils in a red colour (Figure 4.11). This showed that the *praR* mutant produced more cellulose than WT, comparable to the levels of cellulose produced by the cellulose overproducing mutant A1004. Cellulose levels in the *expR* and *cinS* mutants were as in WT. Although cellulose production was altered in a *praR* mutant, its role in nodulation competitiveness was not studied any further. Previous studies had shown that in *R. l.* bv. *viciae* 3841 cellulose did not have a symbiotic function despite its involvement in the formation of a rhizobial biofilm cap on root hairs (Laus *et al.*, 2005; Williams *et al.*, 2008).

PraR and CinS repress the expression of *rosR*

In many rhizobia, the production of EPS is under the control of the transcriptional regulator RosR (Bittinger & Handelsman, 2000; Janczarek & Skorupska, 2007) and this regulator has been shown to affect nodulation competitiveness (Bittinger *et al.*, 1997; Janczarek *et al.*, 2009). The role of *rosR* in *R. l.* bv. *viciae* 3841 is less well studied, because a strain 3841 *rosR* mutant was found to be highly unstable (Alan Williams, unpublished results). It is however clear that RosR plays an important role in the regulation of EPS production in *R. l.* bv. *viciae* 3841, as the mutant showed a very dry phenotype, which pointed towards a lack of acidic EPS production (Alan Williams, unpublished results). *rosR'-lacZ* expression was analysed in the *praR, cinS* and *expR* mutants, showing that *rosR* expression was strongly upregulated in the *praR* and *cinS* mutants, although no difference in expression was seen in the *expR* mutant (Figure 4.12 A). The regulatory effect of PraR on *rosR* was direct, as MBP-PraR bound to the *rosR* promoter (Figure 4.12B). Because of the difficulty with the stability of the mutant, the *rosR* mutation was not transduced into the *praR* mutant for further analysis.

Due to time constraints the effect of mutation of *praR* on the production of acidic EPS and glucomannan production was not further tested. The *praR* mutant colonies looked like the WT suggesting that if there were changes in the levels of acidic EPS, this was not visible at the macroscopic level. The *gmsA* mutation (which abolishes production of glucomannan) was transduced into the *praR* mutant (yielding A1367). In previous studies it was shown that mutation of *gmsA* (A1208) greatly decreased nodulation competitiveness (Williams *et al.*, 2008), and this phenotype was shown to be retained

in the *praR* mutant (Figure 4.13). It remains to be determined whether mutation of *praR* affects the expression of *gmsA,* and if so whether this is due to a direct or indirect effect (for example via RosR). No differences in expression were seen in the microarray analysis, but this would not be expected as in AMS medium polysaccharide production is at a low level.

PraR represses the expression of the extracellular glycanase *plyB*

Previous work showed that ExpR and CinS regulate the expression of the extracellular glycanase *plyB* (Edwards *et al.*, 2009). In strain 3841 PlyB (RL3023) is 91% identical to PlyB from strain 8401. In addition, the 235 bp upstream of both genes were 90% identical. Analysis of *plyB'-lacZ* expression showed that its expression is increased in the *praR* mutant (Figure 1.14). No differential expression of RL3023 was observed in the microarray analysis, but as explained previously this is probably due to the growth conditions used. Microarray analysis was done in exponential growth in AMS minimal medium, while the studies were done in stationary phase in Y mannitol minimal medium.

To examine whether *plyB* is responsible for the increased attachment phenotypes of the *praR* mutant, the gene was mutated (A1365) and introduced into the *praR* mutant (giving A1372). The *plyB-praR* double mutant had reduced biofilm ring formation compared to the *praR* mutant, showing levels similar to WT (results not shown). This fits with the results obtained in strain 8401, where a *plyB* mutant also had a greatly reduced biofilm ring (Edwards *et al.*, 2009). Although *plyB* affected biofilm ring formation in the *praR* mutant, mutation of *plyB* did not affect nodulation competitiveness of the *praR* mutant as the *plyB-praR* double mutant still occupied most of the nodules (Figure 4.15).

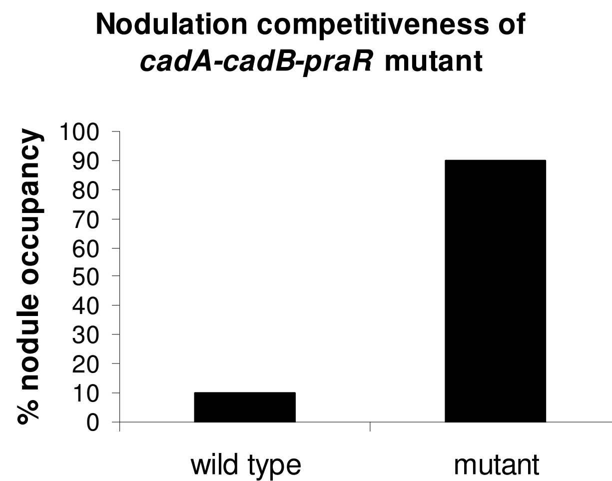


Figure 4.10: Nodulation competitiveness of *cadA-cadB-praR* mutant. Plants were co-inoculated with a 1:1 mixture of WT and mutant. After three weeks, the bacteria were isolated from the nodule and identified based on their antibiotic resistance. Typically about 20% of the nodules contained both kinds of bacteria, and these were omitted from the analysis

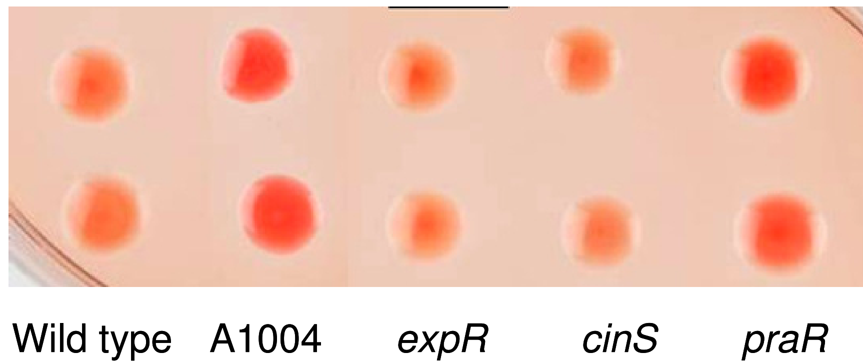
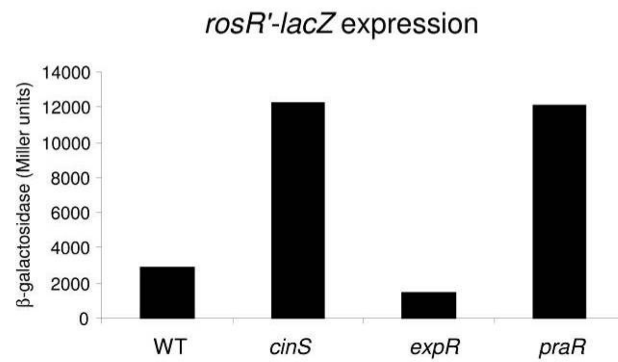


Figure 4.11: Analysis of cellulose production by the *cinS, expR* and *praR* mutants. Colonies were grown on Y mannitol plates containing congo red. A1004: positive control (spontaneous mutant that overproduces cellulose, Martin Krehenbrink).

A)



B)

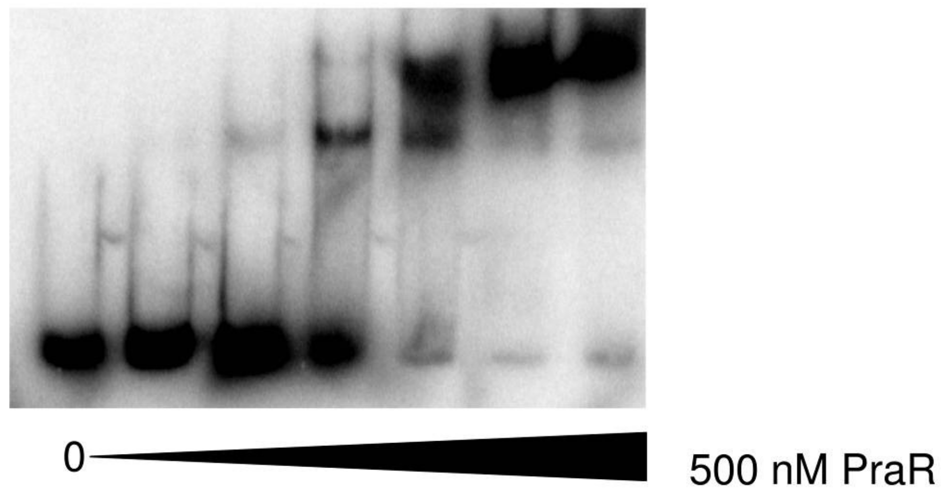


Figure 4.12: *rosR* is regulated by *praR* and *cinS*.. Expression of *rosR'-lacZ* (pIJ11196) was measured by β-galactosidase activity after three days of growth in Y mannitol minimal medium. Strains used were WT, *cinS* mutant (A1245), *expR* mutant (A1246) and *praR* mutant (A1167). B: EMSA analysis of MBP-PraR binding to the *rosR* promoter. The *rosR* promoter was incubated with increasing concentrations of MBP-PraR (twofold dilution series).

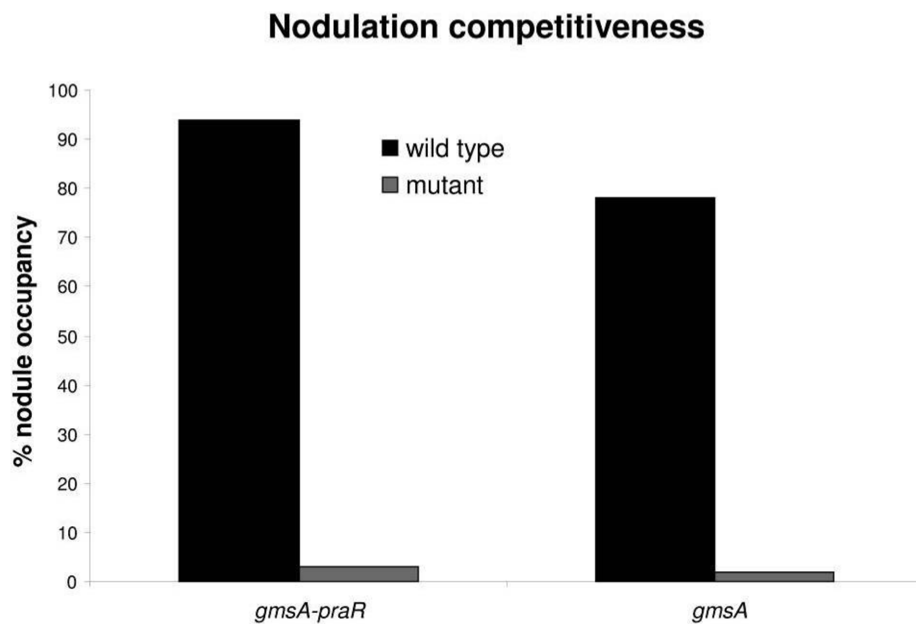


Figure 4.13: Nodulation competitiveness of *gmsA-praR* mutant (A1367). Plants were co-inoculated with a 1:1 mixture of WT and mutant. After three weeks, the bacteria were isolated from the nodules and identified based on their antibiotic resistance. Typically about 20% of the nodules contained both kinds of bacteria, and these were omitted from the analysis

4.2.8 RL0149 encodes a regulator similar to PraR

The microarray analysis revealed the most strongly induced gene in the *praR* mutant to be RL0149 and its product is homologous to PraR (38% identical and 55% similar), especially at the N-terminal DNA-binding domain (Figure 4.16). These two repressors, one of which strongly repressed the expression of the other, reminded of the Cro/CI system in *E. coli* bacteriophage lambda. Bacteriophage lambda uses the Cro and CI repressors to switch between a lytic and a lysogenic lifestyle (see review by Oppenheim *et al.*, 2005). To examine whether the transcriptional regulator encoded by RL0149 is involved in PraR gene regulation, the role of this regulator was investigated in more detail.

Expression studies of RL0149

The expression of RL0149'-*lacZ* was measured in the *cinS, expR* and *praR* mutants. This confirmed that RL0149 expression was increased in the *praR* mutant and also showed that its expression was increased in the *cinS* mutant (A1245), but not in the *expR* mutant (Figure 4.17 A). Introduction of the *expR* mutation into the *cinS* and *praR* mutant backgrounds did not affect the increased expression of RL0149. In the *cinS-praR* mutant expression of RL0149 was at the same level as in the *praR* mutant (Figure 4.17 A). The regulatory effect of PraR on the expression of RL0149 was shown to be direct as MBP-PraR bound to the RL0149 promoter (Figure 4.17 B).

RL0149 did not affect expression of PraR regulated genes

To test if the RL0149 regulator was capable of altering the expression of *praR,* a RL0149 mutant (A1340) was generated. The expression of *praR* and *rhiR* was measured in this mutant (Figure 4.18 A + B), but no difference in expression between WT and the RL0149 mutant was observed.

RL0149 did not interact with CinS, ExpR or PraR

To test if the RL0149 protein interacted with CinS, ExpR or PraR the bacterial two hybrid system developed by Karimova *et al.* (1998) was used. RL0149 was fused to T18 and T25 and analysed together with CinS-T18, T25-CinS, ExpR-T18, PraR-T18 and T25-PraR. RL0149 formed a multimer (β-galactosidase activity: 8526 ± 401 Miller units), but no interactions between RL0149 and CinS, PraR or ExpR were found (Table 4.2). Taken together with the fact that the RL0149 regulator did not regulate the expression of *praR* and *rhiR*, this probably indicated that despite the

similarities between the RL0149 regulator and PraR, it does not contribute to the regulatory mechanism that is used by PraR and CinS.

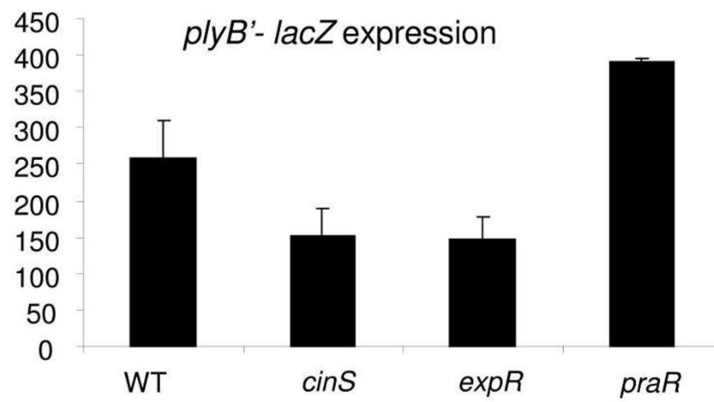


Figure 4.14: *plyB* expression in strain 3841 WT, *cinS* (A1245), *expR* (A1246) and *praR* (A1167) mutants. Expression of *plyB'-lacZ* (pIJ9252) was measured by β-galactosidase activity after three days of growth in Y mannitol minimal medium. Error bars indicate standard deviations.

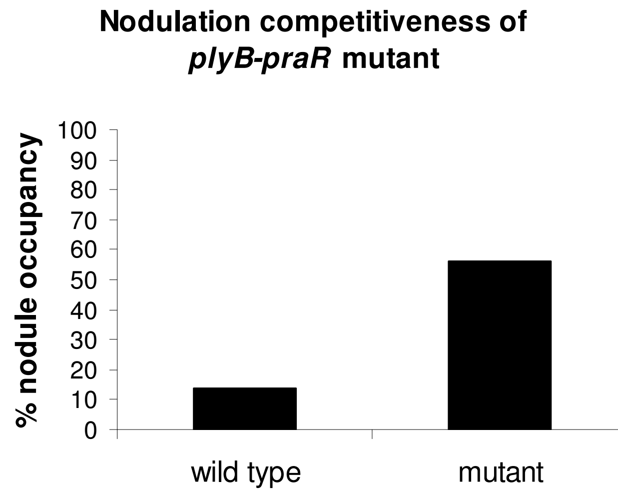


Figure 4.15: Nodulation competitiveness of *gmsA-praR* mutant (A1367). Plants were co-inoculated with a 1:1 mixture of WT and mutant. After three weeks, the bacteria were isolated from the nodule and identified based on their antibiotic resistance. In this experiment 29% of the nodules contained both kinds of bacteria, and these were omitted from the analysis

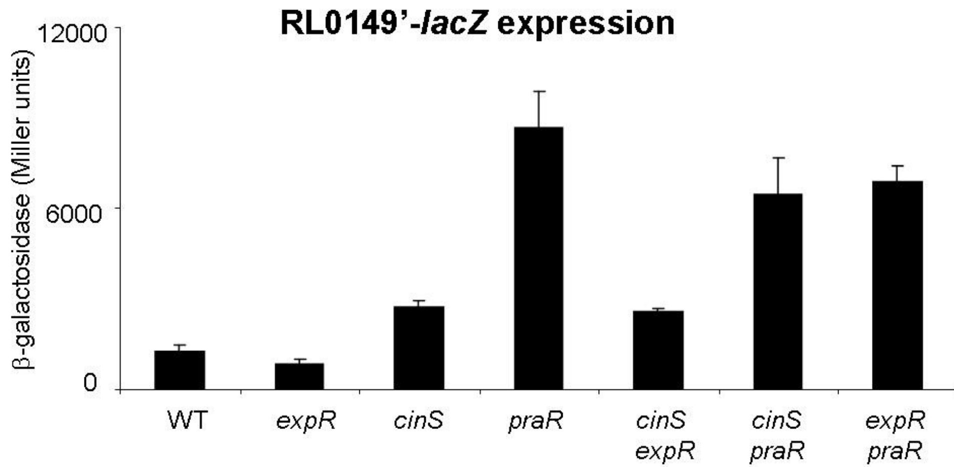
```
PraR            MIENKKKPNPIDIHVGSRIRLRRTMLGMSQEKLGESLGITFQQIQKYEKGTNRVGASRLQ 60
RL0149          ------VPDPVDIIVGRNVRQFRALRRVSQLELGEALGLTFQQIQKYEKGANRVSASKLH 54
                       *:*:** ** .:*  *::  :** :***:**:***********:***.**:*:

PraR            NISNILNVPVSFFFEDAPGEHSSAGGGMEASSSNYVVDFLSSSEGLQLNRAFVKISDPKV 120
RL0149          QIAVFLDVDISALFEGAG--MSPFGSRVELSPDAYALALSYD----KLN----SPAGKEA 104
                :*: :*:* :* :**.*    *. *. :* *.. *.: :  .    :**    . :. :.

PraR            RRKVVELVKALAAEADAD 138
RL0149          VKTIVTLMTGESAETTA- 121
                 :.:* *:.. :**: *
```

Figure 4.16: Alignment of RL0149 and PraR. Alignment was done using ClustalW. The predicted DNA binding domain is marked in bold. All predicted amino acids are shown. '*': identical residues, ':': conserved substitution, '.': semi-conserved substitution

A)



B)

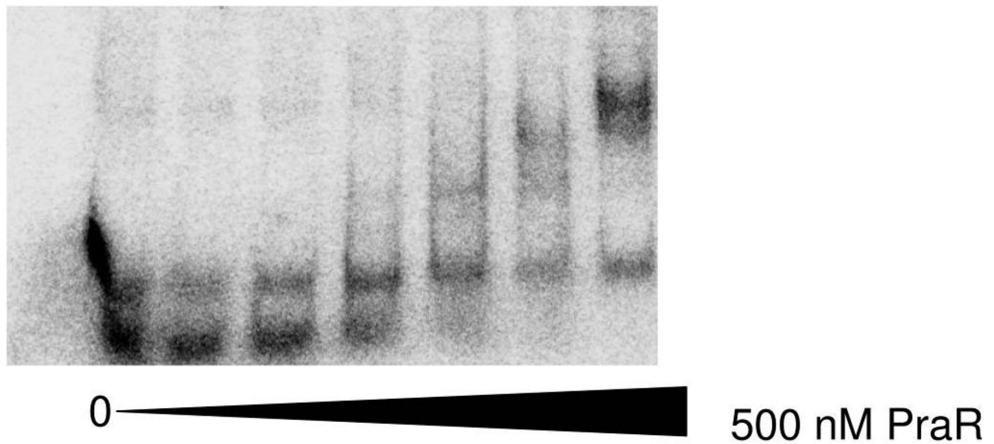
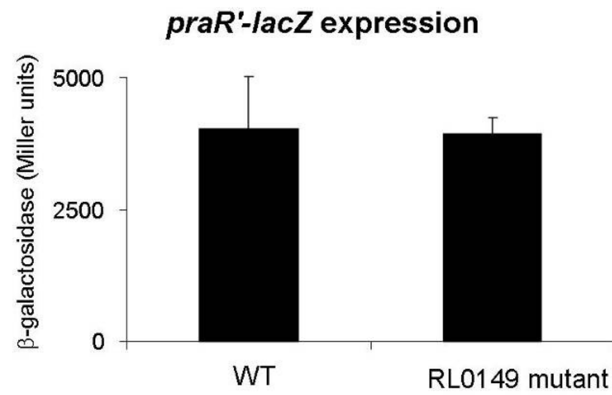


Figure 4.17: Regulation of RL0149 by PraR. A: Expression of RL0149 was measured by measuring β-galactosidase activities of a RL0149'-*lacZ* promoter fusion construct (pIJ11114) in WT, *expR* (A1246), *cinS* (A1245), *praR* (A1167), *cinS expR* (A1232), *cinS praR* (A1312) and *expR praR* (A1313) mutants. B: the RL0149 promoter was incubated with increasing [MBP-PraR] (twofold dilution series).

A)



B)

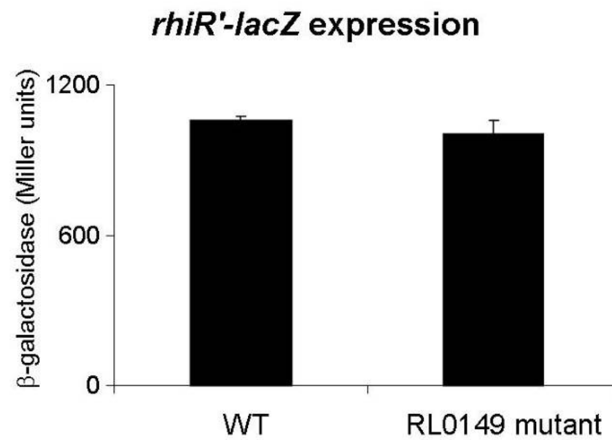


Figure 4.18: Expression of *rhiR* and *praR* in the RL0149 mutant. A) Expression of *praR'-lacZ* (pIJ11112) in WT and RL0149 mutant (A1340). B) Expression of *rhiR'-lacZ* (pIJ9104) in WT and RL0149 mutant (A1340). Expression was measured by the β-galactosidase activity.

	T25-RL0149 XP458	**RL0149-T18**
CinS	218 ± 5	117 ± 3
ExpR	248 ± 26	n.d.
PraR	209 ± 11	100 ± 7
RL0149	8526 ± 401	8526 ± 401
EV	165 ± 73	124 ± 2

Table 4.2: Bacterial two hybrid analysis of interactions between RL0149 and CinS, ExpR, PraR. Interactions between CinS-T18 (pIJ11159), ExpR-T18 (pIJ9716), PraR-T18 (pIJ11132), RL0149-T18 (pIJ11160) pT18, T25-CinS (pIJ9717), T25-PraR (pIJ11133), T25-RL0149 (pIJ11151) and pT25 were examined by bacterial two hybrid analysis. Positive interactions result in the activation of β-galactosidase activity. n.d.: not determined

4.3 Discussion

Mutation of *praR* increased nodulation competitiveness in *R. l.* bv. *viciae* strain 3841 and also increased biofilm rings and attachment to pea root hairs. This enhanced root hair attachment is likely to be the cause of the nodulation phenotype. To investigate which factors determine this, the PraR regulon was investigated. This revealed that in a *praR* mutant there is increased expression of several genes whose products are likely to be involved in attachment. Among these were RapC and RapA2 (*Rhizobium* adhesion proteins), CadA and CadB (cadherin autoaggregation proteins) and proteins involved in polysaccharide production and processing (RosR and PlyB). The *Rhizobium* adherence domains that are present in Rap proteins are also present in the extracellular glycanase PlyB, which could indicate that PlyB uses these domains to bind to EPS to activate its catalytic domain (Finnie *et al.*, 1998). PlyB is active against EPS from *R. leguminosarum* and *R. etli* (Zorreguieta *et al.*, 2000).

Other tested genes that had increased expression were not involved in attachment but could be involved in life in the rhizosphere and infection. For example, the *rhiABC* genes are very highly expressed in the rhizosphere but their function is currently unknown (Cubo *et al.*, 1992). Aquaporin Z (RL3302) is thought to be involved in glycerol uptake and therefore might help to cope with osmotic stresses that are encountered upon infection. Three genes from the *che2* chemotaxis cluster were found to be upregulated. Although previous work had shown that this cluster is not involved in competitive nodulation or chemotaxis to any of the components tested in this study (Miller *et al.*, 2007), it is interesting to note that other work in our lab has shown that a *che2* mutant formed an abnormal biofilm *in vitro* (Williams, 2006). It is possible that the *che2* cluster is responsible for the recognition of rhizobial proteins, thus contributing to the formation of a normal biofilm and possibly root hair attachment. The effects of the *rhi* genes, *cadA, cadB, rapC, rapA2* and *plyB* were examined for their role in nodulation competitiveness, but not one of these genes was shown to be responsible for this on its own. Interestingly, these genes are highly conserved in different biovars of *R. leguminosarum* and the closely related *R. etli,* but not in other rhizobial species. PraR could therefore function to optimise interactions between these rhizobia and their hosts.

Mutation of *praR* increased the expression of *rosR,* which is conserved in many rhizobia and involved in the regulation of polysaccharide production (Bahlawane *et*

al., 2008; Bertram-Drogatz *et al.*, 1998; Bittinger & Handelsman, 2000; Janczarek & Skorupska, 2007). Although a clear increase of cellulose production in the *praR* mutant could be demonstrated, the effect on acidic EPS and glucomannan production will require further study. Despite the fact that *S. meliloti* ExpR and *R. leguminosarum* ExpR seem to function by a different regulatory mechanism, they seem to be involved in regulating similar cellular processes in both species. Both regulators were shown to regulate genes involved in polysaccharide production and chemotaxis. *S. meliloti* ExpR is also involved in the regulation of nitrogen fixation, metabolism and metal transport (Hoang *et al.*, 2004), but this does not appear to be the case in *R. leguminosarum.*

The observation that a *praR* mutant infects peas more efficiently stresses the importance of the regulation of *praR* repression in the rhizosphere. Microarray analysis showed that in the rhizospere expression of *praR* is reduced, while expression of the *cinIS* operon is increased (Ramakrishnanan Karunakaran, unpublished results). PraR, CinS and ExpR all repress *praR* expression and CinS functions as a PraR antirepressor. This indicated that CinS and ExpR also play some role in the regulation of nodulation competitiveness, although in the conditions used, no change in nodulation competitiveness was seen for the *cinS* or *expR* mutants. From the microarray analysis it was clear that the regulatory effects of CinS, ExpR and PraR are very mild, at least under the conditions used. These moderate effects could however be of crucial importance in the rhizosphere. It is likely that mutation of *praR* also has some disadvantages for the rhizobia, as otherwise it would be expected that *R. l.* bv. *viciae* strain 3841 isolates would be dominated by *praR* mutants. Since this is not the case, *praR* mutation could reduce the survival rate of the rhizobia in the soil.

The most strongly upregulated gene in the *praR* mutant was RL0149, which encodes a lambda-repressor like transcriptional regulator. Based on transcriptional studies and bacterial two hybrid analysis, no involvement of the RL0149 regulator in PraR-mediated gene regulation could be demonstrated. The expression pattern of RL0149 in the *cinS, praR* and *expR* mutants was different from any of the patterns observed in Chapter 3, as it was increased in the *cinS* and *praR* mutants, but unaltered in the *expR* mutant. A similar expression pattern was observed for *rosR* expression and for both RL0149 and *rosR* it was shown to be a direct regulatory effect of PraR on the promoters of these genes. The fact that RL0149 and *rosR* are both repressed by CinS and PraR indicated that CinS can not function as an anti-repressor for PraR on this promoter. This is similar to the situation for *praR* expression. For RL0149 and *rosR*,

ExpR is unlikely to be the factor that is stopping CinS from acting as an anti-repressor, as no regulatory effect of ExpR on RL0149 expression was found. Possibly another regulator fulfils the role of ExpR on the *rosR* and RL0149 promoters.

The gene that was most strongly repressed in the *praR* mutant was RL3670 (see Supplementary table 4), which encodes a protein of 80 amino acids with no similarity to any other proteins in the databases. Considering the role of CinS in the antirepression of PraR, and the opposing effects of mutation of *praR* on the expression of RL3670 and RL0149, it might be possible that RL3670 encodes an antirepressor of RL0149.

4.4 Summary

- A *praR* mutant displayed increased nodulation competitiveness when co-inoculated with WT *R. l.* bv. *viciae* strain 3841
- A *praR* mutant attached more strongly to abiotic surfaces and root hairs
- Microarray analysis of the *praR, cinS* and *expR* mutant revealed four classes of genes that were differentially expressed.
    - Group A: ↑ in *praR* mutant, ↓ in *cinS* and *expR* mutants
    - Group B: ↑ in *praR, cinS* and *expR* mutants
    - Group C: ↓ in *praR* mutant, ↑ in *cinS* and *expR* mutants
    - Group D: altered in *praR* mutant, unaltered in *cinS* and *epxR* mutants
- The microarray analysis did not show all regulatory effects of PraR.
- Mutation of *praR* increased the expression of proteins involved in attachment to root hairs.
    - ↑ *Rhizobium* adhesion proteins RapC and RapA2
    - ↑ cadherin proteins CadA and CadB
    - ↑ cellulose production
    - ↑ global EPS regulator RosR
    - ↑ glycanase *plyB*
- Not one of the tested genes (*rhi* genes, *rapC, rapA2, cadA, cadB* and *plyB*) was responsible for the nodulation phenotype.
- It remains to be identified whether PraR affects the production of acidic EPS and glucomannan.
- RL0149 is a transcriptional regulator that is very similar to PraR.
- The expression pattern of RL0149 was peculiar:
    - RL0149 was very strongly upregulated in the *praR* mutant
    - RL0149 was upregulated in the *cinS* mutant, but its expression was unaltered in the *expR* mutant.
- The RL0149 regulator is probably not involved in gene regulation by PraR, CinS or ExpR:
    - Mutation of RL0149 did not affect expression of *rhiR* or *praR.*
    - The RL0149 regulator did not interact with CinS, PraR or ExpR, although it was shown to form multimers.

Chapter 5: Materials and methods

5.1 Microbiological methods

Media and growth conditions

R. leguminosarum strains were grown at 28˚C in tryptone-yeast (TY) medium (Beringer, 1974), Y minimal medium (Sherwood, 1970) containing mannitol (0,2 % w/v) or acid minimal salts medium (AMS) (Poole *et al.*, 1994) containing 10 mM NH_4Cl and 30 mM glucose. Yeast extract mannitol (YEM) medium was used for swarming assays (1g yeast extract, 10 g mannitol, 0.5 g K_2HOP_4, 0.4 g $MgSO_4.7H_2O$, 0.1 g NaCl, 2 g $CaCO_3$, adjust to pH7, distilled water up to 1 l). *E. coli* was grown at 37˚C in L medium (Sambrook *et al.*, 2001) or SOB medium (Difco Bacto tryptone 20 g, Difco Bacto yeast extract 5 g, NaCl 0.5 g, KCl 0.186g, adjust to pH7 with 10M NaOH, distilled water up to 1 l). Liquid cultures were grown in a rotary shaker at 250 rpm and solid medium contained 1% agar unless otherwise specified.

Antibiotics were added as appropriate to maintain selection for plasmids and to select for transconjugants and transductants: apramycin (Apra): 50 μg/ml , ampicillin (Amp) 400 μg/ml, gentamicin (Gm): 20 μg/ml, kanamycin (Km): 50 μg/ml, lividomycin (Liv): 20 μg/ml, neomycin (Neo): 50 μg/ml, spectinomycin (Spec): 200 μg/ml, streptomycin (Strep): 400 μg/ml, tetracycline (Tet): 5 μg/ml for TY or L, 2 μg/ml for minimal medium. For blue-white screening of *E. coli*, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl β-D-1-thiogalactopyranoside) were added to LB agar (40 μg/ml).

Triparental matings

Plasmids were mobilised into *Rhizobium* strains by triparental mating, using pRK2013 as a helper plasmid (Figurski & Helinski, 1979). Donor, helper and recipient strains were mixed onto TY agar plates and incubated at 28 ºC for 16h, after which they were replica-plated onto selective plates. The replica plates were incubated for another 3 days at 28 ºC.

Transductions

Phage preparations were made using strains grown on a TY slope for 3 days, which were resuspended in 3 ml of sterile water. A serial dilution of phage RL38 (Buchanan-Wollaston, 1979) was made, and 100 µl of this was added to 100 µl of the bacteria. The mixture was added to 5 ml of soft TY agar (0.75%), spread onto a TY plate and incubated at 28 ºC for 24h. At this stage plaques were visible and 10 ml of sterile water was added to a plate with near confluent plaques to elute the phage. After three hours the phage suspension was removed from the plate by a syringe, filter sterilised and 35 µl of chloroform was added to prevent microbial contamination. The phage was stored at 4 ºC.

For transductions, recipient strains were grown on a TY slope for 3 days and resuspended in 3 ml of sterile 160 mM NaCl solution. The bacteria were centrifuged (16 000 g, 1 min), washed with 500 µl 160 mM NaCl solution, centrifuged (16 000 g, 1 min) and resuspended in 1 ml of water. 1 µl, 10 µl and 100 µl of phage was added to 100 µl of the bacteria and the mixture was incubated for 1 h at room temperature (RT) before plating onto selective medium. Transductants were restreaked several times to remove remaining phage.

Biofilm ring assays

Strains were pregrown in TY medium (28 ºC) without antibiotics, transferred to Y mannitol minimal medium without antibiotics and grown for five days shaken at 250 rounds per minute (rpm) at 28 ºC.

Growth curves

Strains were pregrown for three days in TY medium (28 ºC) and transferred to fresh medium (1/100). Strains were then incubated shaking in a Tecan (GENios) device, while measurements of OD_{600} were taken each 30 min.

β-galactosidase activity assays

β-galactosidase activities were determined as described by Miller *et al.*(1972). 1 ml of culture was transferred into a tube and centrifuged (16 000 g, 1 min). The supernatant was discarded and the bacteria were washed in 0.5 ml Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 0.27 % β-mercaptoethanol (v/v)), centrifuged (16 000 g, 1 min) and resuspended in 1 ml of Z buffer. 300 µl of cells were transferred into a microtiterplate and the OD_{600} was determined by measuring

with a Titertek multiscan device. The remaining 700 µl of cells were lysed (by adding for *Rhizobium*: 30 µl of chloroform, 15 µl of 0.1% sodium dodecyl sulphate (SDS) and for *E. coli*: 30 µl toluene, 30 µl 0.1% SDS followed by a 1 hour incubation at 37 º C while shaking). The lysed cells were diluted as appropriate into Z buffer to make a final volume of 500 µl (usually 1/5 dilution) and incubated for 5 min at 28 ºC. 100 µl of 4 mg/ml 2-nitrophenyl-β-D-galactopyranoside (ONPG) was added and the reaction was incubated at 28 ºC until a yellow colour developed. The reaction time was noted and the reaction was stopped by adding 250 µl of 1.5 M Na_2CO_3. 300 µl of the reaction was transferred into a microtiterplate and the OD_{420} was determined by measuring with a Titertek multiscan device. Miller units were calculated as follows:

$$\text{Miller units} = OD_{600} / (OD_{420} * \text{time (min)} * \text{dilution factor})$$

Gfp measurements

Cultures were pregrown in TY medium and then transferred to a fresh culture. 100 µl of culture were transferred to a microtiterplate and the OD_{600} and fluorescence (excitation at 485 nm, emission at 535 nm) was determined using a Tecan Safire microtiterplate reader. Gfp units were calculated as follows:

$$\text{Gfp units} = \text{fluorescence} / OD_{600}$$

AHL detection assays

RhiI and RaiI-made AHLs were detected by a *C. violaceum* bioassay (McClean *et al.*, 1997). *Rhizobium* strains were pregrown on TY agar at 28 ºC. *C. violaceum* precultures were inoculated from a -70 ºC glycerol stock in TY medium and incubated overnight. 100 µl of *C. violaceum* was added to 1% TY agar and poored on top of a TY plate. *Rhizobium* colonies were scooped off the plates with a loop, put on top of the *C. violaceum* plates and incubated overnight at 28 ºC after which the size of the purple violacein halo was determined.

R. leguminosarum strain A34 was used as a biosensor strain that is sensitive to CinI-made AHLs. Strain A34 was suspended in 10 ml of TY broth (to give an OD_{600} of ≈0.4), which was added to 200 ml of cooled TY agar and immediately poured as a thin layer onto a Petri dish. The agar was allowed to set before being overlaid with a thin layer of TY agar. Growth inhibition was assessed by inoculating rhizobia onto the surface and measuring haloes of growth inhibition following 2 days of growth at 28°C.

Swarming assays

Swarming assays were carried out as described (Daniels *et al.*, 2006). Rhizobia were spot inoculated on 0.7 % YEM agar plates (1 cm thick) and incubated for 5 days (28 ºC), after which the size of the colony was determined.

5.2 DNA manipulations

Purification of DNA

Genomic *Rhizobium* DNA was prepared as described by Chen & Kuo (1993). 1.5 ml of culture was centrifuged (16 000 g, 3 min) and the cell pellet was resuspended in 200 µl lysis buffer (40 mM Tris-acetate pH7.8, 20 mM sodium acetate, 1 mM ethylenediaminetetraacetic (EDTA), 1 % SDS). To remove proteins and cell debris, 66 µl 5M NaCl was added, followed by mixing and centrifugation (16 000 g, 10 min). The clear supernatant was transferred into a fresh tube and an equal volume of $CHCl_3$ was added. The solutions were mixed by inverting the tube 50 times, centrifuged (16 000 g, 10 min) and the extracted supernatant was transferred to a new tube. The DNA was precipitated by adding 2.5 X volume of EtOH and centrifugation (16 000 g, 10 min). The pellet was washed with 70 % EtOH and resuspended in 50 µl 10 mM Tris/HCl pH 8 or water.

Plasmids were extracted from *E. coli* by alkaline lysis (Kieser *et al.*, 2000). The cell pellet from 1.5 ml of culture was resuspended by vortexing in 100 µl solution I (50 mM Tris/HCl, pH 8; 10 mM EDTA). 200 µl solution II (200 mM NaOH; 1 % SDS) was added to lyse the cells and the tubes were inverted ten times. A volume of 150 µl solution III (3 M potassium acetate, pH 5.5) was added to precipitate cell debris and mixed by inverting the tube five times. The tube was centrifuged for 5 min at 16 000 g. The supernatant was mixed with 400 µl phenol/chloroform, vortexed briefly and then centrifuged for 5 min at 16 000 g. The upper phase was then transferred to a tube, 600 µl of ice cold isopropanol was added and DNA was precipitated by placing the tube on ice for 10 min and then centrifugation for 5 min at 16 000 g. The pellet was washed with 200 µl 70 % EtOH, centrifuged for 1 min at 16 0000 g, air dried and resuspended in 50 µl 10 mM Tris/HCl pH 8 or water.

Cloning

Restriction digests were done according to the manufacturer's instructions. After digestion the reaction was stopped by adding an equal volume of phenol-chloroform (1:1), vortexing and centrifugation for 5 min at 16 000 g. The aqeous phase was transferred to a new tube and the DNA was precipitated by adding 2.5 x volume of EtOH and 1/5 x volume of 5M sodium acetate, incubating for 10 minutes on ice and centrifugation for 15 min at 16 000 g. The pellet was washed with 70 % EtOH, centrifuged for 5 min at 16 000 g and resuspended in 25 µl of water. Ligations were done according to the instructions of the manufacturer of the ligase used.

The size of DNA fragments was analysed by electrophoresis. Samples were prepared by adding 1/10 volume of loading buffer (for 20 ml: 5.25 ml water, 0.025 g xylene cyanol, 0.025 % bromophenol blue, 1.25 ml 10% SDS, 12.5 ml glycerol). Samples were loaded in a horizontal 1% agarose gel (TBE: 50 mM Tris/borate pH8.5, 2.5 mM EDTA) and run at 5V/cm until the bands were separated. The gel was stained in a solution of ethidium bromide (1 µg/ml) for 15 min and viewed under a ultraviolet (UV) transilluminator. When required, DNA was purified from excised gel sections using the QIAquick gel extraction kit (Qiagen).

Gateway cloning was done by using BP Clonase and LR Clonase (Invitrogen) following the manufacturer's instructions.

PCR amplifications

PCR reactions were done by using either Amplitaq Gold, Phusion or GoTaq Green, as described by the instructions of the manufacturer. Typically, primers were designed to have an temperatures between 60-65 ºC.

Transformations

Chemically competent cells were prepared by inoculation of an overnight culture of *E. coli* cells into 50 ml of L medium. This culture was grown until an OD_{600} of 0.6 and centrifuged for 10 min at 6000 g (4 ºC). The supernatant was discarded, the bacteria were resuspended in 16 ml of buffer 1 (100 mM RbCl, 50 mM $MnCl_2$, 10 mM $CaCl_2$, 15 % glycerol) and incubated for 15 min on ice. The bacteria were centrifuged for 10 min at 6000 g (4 ºC) and the supernatant was discarded. The bacteria were resuspended in 4 ml of buffer 2 (10 mM RbCl, 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS) pH5.9, 7.5 mM $CaCl_2$, 15 % glycerol) and

incubated for 15 min on ice. Then 100 µl aliquots of the cells were frozen on dry ice and stored at -70 ºC until use.

To introduce plasmids into *E. coli,* 100 µl of competent cells were defrosted on ice, added to 1 µl of plasmid DNA or a ligation mixture and incubated for 20 min on ice. The samples were heat-shocked at 42 ºC for 45 seconds, incubated on ice for 1 min and 500 µl of L medium was added. The cells were incubated for 1 h at 37 ºC, plated onto selective plates and incubated overnight at 37 ºC.

Electrocompetent BW25113/pIJ790 cells were prepared by incoculation of an overnight culture of *E. coli* cells into 50 ml of SOB medium (28 ºC). This culture was grown until an OD_{600} of 0.6 and centrifuged (6000 g, 10 min) at 4 ºC. The supernatant was discarded and the bacteria were resuspended in 10 ml of ice-cold 10 % glycerol. The cells were centrifuged as before and washed in 10 % glycerol a further two times. After decanting the supernatant from the final wash cells were resuspended in the remaining ~ 100 µl of 10 % glycerol, frozen on dry ice and stored at -70 ºC.

To introduce plasmids by electroporation, 50 µl electrocompetent cells were mixed with ~ 100 ng plasmid DNA per transformation in a 0.2 cm ice-cold electroporation cuvette using a GenePulser II (Bio-Rad) set to: 200 Ω, 25 mF and 2,5 kV. The expected time constant was 4.5 – 4.9 ms. After electroporation, 1 ml ice cold LB was immediately added to the shocked cells and incubated with shaking for 1 h at 28 ºC. Transformants were selected by spreading onto LB agar containing the appropriate antibiotics.

DNA sequencing

DNA sequencing was used to confirm the correct sequence of plasmids and PCR fragments. DNA sequencing was carried out using ABI BigDye® 3.1 dye-terminator reaction mix (Applied Biosystems) with concentrations of DNA template and primer according to the manufacturer’s instructions. Sequence analysis was carried out by the John Innes Centre Genome Laboratory. Sequence chromatograms were analysed using the Chromas Lite software.

5.3 Construction of strains and plasmids

Strains, plasmids and primers used in this study are listed in Table 1, 2 and 3 respectively. Generation of strains and plasmids was done as follows.

***cinS* mutant**

A *R. l.* bv. *viciae* 3841 *cinS* mutant (A1229) was generated using the Redirect protocol (Gust *et al.*, 2004). A pLAFR1 cosmid containing the *cin* QS system (DG10) was isolated from a *R. l.* bv. *viciae* 3841 cosmid library (Kannenberg *et al.*, 1992) using a PCR screen with primers cinS-F and cinS-R (cinS-F/R). After digestion of DG10 with *Not*I, a 12 kb fragment containing the *cinRI* region was subcloned into SupercosI. The resulting plasmid pIJ11040 was introduced into BW25113/pIJ790 cells and targeted with an apramycin resistance cassette (amplified from pIJ773 using primers Redirect_cinS-F/R), yielding pIJ11041. Correct replacement of the *cinS* gene by the apramycin cassette was confirmed by PCR using primers (cinS-F/R) outside of the *cinS* open reading frame (ORF). pIJ11041 was then introduced into *E. coli* DH5α by electroporation, and transferred by conjugation to WT *R. l.* bv. *viciae* strain 3841. A double cross-over event was isolated based on apramycin resistance and kanamycin sensitivity and checked by PCR using primers cinS-F/R (A1229). The mutation was transduced into strain 3841 selecting for apramycin resistance to give strain A1245.

***expR* and *rapC* mutants**

To make *expR* and *rapC* mutants, pK19mob was inserted into the genes by a single cross-over event (Schafer *et al.*, 1994). An internal fragment of the gene was amplified by PCR and cloned into pK19mob using the *Xho*I and *Hin*dIII restriction sites introduced on the primers. For the *expR* mutant, the internal fragment (556 bp) was amplified using primers expR_pK19-F/R, cloned into pGEM T-easy (pIJ9996) and subcloned into pK19mob (pIJ11007). The *expR* mutation (A1216) was transduced into strain 3841 selecting for Km resistance to give strain A1245. For the *rapC* mutant, the internal fragment (347 bp) was amplified using primers rapC_pK19-F/R and cloned into pK19mob (pIJ11224). The resulting plasmids were conjugated into *R. l.* bv. *viciae* 3841 and a single-crossover event was selected by plating on neomycin (400 μg/ml). Correct integration of the plasmids into the chromosome were verified by PCR analysis, using one primer outside the internal fragment, and one vector specific primer (M13-F and M13-R).

***rhiI, praR, cinI* mutants**

A *rhiI* mutant (A848) was made by recombining the *rhiI*15::Tn*5* allele on pIJ7790 (Rodelas *et al.*, 1999) into 3841 as described previously (Ruvkun & Ausubel, 1981), followed by transduction (A850). In a similar way, a *rhiR* mutant (A904) was made by recombining the *rhiR1::*Tn*5* allele on pIJ1242 (Rodelas) into 3841 (Maria Sanchez-Contreras) and transduction (A920).

A library of Tn*5*-induced mutant colonies of *R. l.* bv. *viciae* strain 3841 was screened to identify mutants producing altered levels of AHLs. This was done by growing the rhizobia on a lawn of the biosensor strain *C. violaceum* CV026, which detects short-chain AHLs. One mutant was identified (A963), which caused increased production of the indicator pigment violacein (Craig McAnulla). The insertion point of the Tn*5* in A963 was identified by cloning *Eco*RI digested genomic DNA into pBluescript, selecting for kanamycin resistance (pIJ9758) and sequencing from the ends using primers M13-F/R and found to be 126 nucleotides after the start of the ORF. The *praR* mutation was transduced into strain 3841 selecting for kanamycin resistance to give strain A1132, and the Km-resistance cassette was exchanged for a Spec-resistance cassette using plasmid pJQ173 (Quandt *et al.*, 2004) to give A1167.

To identify a *cinI* mutant, pools of mutants from library were screened with PCR primers cinI_Tn5-F to identify a mutant with a Tn*5* insertion in *cinI.* The mutant (A993) did not produce CinI-made AHLs as assayed by a bioassay against a lawn of *R. leguminosarum* strain A34, that is inhibited in growth by CinI-made AHLs (Schripsema *et al.*, 1996). The mutation was transduced to strain 3841 selecting for kanamycin resistance, giving strain A994 (Maria Sanchez-Contreras).

Strain	Description	Reference
300	WT *R. leguminosarum* bv. *viciae* 3841	(Johnston & Beringer, 1975)
3841	WT *R. leguminosarum* bv. *viciae* 3841	(Johnston & Beringer, 1975)
8401	WT *R. leguminosarum* strain 8401	(Lamb *et al.*, 1982)
A34	WT *R. leguminosarum* strain 8401/pRLJI1	(Downie *et al.*, 1983)
A552	8401 *cinR::*Tn*5*	(Lithgow *et al.*, 2000)
A789	8401 *raiR::*Tn*5*	(Wisniewski-Dye *et al.*, 2002)
A850	3841 *rhiI*::Tn*5*	Maria Sanchez-Contreras
A920	3841 *rhiR*::Tn5 transduced	Maria Sanchez-Contreras
A922	3841 *rhiR*::Tn*5*	Maria Sanchez-Contreras
A924	3841 *cinR*::Tn5 transduced	(McAnulla *et al.*, 2007)
A963	3841 *praR::*Tn*5*	Maria Sanchez-Contreras
A994	3841 *cinI::*Tn*5*	Maria Sanchez-Contreras
A1004	3841 cellulose overproducer	Martin Krehenbrink
A1102	8401 *cinS*ΩSpec^R	(Edwards *et al.*, 2009)
A1132	3841 *praR::*Tn*5* transduced	Craig McAnulla
A1167	3841 *praR::*Tn*5* (Spec)	Anna Swiderska
A1206	3841 *rapA2*::Spec	Anna Swiderska
A1208	3841 *gmsA*::Tn5 (Gm)	(Williams *et al.*, 2008)
A1216	3841 *expR*ΩpK19mob	This work
A1229	3841 Δ*cinS*ΩApra^R	This work
A1232	3841 Δ*cinS*ΩApra^R *expR*ΩpK19mob	This work
A1245	3841 Δ*cinS*ΩApra^R transduced	This work
A1246	3841 *expR*ΩpK19mob transduced	This work
A1312	3841 *praR::*Tn*5* (Spec) Δ*cinS*ΩApra	This work
A1313	3841 *praR::*Tn*5* (Spec) *expR*ΩpK19mob	This work
A1314	3841 *cinI::*Tn*5* *praR::*Tn*5* (Spec)	This work
A1325	300 Δ*cinS*ΩApraR	This work
A1326	300 *expR*ΩpK19mob	This work
A1328	300 *praR*::Tn*5* (Spec) *rapA2*::Tn*5*	This work
A1344	300 RL0728::Tn5	This work
A1345	300 *praR::*Tn*5* (Spec)	This work
A1254	3841 *cadB*::Tn*5*	This work
A1362	3841 *rapC*ΩpK19mob	This work
A1263	3841 *cadA*::Tn*5* (Gm)	This work
A1363	3841 *praR*::Tn*5* (Spec). *cadB*::Tn*5*	This work
A1367	300 *praR*::Tn*5* (Spec) *gmsA::*Tn*5* (Gm)	This work
A1369	300 *praR*::Tn*5* (Spec) RL0728::Tn*5*	This work

Strain	Description	Reference
A1370	300 *praR*::Tn*5* (Spec) *rhiR*::Tn*5*	This work
A1372	300 *praR*::Tn*5* (Spec) *plyB*::Tn*5*	This work
A1374	300 *praR*::Tn*5* (Spec) *rapC*ΩpK19mob	This work
A1377	300 *praR*::Tn*5* (Spec) *motA*-	This work
A1378	300 *motA*-	This work
A1365	3841 *plyB*::Tn*5*	This work
A1383	3841 *praR*::Tn*5* (Spec), *cadB*::Tn*5*, *cadA::*Tn*5* (Gm)	This work
C. violaceum CV026	AHL detection strain	(McClean *et al.*, 1997)
E. coli Bl21 (DE3)	Host for heterologous protein expression	New England Biolabs
E. coli BW25113	K12 derivative: Δ*araBAD*, Δ*rhaBAD*	(Gust *et al.*, 2004)
E. coli BTH101	Host for bacterial-two-hybrid analysis	(Karimova *et al.*, 1998)
A1381	3841 *praR::*Tn*5* (Spec), *rhiI::*Tn*5*	This work
R. etli CNPAF512	WT *R. etli* CNPAF512	Jan Michiels
FAJ4006	*R. etli cinI* mutant	(Daniels *et al.*, 2002)
FAJ4007	*R. etli cinR* mutant	(Daniels *et al.*, 2002)
RU2307	3841 GOGAT mutant	Jay Mullay
RU2386	3841 GOGAT mutant, spontaneous *hfq* mutation	Jay Mullay
3841 *motA*-	3841 *motA* mutant	Michael Hynes
A1278	3841 RL0728::Tn5	Fang Xie
A1340	3841 RL0149::Tn5	This work
A1253	3841 *cadA*::Tn*5*	This work
A1261	3841 *cadA*::Tn*5* transduced	This work
A1254	3841 *cadB*::Tn*5*	This work
A1264	3841 *cadB*::Tn*5* transduced	This work
A1263	3841 *cadA*::Tn*5* (Gm)	This work
A1224	300 *rhiR*::Tn*5*	Anna Swiderska
A1208	300 *gmsA*::Tn*5* (Gm)	(Williams, 2006)

Table 5.1: Strains used in this study.

Plasmid	**Description**	**Reference**
DG10	pLAFR1 cosmid containing *cin* QS locus, Tet^R	This work
pBBR1-MC3	Broad-host range vector, Kan^R	(Kovach *et al.*, 1995)
pBBR1-MC5	Broad-host range vector, Gm^R	(Kovach *et al.*, 1995)
pBluescript	Cloning vector, Amp^R	Stratagene
pDONR207	Gateway donor vector, Gm^R	Invitrogen
pGEM T-easy	Vector for T/A cloning, Amp^R	Promega
pK19mob	Integrative vector for mutant generation, Km^R	(Schafer *et al.*, 1994)
pKT230	Broad-host range vector, Kan^R	(Bagdasarian *et al.*, 1981)
pLAFR1	Broad-host range cosmid vector, Tet^R	(Friedman *et al.*, 1982)
pLMB-hfq	cloned *hfq* in pLMB, Tet^R	Jay Mulley
pMP220	Broad-host range *lacZ* expression vector, Tet^R	(Spaink *et al.*, 1987)
pRK2013	helper plasmid for triparental conjugation, Km^R	(Ditta *et al.*, 1980)
pRU1156	Broad-host range *gfp* expression vector, Tet^R	(Karunakaran *et al.*, 2005)
pT18	vector containing T18 fragment of *E. coli cya,* Amp^R	(Karimova *et al.*, 1998)
pT25	vector containing T25 fragment of *E. coli cya,* $Chlor^R$	(Karimova *et al.*, 1998)
pT18-zip	Positive control bacterial-two-hybrid system, leucine zipper part II, Amp^R	(Karimova *et al.*, 1998)
pT25-zip	Positive control bacterial-two-hybrid system, leucine zipper part I, Cm^R	(Karimova *et al.*, 1998)
SupercosI	Cosmid vector, Km^R, Amp^R	Stratagene
pHM-GWA	Gateway expression vector, Amp^R	(Busso *et al.*, 2005)
pJQ173	Plasmid used for changing Km^R to $Spec^R$ in Tn*5*	(Quandt *et al.*, 2004)
pJQ175	Plasmid used for changing Km^R to Gm^R in Tn*5*	(Quandt *et al.*, 2004)
pIJ773	*aac(3)IV* ($Apra^R$) + *oriT*	(Gust *et al.*, 2004)
pIJ790	λ-RED (*gam, bet, exo*), *cat, araC, rep101* ^{ts}	(Gust *et al.*, 2004)
pIJ7794	*rhiI'-lacZ* in pMP220, Tet^R	(Rodelas *et al.*, 1999)
pIJ9104	*rhiR'-lacZ* in pMP220, Tet^R	(Lithgow *et al.*, 2000)
pIJ9252	*plyB'-lacZ* in pMP220, Tet^R	(Edwards *et al.*, 2009)
pIJ9272	*raiR'-lacZ* in pmp220, Tet^R	(Wisniewski-Dye *et al.*, 2002)
pIJ9493	*expR* in pBBR1-MC5, Gm^R	(Edwards *et al.*, 2009)
pIJ9611	*cinI'-gfp* in pRU1156, Tet^R	Anne Edwards
pIJ9655	cloned *cinI* in pKT230, Liv^R, Km^R	(Edwards *et al.*, 2009)
pIJ9692	cloned *cinS* in pKT230, Liv^R, Km^R	(Edwards *et al.*, 2009)
pIJ9716	*cinS* in pT25, Cm^R	Anne Edwards
pIJ9717	*expR* in pT18, Amp^R	Anne Edwards

Plasmid	**Description**	**Reference**
pIJ9758	*Bam*HI fragment containing *praR*::Tn*5* in pBluescript, Amp^R, Km^R	Anna Swiderska
pIJ9769	*expR* in pBBR1-MC3, Km^R	(Edwards *et al.*, 2009)
pIJ9996	internal fragment of *expR* in pGEM T-easy, Amp^R	This work
pIJ11007	800 bp fragment of *expR* in pK19mob, Km^R	This work
pIJ110033	*cinS* in , Amp^R	This work
pIJ11040	12kb containing *cin* QS system in Supercos1, Amp^R, Km^R	This work
pIJ11041	12kb containing cin QS system, *cinS* replaced by *aac(3)IV* Amp^R, Km^R	This work
pIJ11043	*cinS* in pET21a, Amp^R	This work
pIJ11048	*cinS-his$_6$* in pGEM T-easy, Amp^R	This work
pIJ11051	*cinS-his$_6$* in pBBR1-MC3, Km^R	This work
pIJ11052	*cinS-his$_6$* in pBBR1-MC5, Gm^R	This work
pIJ11108	*praR* promoter in pGEM teasy, Amp^R	This work
pIJ11109	*praR* promoter + *praR* ORF in pGEM teasy, Amp^R	This work
pIJ11112	*praR*'-lacZ in pMP220, Tet^R	This work
pIJ11113	*praR* in pMP220, Tet^R	This work
pIJ11132	*praR* in pT18, Amp^R	This work
pIJ11133	*praR* in pT25, $Chlor^R$	This work
pIJ11151	RL0149 in pT25, Cm^R	This work
pIJ11152	*praR* in pDONR207, Gm^R	This work
pIJ11155	*praR* in pHM-GWA, Amp^R	This work
pIJ11158	RL0149'-*lacZ* in pMP220, Tet^R	This work
pIJ11159	*cinS* in pT18, Amp^R	This work
pIJ11160	RL0149 in pT18, Amp^R	This work
pIJ11163	RL4665'-*lacZ* in pMP220, Tet^R	This work
pIJ11164	RL4371'-*lacZ* in pMP220, Tet^R	This work
pIJ11165	pRL110096'-*lacZ* in pMP220, Tet^R	This work
pIJ11167	pRL110060'-*lacZ* in pMP220, Tet^R	This work
pIJ11168	RL1940'-*lacZ* in pMP220, Tet^R	This work
pIJ11169	RL3302'-*lacZ* in pMP220, Tet^R	This work
pIJ11170	RL2423'-*lacZ* in pMP220, Tet^R	This work
pIJ11171	RL3074'-*lacZ* in pMP220, Tet^R	This work
pIJ11175	RL2169'-*lacZ* in pMP220, Tet^R	This work
pIJ11177	RL2331'-*lacZ* in pMP220, Tet^R	This work
pIJ11194	*expR* in pT25, $Chlor^R$	This work

Plasmid	Description	Reference
pIJ11196	*rosR'-lacZ* in pMP220, Tet^R	This work
pIJ11198	pRL110097'-*lacZ* in pMP220, Tet^R	This work
pIJ11224	internal fragment of *rapC* in pK19mob, Km^R	This work

Table 5.2: Plasmids used in this study

Primer	Sequence
cadA-F	CCGAGTTCGAATCTTACGACG
cadB-F	CTGCAAGTCCCACCTCGGTG
cinI_gelshift-R	TTCTTGCGCAGGCGAAAC
cinI_mutant-F	TGCGCATCATCAATCCCTAG
cinS_pET15-F	CAGCCATATGAACCGCCTCGCTGAA
cinS_pET15-R	CTAGCTCGAGTCAGCTGAAGCTGCTCTT
CinS_T18-F	TTTTGGTACCTATGAACCGCCTCGCTGAAAC
CinS_T18-R	TTTTGGTACCCTGCTGAAGCTGCTCTTCAGC[c]
cinS-F	GAAATCGAATGTCTGCACTGGACG
cinS-R	GTGTCAAATTTCCGATTTTTCGCGTC
expR_check	GTGAATATTAATTCGTTA
expR_pK19-F	TTTTAAGCTTGCTTCGAATATT[a]
expR_pK19-R	TTTTCTAGAGTGGTTTGAGATCTTCAGCATC[b]
expR_T18-F	CACGTCGAACCTCGAGTGCATCTG[d]
expR_T18-R	GAGAAGCGGAATTGCTCAAGCTTATCAG[a]
M13-F	TGTAAAACGACGGCCAGT
M13-R	CAGGAAACAGCTATGACC
P310	
plyB-F	GTAATTCGAGAACAAGGCG
pMP220-F	GAACGGCCTCACCCCAA
pMP220-R	ATCAACGGTGGTATATCCAGTGATTTT
praR_B2H-F	TTTTGGTACCTATGATTGAAAACAAGAAGCCGAATC[c]
praR_B2H-R	TTTTGGTACCCTGTCGGCGTCGGCTTCAG[c]
praR_full-R	CAGAAGGACATAAATATATCTTTATATC
praR_GW-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGATTGAAAACAAGAAGAAGC
praR_GW-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGTCGGCGTCGGCTTC
praR-F	GAGGTGCTGCTGATTTTGATTG
praR-R	GTATTTCTGGATCTGCTGGAAGGTG
pRL110060-F	TTTTGAATTCGAAACTGACCGCACGCC
pRL110060-R	TTTTCTGCAGGCACATGGCGATAGGcATC
pRL110096-F	TTTTGAATTCCAATGCAATATGTCCTCCGA
pRL110096-R	TTTTCTGCAGCAACTACATGCTTGGTGCAATG
pRL110097-F	TTTTGAATTCGCGTCTGCTTCTGGTCGAG
pRL110097-R	TTTTCTGCAGCAATGCAATATGTCCTCCGA
pT18-R	CTCGAAATCGGTGATCACG
pT25-F	AGCAACCACGCAGGCTACGAG
rapC_pK19-F	TTTTAAGCTTAACTTCTTCGATGGCGAGCG
rapC_pK19-R	TTTTTCTAGACAGGAAGAGCGTGCCAGC

Primer	Sequence
Redirect_cinS-F	CCGCTCGGTCGTGACCCAATTCCTGGAGATGGCAGCATGAT TCCGGGGATCCGTCGACC
Redirect_cinS-R	GCTCGTCTTCAGGCGGGGCGGAGGGGAACCACACCCTCATG TAGGCTGGAGCTGCTT
rhiR-F	TTTTGAATTCGCCTAAACTTTCGCTTCTGACG[f]
rhiR-R	TTTTGAATTCGGCTGACTCTGACAGGAAATCG[f]
RL0149_B2H-F	TTTTGGTACCTGGTGGTTTCGGCTGATTC
RL0149_B2H-R	TTTTGGTACCCTGGTGGTTTCGGCTGATTC
RL0149-F	TTTTGAATTCCGGAATCGTAAAGC
RL0149-R	TTTTGAATTCGAAAGTCAGTCCGAGTGCTTC
RL1940-F	TTTTGAATTCCTAGTGGCTTCCTCTGGATGC
RL1940-R	TTTTCTGCAGATTTCGTCGATCTCATCGC
RL2169-F	TTTTGAATTCTATATTGGGGTCCGTTCGAG
RL2169-R	TTTTCTGCAGTGGGCACAGAAGCAAGAATATC
RL2331-F	TTTTGAATTCCGATCAAAAGACCGAAACG
RL2331-R	TTTTCTGCAGGGTCAATGAGATGTRCGAGTTACTACC
RL2423-F	TTTTGAATTCGGAACGCGACCTGCTCC
RL2423-R	TTTTCTGCAGGAAGAGGGTAGATGGCGCC
RL3074-F	TTTTGAATTCGATGCGGCGAAGATCAG
RL3074-R	TTTTCTGCAGAAGCGCTGCTGACATCATC
RL3302-F	TTTTGAATTCTGTTCGAGGCAGGAGTCG
RL3302-R	TTTTCTGCAGCAGAAcGTGCCGAGAAATTC
RL4371-F	TTTTGAATTCTCGAACCGAAGATGCTGAG
RL4371-R	TTTTCTGCAGGAAGTCGACACAGCAGCG
RL4665-F	TTTTGAATTCAGGTATGCCGCATTCGAG
RL4665-R	TTTTCTGCAGGGTCTTGTGAAcGGCAAGC
rosR-F	TTTTGAATTCGGCAAATGGCAAACACGC
rosR-R	TTTTCTGCAGATCCACAAGCAGCTCCG
Selex_temp	GATGAAGCTTCCTGGACAAT-$(N)_{20}$-GCAGTCACTGAAGAATTCTG
Selex-F	GATGAAGCTTCCTGGACAAT
Selex-R	CAGAATTCTTCAGTGACTGC
T25_cinS-F	CAATTCCTGGAGAGGTACCCATGAAC[c]
T25_cinS-R	GAACGGTACCCTCAGCTGAAG[c]
T25_expR-F	TTTTGGTACCTGTGAATATTAATTCGTTAATTCAATTACTTG[c]
T25_expR-R	TTTTGGTACCTCTAACTTATCAGGCCATGACGG[c]
T7-F	AATACGACTCACTATAGG
T7-R	GCTAGTTATTGCTCAGCGG

Table 5.3: Primers used in this study. Restriction sites are underlined: [a]: *Hin*dIII, [b]: *Xba*I, [c]: *Kpn*I, [d]: *Xho*I, [e]: *Nde*I, [f]: *Eco*RI.

***plyB*, RL0149, *cadA* and *cadB* mutants**

A library of Tn*5*-induced mutant colonies of *R. l.* bv. *viciae* strain 3841 was screened by PCR to identify mutants in genes of interest using one transposon specific primer (P310) and one gene specific primer (see Table 5.4), using an intelligent pooling strategy.

Gene	Primer	Strain
cadA (pRL100309)	cadA-F	A1253
cadB (RL2169)	cadB-F	A1254
plyB (RL3023)	plyB-F	A1365
RL0149	RL0149-F	A1340

Table 5.4: Generation of mutants using Tn*5* library

Double mutants

Strains in the *R. l.* bv. *viciae* strain 300 genetic background were generated by transduction of the mutations into strain 300 or A1345, selecting for the appropriate antibiotic resistance. Double mutants were generated as follows. A1232 was generated by transducing the A1229 mutation into A1246 selecting for apramycin resistance. A1312 was generated by transducing the A1229 mutation into A1167 selecting for apramycin resistance. A1313 was generated by transducing the A1216 mutation into A1167 selecting for Km resistance. A1314 was generated by transducing the A994 mutation into A1167 selecting for Km resistance. A1381 was generated by transducing the A850 mutation into A1167 selecting for Km resistance. The *cadA-cadB-praR* triple mutant was generated as follows. First the *cadB::*Tn*5* mutation was transduced into A1345, selecting for Km resistance (A1363). The Km resistance cassette of strain A1253 was exchanged for a Gm resistance cassette using plasmid pJQ175 (A1263) and this *cadA::*Tn*5* (Gm) mutation was transduced into strain A1363, giving strain A1383.

Other plasmids

Promoter-*lacZ* fusion constructs were made by cloning the promoters of the target genes into pMP220 using *Eco*RI and *Pst*I. Full length cloned *praR* was also introduced into pMP220 using *Eco*RI. Promoters were amplified using the appropriate primers and digested using the appropriate restriction enzymes (see Table 5.5). The *praR*

promoter and the full length *praR* gene were cloned into GEM-Teasy (pIJ11108 and pIJ11109) and subcloned into pMP220. The other genes were cloned directy into pMP220. Where necessary, the correct orientation of the insert was determined by PCR analysis by using one vector-specific (pMP220 F/R) and one gene-specific primer.

Plasmid	**Promoter**	**Primer**	**Restriction enzymes**
pIJ11112	RL0390 (*praR*)	praR-F/R	*Eco*RI
pIJ11113	Full length *praR*	praR-F/praR_full-R	*Eco*RI
pIJ11158	RL0149	RL0149-F/R	*Eco*RI, *Pst*I
pIJ11163	RL4665	RL4665-F/R	*Eco*RI, *Pst*I
pIJ11164	RL4371	RL4371-F/R	*Eco*RI, *Pst*I
pIJ11165	pRL110096	pRL110096-F/R	*Eco*RI, *Pst*I
pIJ11167	pRL110060	pRL110060-F/R	*Eco*RI, *Pst*I
pIJ11168	RL1940	RL1940-F/R	*Eco*RI, *Pst*I
pIJ11169	RL3302	RL3302-F/R	*Eco*RI, *Pst*I
pIJ11170	RL2423	RL2423-F/R	*Eco*RI, *Pst*I
pIJ11171	RL3074 (*rapC*)	RL3074-F/R	*Eco*RI, *Pst*I
pIJ11175	RL2169	RL2169-F/R	*Eco*RI, *Pst*I
pIJ11177	RL2331	RL2331-F/R	*Eco*RI, *Pst*I
pIJ11196	*rosR*	rosR-F/R	*Eco*RI, *Pst*I
pIJ11198	pRL110097	pRL110097-F/R	*Eco*RI, *Pst*I

Table 5.5: Construction of promoter-*lacZ* fusion constructs.

The *cinI'-gfp* promoter fusion construct (pIJ9611) was made by amplifying the *cinI* promoter (CinI-F/R) and cloning into pRU1156 using *Hin*dIII.

For the bacterial-two-hybrid constructs, the *cinS, expR, praR* and RL0149 ORFs were amplified using the appropriate primers (see Table 5.6) and cloned into pT18 and pT25 using the appropriate restriction enzymes.

Plasmid	Gene	Vector	Primer	Restriction enzymes
pIJ11132	*praR*	pT18	praR_B2H-F/R	*Kpn*I
pIJ11133	*praR*	pT25	praR_B2H-F/R	*Kpn*I
pIJ9716	*cinS*	pT25	T25_cinS-F/R	*XhoI,HindIII*
pIJ9717	*expR*	pT18	expR_T18-F/R	*KpnI*
pIJ11159	*cinS*	pT18	cinS_T18-F/R	*KpnI*
pIJ11194	*expR*	pT25	T25_expR-F/R	*Kpn*I
pIJ11151	RL0149	pT25	RL0149_B2H-F/R	*Kpn*I
pIJ11160	RL0149	pT18	RL0149_B2H-F/R	*Kpn*I

Table 5.6: Cloning of bacterial two hybrid constructs.

Constructs used for protein purification were made as follows. The *cinS* ORF was amplified using primers cinS_pET21-F/R and cloned into the *Nde*I and *Xho*I restriction sites of pET21a (Novagen), to give pIJ11043. The gene encoding CinS-His$_6$ was amplified from pIJ11043 using primers T7-F/R and cloned into pGEM T-easy (pIJ11048), after which it was subcloned into pBBR1-MC3 and pBBR1-MC5 with *Eco*RI. Correct orientation of the insert was determined by PCR analysis with one vector- and one gene-specific primer (pIJ11051 and pIJ11052). The *praR* ORF was amplified using primers praR_GW-F/R and cloned into pDONR207 (Invitrogen) making pIJ11152, after which it was moved into pHM-GWA (Busso *et al.*, 2005) using the Gateway technology (Invitrogen) to give plasmid pIJ11155. This fused the *praR* OFR to the C-terminus of His$_6$-tagged maltose binding protein.

For generation of the N-terminal His6-tagged fusion of CinS, the *cinS* ORF was amplified using primers cinS_pET15-F/R and cloned into pGEMT-easy (pIJ11029), after which it was subcloned into pET15b using *NdeI* and *XhoI* (pIJ11033).

5.4 Microarray analysis

RNA purification

RNA was purified from *Rhizobium* cultures using the RNeasy Mini kit (Qiagen) as described in the manufacturer's instructions. *Rhizobium* cultures were grown in AMS minimal medium to exponential phase (OD_{600} 0.7 – 0.8). 12 ml samples were rapidly mixed with 24 ml RNAlater (20 mM EDTA, 25 mM citric acid$_3$, 5.3 M $(NH_4)_2SO_4$, pH

5.2) and stored until further usage. The cells in the RNAlater solution were pelleted by centrifugation for 10 min at 16 000 g (4°C). Pellets were air dried (15 min), re-suspended in 250 µl 10 mM Tris-HCl pH 8 and transferred to a ribolyser tube (2 ml screw-cap eppendorf carrying ≈ 400 µl sand) with 700 µl RLT buffer (Qiagen) and 1% β-mercaptoethanol (v/v). Cells were lysed using a Ribolyser (Hybaid) by two rounds of lysis (speed 5.5 for 30 s, 3 min incubation on ice between rounds) before centrifugation (16 000 g, 3 min, 4°C). The supernatant was transferred to an RNase-free 1.5 ml Eppendorf tube, 450 µl EtOH was added and the solution was mixed by pipetting thoroughly. The solution was then applied to an RNeasy spin column. On-column DNase treatment (DNase kit from Qiagen), washing and elution steps were performed according to the manufacturer's protocol. Purified RNA was quantified using a Bio-Rad Experion device, with RNA Stdsens chips, RNA ladder and reagents as described in the manufacturer's instructions. Data were analysed using the Experion 3.0 software package.

cDNA first strand synthesis and CyDye labelling

10 – 15 µg purified total RNA was used as a template for first-strand synthesis of cDNA using the CyScribe post-labelling kit (GE Healthcare), with random nonamer oligonucleotides. 11.5 µl RNA (diluted with RNase-free water or concentrated using a SpeediVac at 37°C where necessary) was incubated with 1 µl random nonamers (for 5 min at 70°C, then for 10 min at RT) to allow primers to anneal. Extension reactions (1 x CyScript buffer, 10 mM DTT, 0.3 mM dATP, 0.3 mM dGTP, 0.3 mM dCTP, 0.05 mM dTTP, 0.25 mM amino-allyl-UTP, 1 µl CyScript reverse transcriptase) were done at 42°C for 6 h. To degrade remaining RNA, 2 µl 2.5 M NaOH was added to each sample, vortexed and incubated (37°C , 15 min) before neutralising with 10 µl 2 M HEPES. Amino-allyl labelled cDNA was purified using the CyScribe GFX purification kit as described in the manufacturer's instructions (using 80% EtOH during the wash stages instead of the provided wash buffer) and eluted in 60 µl 0.1 M sodium bicarbonate pH 9.0. The eluted cDNA was mixed with the appropriate CyDye NHS ester and incubated overnight in the dark. Unreacted CyDyes were inactivated by the addition of 15 µl 4M hydroxylamine (15 min, RT). CyDye-labelled cDNA was purified using the CyScribe GFX purification kit as described in the manufacturer's instructions and eluted in 2 x 40 µl of the supplied elution buffer at 65°C. CyDye-labelled cDNA was quantified by measuring the absorbance of each sample at 260 nm, 550 nm and 650 nm using a Nanodrop spectrophotometer.

Microarray hybridisation and analysis

The UltraGAPSTM slides (Corning) spotted with oligonucleotides representing the genes of *R. leguminosarum* bv *viciae* 3841 were blocked before hybridisation to reduce non-specific binding of probe to free functional groups. Slides were immersed for 15 min (with gentle agitation at 100 rpm) in a solution prepared as follows: 500 mg succinic anhydride was completely dissolved in 31.5 ml 1-methyl-2-pyrrolidinone, followed by the addition of 3.5 ml 0.2 M sodium borate (pH 8.0). Slides were washed by immersion in 50 ml MilliQ filtered sterile water for 1 min (with agitation at 100 rpm), immersed 5 times in EtOH before drying by centrifugation for 5 min at 800 rpm. For each microarray experiment, equal quantities of Cy3- and Cy5-labelled cDNA (20 – 70 pmol) were combined in a DNase–free Eppendorf, concentrated to 2 - 5 µl in a SpeediVac and re-suspended to a final volume of 65 µl in hybridisation buffer (25% deionised formamide, 5x saline sodium citrate (SSC), 0.1% SDS, 10 ng calf thymus DNA) at 42°C. Target cDNA was denatured for 2 min at 95°C and cooled for 2 min at RT, 2 min. A UltraGAPSTM slide was loaded into a SlideBoosterTM SB800 (Advalytix, Implen) chamber onto 45 µl AdvaSon coupling fluid (Advalytix) as described in the manufacturer's protocol. A Lifterslip (Implen, 25 mm x 60 mm) was fitted onto the top of the microarray slide and the denatured target cDNA (65 µl) was loaded by capillary action under the edge. 500 µl humidifying buffer was loaded into each well in the chamber to maintain humidity during the hybridisation (with program settings: mixing power 27, Pulse/Pause ratio 5:5, 18 hr, 42°C).

Following hybridisation, slides were washed in a series of increasingly stringent buffers, with agitation at 100 rpm, to remove unbound probe. 20 x SSC buffer was made up by dissolving 175.3 g NaCl and 88.2 g sodium citrate into 800 ml RNase-free water and adjusting the pH to 7 with HCl. Washes were done as follows: 2x SSC, 0.1% (v/v) SDS at 42°C for 7 min; 0.2x SSC, 0.1% (v/v) SDS at 42°C for 5 min, repeated twice in fresh buffer; 0.2x SSC at RT for 4 min, repeated twice in fresh buffer; 0.1x SSC at RT for 1 min. Each slide was then dipped 5 times in sterile MilliQ filtered water and then 3 times in isopropanol and centrifuged (5 min, 800 rpm) to dry. Hybridised microarray slides were scanned using an Axon GenePixR 4200A scanner and visualised using the Genepix Pro software. Preview scans were set to 30% power and photomultiplier tube (PMT) gain 600 for both channels. PMT gain was adjusted to balance the signals, where necessary. Final scans were performed at 70% power to prevent saturation of the signals. Axon scanned images were initially processed using Bluefuse software (Cambridge BlueGnome). This file was then analysed further using

the Genespring GX 7.0 analysis platform (Agilent). This involved data transformation (measurements less than 0.01 set to 0.01), Lowess normalisation (per spot and per chip, intensity dependent) dye-swap where necessary. Statistical significance was determined using the T-test calculations provided by Genespring and reported as significant at $P<0.10$.

5.5 Protein experiments

Protein purification

Recombinant CinS-His_6 and MBP-PraR were purified from *E. coli* BL21(DE3) cells carrying plasmids pIJ11043 and pIJ11155 respectively. Cells were grown in 3 l of L medium until an OD_{600} of 0.6, after which protein expression was induced with 0,5 mM IPTG at 30 ºC for 4 h. Cell pellets were resuspended in 40 ml lysis buffer (10% glycerol, 1mM phenylmethanesulfonyl fluoride, 50 mM Tris-HCl pH 8.0), lysed with a French press, and the cell lysate was centrifuged (20 min at 40,000 g). The supernatant was loaded onto a 1 ml Ni^{2+}-loaded Hi-Trap Chelating HD column (GE Healthcare), washed with 200 mM NaCl, 20 mM imidazol, 25 mM K_2HPO_4 pH 8, and eluted with an imidazol gradient. The eluted proteins were dialysed into storage buffer (50% glycerol, 50 mM NaCl, 0,1 mM EDTA, 1 mM DTT, 50 mM Tris-HCl pH 8 for CinS and 20% glycerol, 100 mM NaCl, 1 mM DTT, 25 mM Tris-HCl pH 8 for MBP-PraR), and stored at -20 ºC and -80 ºC respectively. Protein concentrations were detemined by a Bradford assay (Bio-Rad).

Protein gel electrophoresis

Protein purity was examined by denaturing SDS polyacrylamide gel electrophoresis (SDS-PAGE) as described (Sambrook *et al.*, 2001). Protein samples were prepared by adding 1/5 volume of protein loading buffer (400 mM Tris-HCl pH 5.8, 40 % glycerol (v/v), 4% SDS (w/v), 4 mM EDTA, 20% β-mercaptoethanol (v/v)) and boiling for 5 min. After the samples had cooled down to RT they were centrifuged (16 000 g, 1 min) to remove debris. Samples were loaded on a vertical polyacrylamide Mini-Protean gels (Biorad): the running gel was made up of 16% (w/v) acrylamide-bisacrylamide (37.5:1), 375 mM Tris, 0.1% SDS, 25 mM EDTA, 0.04 % 1,2-bis(dimethylamino)- ethane (TEMED) (v/v), 0.1 % ammonium persulphate (APS) (w/v). The stacking gel was made up of 5% (w/v) acrylamide-bisacrylamide (37.5:1), 125 mM TrisHCl pH5.8, 0.05 % SDS (w/v), 0.1 % TEMED (v/v), 0.04% APS (w/v). Gels were run in running buffer (25 mM TrisHCl, 192 mM glycine, 0.1 % SDS (w/v))

at 100 mA until the bands had separated, after which the gel was stained in protein stain solution. The protein stain solution was prepared by mixing one part of protein stain solution I (330 mg Brilliant blue G, 70 mL methanol) with two parts of protein stain solution II (85 g $(NH_4)_2SO_4$, 15 mL 85% H_3PO4, 330 mL H_2O).

Western blotting

Following SDS-PAGE, gels were washed in transfer buffer. Two sheets of Whatman 3MM filter paper were cut to the size of the gel and soaked in transfer buffer (2.4g/l tris, 11.4 g/l glycine, 10 % methanol). These were laid onto the bottom plate of the blot cassette, avoiding the trapping of air bubbles. The gel, soaked in transfer buffer, was laid on top of this and covered with a sheet of Protran nitrocellulose (Whatman GmbH) and two sheets of Whatman 3MM filter paper. Protein gels were transferred onto nitrocellulose in transfer buffer by electroblotting (100 mA, 4 ºC, 1 h). After blocking of the membrane in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 1% BSA the membrane was incubated with anti-CinS antiserum (raised against purified CinS-His_6, Biogenes) (1:20 000 in TBS, 1% BSA), followed by incubation with anti-rabbit antiserum (1:10 000 in TBS, 1% BSA). After these 3 30 min washes with TBS + 0.1% Tween 20, the CinS protein was visualised using alkaline phosphatase activity using 5-bromo-4-chloro-3-indolyl phosphate (B5655-25TAB, Sigma) according to the manufacturer's instructions.

Q-Tof analysis

Q-ToF analysis of CinS-His_6 was done after dialysis of the protein against 20 mM Tris-HCl pH8 (final concentration 4 mg/ml). The protein was diluted in 50% methanol, 1% formic acid and applied to the Q-ToF by electrospray using a Picotip emitter (NewObjectives). Scans of 2 s were collected over several minutes and the spectra were combined yielding a single combined spectrum. The spectrum was processed by background subtraction, smoothing, and centering using the options in Masslynx (Waters).

CD analysis

CinS-His_6 was dialysed overnight at 4°C into 20 mM potassium phosphate buffer pH 8. Far-UV (180-260 nm) CD spectra were recorded at 20°C using a J-710 spectropolarimeter (Jasco Ltd.) and a 0.5 mm path length cell. Spectra were the average of three accumulations at 100 $nm.min^{-1}$ and 0.5 nm resolution.

DLS analysis

Dynamic light scattering analysis was done after dialysis of CinS-His$_6$ against 100 mM Tris-HCl pH 8, 100 mM NaCl buffer. The purified CinS-His6 was concentrated to around 10 mg/ml using an Ultrafree 10 kDa cutoff concentrator (Millipore) and particles were removed by filtration through a 0.1 mm Ultrafree filter (Millipore). The sample was introduced into a 12 µl microsampling cell and inserted into a Dynapro-MSTC molecular-sizing instrument at 20 ºC (Protein Solutions Inc.). Fifteen scattering measurements were taken and the resulting data were analysed using the DYNAMICS software package (Protein Solutions Inc.).

Absorption of CinS-interacting proteins from *R. leguminosarum* cell lysate

Purified CinS-His$_6$ was covalently attached to CNBr-activated beads (GE Healthcare). First, the protein was dialysed against coupling buffer (500 mM NaCl, 100 mM $NaHCO_3$ pH 8.3) and the concentration of the protein was adjusted to 3 mg/ml. CNBr-activated sepharose beads were activated by resuspending them in 1 mM HCl, washed with coupling buffer and incubated with CinS-His$_6$ overnight at 4 ºC. The beads were washed with coupling buffer, and residual active groups on the beads were inactivated by 2 h incubation at RT in blocking buffer (500 mM ethanolamine-HCl pH 8.5). The beads were then washed, first with coupling buffer, then four times alternating between wash buffer 1 (1 M NaCl, 100 mM $CH_3COO^-Na^+$, pH 4.0) and wash buffer 2 (1 M NaCl, 100 mM $NaHCO_3$, pH 8.3), and a final wash with coupling buffer. A *R. leguminosarum* cell lysate was prepared by harvesting the cells of a 4 l TY culture grown for two days. The cell pellet was resuspended in 100 ml 500 mM NaCl, 100 mM Tris-HCl, pH 8.0, lysed with a French press and centrifuged (20 min, 40,000 g). The supernatant was incubated with the CinS-beads for 30 min at RT, after which the beads were recovered by centrifugation (5 min, 5000 g). The beads were then washed ten times by resuspension in 50 ml 100 ml 500 mM NaCl, 100 mM TrisHCl, pH 8.0 and recovered by centrifugation. Interacting proteins were released with 500 mM NaCl, 200 mM glycine, pH 2.8 and the eluate was quickly neutralised by adding an equal volume of 1M K_2HPO_4 (pH 8.0). Proteins in the different washes and the eluted fraction were concentrated using acetone precipitation for analysis by SDS-PAGE. Bands were cut out of the gel with a razor blade and analysed by Maldi-ToF after in-gel trypsin-digestion.

5.6 Protein-DNA/RNA interaction analysis

EMSA analsyis

RNA fragments were prepared by *in vitro* transcription of PCR fragments containing a T7 promoter (TGTAATACGACTCACTATAGGGCGAGAATTCGAGCTC at 5' end of sRNA-specific forward primer sequence) and terminator (TTTAAGCTTTCGATGCTGAAGTAGTCCCGCTCAAG at 3' end of sRNA-specific reverse primer sequence). Reaction conditions for the *in vitro* transcription reaction were as described in the instructions of the manufacturer of the T7 RNA polymerase (Promega). [α-^{32}P]-rCTP was added to the reaction mixture to incorporate radioactive label into the RNA fragments, according to the instructions of the manufacturer. CinS-His_6 was bound to the sRNA's in 20 µl EMSA buffer containing 1 µM CinS-His_6. Three different EMSA buffers were tested: EMSA buffer 1 (100 µM EDTA, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 100 mM KCl, 100 µM DTT, 3 % glycerol, 50 ng yeast tRNA, 20 mM Tris-HCl, pH 8), EMSA buffer 2 (5 mM Mg-acetate, 100 mM NH_4Cl, 100 µM DTT, 3 % glycerol, 50 ng yeast tRNA, 10 mM Tris-HCl, pH 7.5), EMSA buffer 3 (500 mM KCl, 5 mM DTT, 3 % glycerol, 50 ng yeast tRNA, 100 mM HEPES, pH 8). After incubation at RT for 20 min, the binding reactions were loaded on native polyacrylamide gels (5 % acyrlamide-bisacrylamide (37.5 : 1), 1 x TBE) and run in TBE buffer at 40 mA V for 90 min. EMSA data were collected by exposure of the dried gel onto photographic film.

For analysis of binding of CinS-His6 and MBP-PraR to DNA, promoter fragments were prepared by PCR and end-labelled using [γ^{32}-P]-ATP and T4 polynucleotide kinase (New England Biolabs) (Le *et al.*, 2009). MBP-PraR was bound to DNA in 20 µl EMSA buffer (20 mM TrisHCl pH8, 200 ng salmon sperm DNA, 1 mM EDTA, 100 mM NaCl, 0.5 mM DTT, 5 mM $MgCl_2$, 8% (v/v) glycerol) containing 0.1 nM radiolabelled DNA (approximately 8000 c.p.m.) and varying amounts of MBP-PraR and CinS-His_5. After incubation at RT for 20 min, the binding reactions were loaded on native polyacrylamide gels (5 % acyrlamide-bisacrylamide (37.5 : 1), 1 x TBE) and run in TBE buffer at 100 V for 45 min in a Biorad Mini-Protean gel system. The effect of CinS-His_6 on binding of MBP-PraR to the promoters was assayed by adding the protein after the first incubation and incubating for another 15 min. EMSA data were collected and analysed on a PhosphorImager (FujiFilm) using Multi Gauge image analysis software (FujiFilm).

SELEX analysis

The recognition sequence of PraR was selected using SELEX (Systematic Evolution of Ligands by Exponential Enrichment), using purified MBP-PraR. A 62-mer double stranded oligonucleotide was synthesised containing 20 sequential completely degenerate nucleotides (selex_temp). Two primers complementary to the conserved regions on this sequence (selex-F/R) were also synthesised (Oliphant *et al.*, 1989). Before the first binding, the template was converted to double stranded DNA by a 20 min incubation (72ºC) with Taq polymerase, deoxynucleotides and the selex-R primer. 20 µg MBP-PraR was immobilised on Ni^{2+}-agarose beads by a 20 min incubation (RT) in binding buffer (25 mM TrisHCl pH 8.0, 1 mM MgCl2, 10% (v/v) glycerol, 250 mM NaCl) and excess MBP-PraR was removed by two washes with binding buffer. After incubation of the beads with the doublestranded selex template (20 min, RT), the beads were centrifuged and washed five times with binding buffer containing 0.1 mg/ml sonicated salmon sperm DNA and resuspended in 50 µl 10 mM Tris-HCl pH 8.0, 1mM EDTA. To remove the bound DNA fragments from the protein, the beads were boiled and centrifuged. 2 µl of the supernatant were used for amplification with primers selex-F/R and products were analysed by agarose gel electrophoresis (2% w/v). This procedure was repeated 10 times, and after this the products of the amplification reaction were cloned into pGEM T-easy and sequenced.

5.7 Bacterial two hybrid analysis

Bacterial two hybrid analysis was done as described by Karimova *et al.* (1998) Bacterial two hybrid plasmids were introduced into *E. coli* BTH101 by transformation and plated on McConkey agar (1% maltose, 0.5 mM isopropyl-b-D-thiogalactopyranoside (IPTG)). After pregrowth in L medium, cultures were grown in the presence of 0.5 mM IPTG at 20 ºC for 24 h and β-galactosidase expression was measured as described previously.

5.8 Plant experiments

Nodulation tests

Pea (*Pisum sativum*) germination was done by washing of the seeds with 70 % EtOH (1 min) followed by 20 min sterilisation in 1 % bleach. After imbibing in water for 3 hours, the seeds were placed on water agar in the dark to germinate. Germinated seeds were placed in sterile flasks containing FP agar and kept in the dark until the shoot could be pulled through the foam plug. The flasks were then wrapped in black plastic and the plants were moved to a growth chamber for three weeks.

Nodulation competitiveness tests

Peas were germinated as described above. Peas were transferred to a sterile flask containing 50 % silica sand/vermiculite mix. The mutation to be tested for competitiveness was transduced into *R. l.* bv. *viciae* 300 (which carries no antibiotic resistance marker). The strain to be tested and *R. l.* bv. *viciae* 3841 were suspended in water, the concentration was adjusted to $OD_{600} = 10^{-5}$ and then 5 ml of each strain were mixed. The resulting 10 ml were inoculated onto one flask after 5 days of growth. The flasks were then wrapped in black plastic and transferred to a growth chamber. After 28 days the peas were removed from the sand/vermiculite mixture, washed and the nodules were harvested. The nodule surface was sterilised by a short wash with 70 % EtOH, followed by 1 min sterilisation in 10 % bleach and 5 washes to remove the bleach. The nodules were transferred into the wells of microtiter plates, each containing 50 µl of 10% glycerol and crushed with a sterilised metal rod. Aliquots of 5 µl were spotted onto selective TY plates (one containing Strep, one containing the antibiotic for which the mutant carried the antibiotic resistance gene). Plates were incubated for 3 days at 28 ºC and scored.

Chapter 6: General discussion

Previous work had shown that a small regulatory gene, *cinS*, is encoded in the *cin* locus, downstream of and co-transcribed with the AHL synthase gene *cinI* (Edwards *et al.*, 2009). All known regulatory effects of the *cin* QS system were mediated via CinS and therefore the molecular mechanism by which CinS regulates gene expression was investigated (Chapter 2 and 3). I looked for proteins that interacted with CinS using a pull-down assay with total *Rhizobium* cell lysate, which identified the transcriptional regulator PraR. Using *in vitro* gel shift assays and *in vivo* transcriptional studies, it was shown that PraR acts as a transcriptional repressor of the QS regulator *rhiR*. CinS functions as an inducer of *rhiR* by acting as an antirepressor of PraR. A PraR binding box was identified and was also found in the promoters of other regulatory targets of CinS. Two additional PraR targets were the QS regulator *raiR* and the glycanase *plyB,* both of which are expressed in a similar manner as *rhiR*. CinS thus functions to couple the induction of the *cin* genes with the induction of the *rhi* and *rai* genes in *R. leguminosarum.*

Because CinS acts via PraR, the expression level of *praR* is very important for gene regulation. In *S. meliloti* the *praR* homologue *phrR* was induced by different stresses and acid pH (Reeve *et al.*, 1998). No induction of *praR* by acid pH was observed in *R. leguminosarum*. Transcriptional fusions showed that mutations in *cinS* and *praR* both induced *praR* expression, although it is not clear at this point why mutation of *cinS* increased *praR* expression. One possibility is that the LuxR-type regulator ExpR stops CinS from acting as an antirepressor on the *praR* promoter. ExpR had been identified in previous work to be involved in CinS-mediated gene regulation (Edwards *et al.*, 2009). Using bacterial two hybrid analysis it was found that ExpR bound to both CinS and PraR, and transcriptional studies showed that ExpR repressed *praR* expression. This repression is thought to lead to the induction of PraR targets like *rhiR, raiR* and *plyB*. To my knowledge, this is the first time a protein has been identified that interacts with both a repressor and its antirepressor. Unfortunately, the possible role for ExpR could not be tested *in vitro*, due to the difficulties in the purification of ExpR.

In the suggested mechanism of *praR* regulation, the relative concentrations of PraR, ExpR and CinS could be crucial. Even slight changes in levels of one of the proteins could have important effects on *praR* expression and on the expression of PraR target genes. In Chapter 4, two genes (*rosR* and RL0149) were identified that were repressed by CinS and PraR, but not by ExpR. This expression pattern could indicate that another regulator exists that stops CinS from acting as a PraR-antirepressor. PraR pull down experiments would be interesting, not only to further study the interactions between CinS-PraR and ExpR-PraR, but also to see whether other regulators interact with PraR.

As ExpR regulates *praR* expression, regulation of *expR* expression and ExpR activity are also important. ExpR has a predicted AHL-binding domain although it appears to function independently of CinI-made AHLs. Other LuxR-type regulators that function independently of AHLs have been identified, namely QscR in *P. aeruginosa,* CarR in *S. marcescens* and OryR and XccR in *Xanthomonas species* (Chugani *et al.*, 2001; Cox *et al.*, 1998). The modes of action of these AHL-independent LuxR-type regulators differ: QscR regulates gene expression by forming inactive dimers with RhlR and LasR. However, this mode of action is unlikely to be the case for ExpR, as CinR is not required for the expression of target genes of ExpR. In addition, the transcriptional data suggest a direct role for ExpR as an independent repressor of *praR* expression, also contesting a post-translational mechanism of action. It is not known how CarR exerts it regulatory effect, but OryR and XccR respond to plant-made metabolites. Considering the role of PraR in nodulation (Chapter 4), it is possible that ExpR responds to signals from the plants for regulation of *praR* expression.

To further characterise the interaction between CinS and PraR, it would be useful to co-crystallise both proteins. So far, attempts to crystallise CinS were unsuccessful, and it might be possible that both proteins need to be present before crystals can be obtained. Many different modes of action for anti-repressors have been reported since their initial discovery in 1970 (Oppenheim *et al.*). Co-crystallisation of the sporulation regulator SinR with its antirepressor SinI from *Bacillus subtilis* showed that SinI functions by forming inactive dimers with SinR (Lewis *et al.*, 1996). In the absence of SinI, SinR multimerises to a tetramer and the SinR tetramer can bind to DNA. Like PraR, SinR is a lambda repressor-like regulator, and SinI shows amino acid homology to SinR, allowing for heterodimerisation to occur. However, there is no amino acid homology between PraR and CinS. Other examples where an anti-repressor inhibits the DNA-binding activity of a repressor have been described: CarA-CarS in

Myxococcus Xanthus (Leon *et al.*, 2010), MexR-ArmR in *P. aeruginosa* (Wilke *et al.*, 2008) and PpsR-AppA in *Rhodobacter sphaeroides* (Masuda & Bauer, 2002). Different modes of actions have been described as well. The RstR-anti-repressor RstC in phage CTXphi functions by inducing the formation of insoluble RstR aggregates (Davis *et al.*, 2002), while ImmA in *B. subtilis* causes cleavage of the ImmR repressor (Bose *et al.*, 2008). Further study of the CinS-PraR interaction could provide more insights into how CinS prevents PraR from repressing gene expression.

Co-purification of proteins might also solve the problems encountered while purifying ExpR. *R. leguminosarum* ExpR was found to be highly insoluble (at different pH's and salt concentrations) and attempts to refold the protein were unsuccessful (Chapter 3). Fusing ExpR to MBP increased solubility, but no activity of the fusion protein was seen in gel shift assays. Even after cleavage of the tag from the fusion protein no DNA-binding activity could be shown. The behaviour of *R. leguminosarum* ExpR during purification differs from that of *S. meliloti* ExpR (58 % sequence identity), which was found to be soluble and active (McIntosh *et al.*, 2008). Despite this, the problems with purification are not completely unexpected, as stability problems are encountered for most other LuxR-type regulators (Urbanowski *et al.*, 2004). As ExpR interacts with PraR and CinS, it might be possible that co-purification with one of these proteins would help in the stabilisation of the protein.

Mutation of *cinS* seemed to be responsible for all phenotypes of a *cinI* mutant, indicating that CinS is the main regulator of the *cin* QS system. Thus far, the only promoter found to be induced by CinR is the *cinIS* operon itself (Edwards *et al.*, 2009). CinS is conserved in *R. etli,* where it regulates swarming. This phenotype was previously attributed to CinI-made AHLs (Chapter 2 and Daniels *et al.*, 2006). By extension, other phenotypes of *cin*-like QS systems are predicted to be CinS-dependent, although this remains to be investigated. Root hair attachment and nodulation have been shown to be controlled by the *mrtI/R* QS genes in *M. tianshanense* (Cao *et al.*, 2009). Considering the role of PraR and CinS in root hair attachment, this might indicate that the CinS homologue has got a similar role in *R. leguminosarum* as in *M. tianshanense.*

One of the most interesting observations in this work was that *praR* mutation causes an increase in nodulation competitiveness. This phenotype was likely due to the increased attachment of the *praR* mutant to pea root hairs. Using different approaches, target genes -encoding proteins involved in polysaccharide metabolism and adhesion-

of PraR were determined (Chapter 4). Attachment studies have focussed on the *praR* mutant and further study of the *cinS* and *expR* mutants is desirable. The *cinS* and *expR* mutants did not show any difference in nodulation competitiveness, but they did produce a thicker biofilm ring in flasks (similar to the one formed by the *praR* mutant). Therefore, the factor(s) that are causing the increased biofilm rings are probably not directly responsible for the nodulation competitiveness phenotype. It is likely that several of the identified factors contribute towards increased attachment in different circumstances. Other PraR-regulated factors were identified that could help to adapt to life in the rhizosphere and during infection. These include the RhiABC proteins (Cubo *et al.*, 1992), an ABC-transporter protein (RL1049), *che2* chemotaxis proteins and an aquaporin (RL3302) that is predicted to be involved in osmotic stress regulation.

It is not known whether CinR regulates an additional set of genes in response to CinI-made AHLs, independently of CinS. A *cinR* mutant microarray would be the best way to study this. This was not yet done as it was clear that the *cinS, expR* and *praR* mutant cultures were not grown under optimal conditions for their respective microarrays (Chapter 4). In future experiments, it would be worthwhile trying to improve the growth conditions and then repeat these microarrays, including one for the *cinR* mutant. Finding conditions that are more suitable for identifying all the regulatory targets of CinS, PraR and ExpR could be challenging. Based on the observations that PraR plays an important role in nodulation competitiveness, preliminary microarrays under rhizosphere conditions have been done for the *cinS* and *praR* mutants (Ramakrishnan Karunakaran, personal communication). Unfortunately these were unsuccessful in identifying PraR-regulated genes. Another option is doing chromatin immunoprecipitation experiments, by pulling down DNA fragments that interact with PraR or ExpR and subsequently hybridising them on tiled microarrays. This method could be more sensitive to pick up direct regulatory targets.

The regulatory mechanism used by CinS and PraR that was described here probably functions to finetune gene regulation in the rhizosphere to improve legume-*Rhizobium* interactions. CinS and PraR are not encoded close to each other in the genome, which is often the case for antirepressor-repressor complexes (Bose *et al.*, 2008; Lewis *et al.*, 1996). However, other situations where repressors and antirepressors are encoded on different loci have been observed previously (Lopez-Rubio *et al.*, 2002; Wilke *et al.*, 2008). CinS is not conserved among the Alpha-proteobacteria, but PraR is (Akiba *et al.*, 2010). This raises questions about how the CinS-PraR system has evolved and

what the function of PraR in other bacteria is. Possibly, other small proteins are present that fulfil the antirepressor role of CinS and it would be interesting to study whether the regulatory mechanism described here for PraR and CinS is more generally conserved.

References

Abedin, M. & King, N. (2008). The premetazoan ancestry of cadherins. *Science* **319**, 946-948.

Ahmer, B. M., van Reeuwijk, J., Timmers, C. D., Valentine, P. J. & Heffron, F. (1998). *Salmonella typhimurium* encodes an SdiA homolog, a putative quorum sensor of the LuxR family, that regulates genes on the virulence plasmid. *J Bacteriol* **180**, 1185-1193.

Akiba, N., Aono, T., Toyazaki, H., Sato, S. & Oyaizu, H. (2010). A *phrR*-like gene, *praR*, of *Azorhizobium caulinodans* ORS571 is essential for symbiosis with *Sesbania rostrata*, and is involved in the expression of *reb* genes. *Appl Environ Microbiol*.

Al-Hussaini, R. & Mahasneh, A. M. (2009). Microbial growth and quorum sensing antagonist activities of herbal plants extracts. *Molecules* **14**, 3425-3435.

Albus, A. M., Pesci, E. C., Runyen-Janecky, L. J., West, S. E. & Iglewski, B. H. (1997). Vfr controls quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* **179**, 3928-3935.

Ames, P. & Bergman, K. (1981). Competitive advantage provided by bacterial motility in the formation of nodules by *Rhizobium meliloti. J Bacteriol* **148**, 728-908 p.

Ampomah, O. Y., Jensen, J. B. & Bhuvaneswari, T. V. (2008). Lack of trehalose catabolism in *Sinorhizobium* species increases their nodulation competitiveness on certain host genotypes. *New Phytol* **179**, 495-504.

Aneja, P., Zachertowska, A. & Charles, T. C. (2005). Comparison of the symbiotic and competition phenotypes of *Sinorhizobium meliloti* PHB synthesis and degradation pathway mutants. *Can J Microbiol* **51**, 599-604.

Appleby, C. A., Tjepkema, J. D. & Trinick, M. J. (1983). Hemoglobin in a nonleguminous plant, *Parasponia*: possible genetic origin and function in nitrogen fixation. *Science* **220**, 951-953.

Armitage, J. P., Gallagher, A. & Johnston, A. W. (1988). Comparison of the chemotactic behaviour of *Rhizobium leguminosarum* with and without the nodulation plasmid. *Mol Microbiol* **2**, 743-748.

Atkinson, S., Throup, J. P., Stewart, G. S. & Williams, P. (1999). A hierarchical quorum-sensing system in *Yersinia pseudotuberculosis* is involved in the regulation of motility and clumping. *Mol Microbiol* **33**, 1267-1277.

Atkinson, S., Chang, C. Y., Patrick, H. L., Buckley, C. M., Wang, Y., Sockett, R. E., Camara, M. & Williams, P. (2008). Functional interplay between the *Yersinia pseudotuberculosis* YpsRI and YtbRI quorum sensing systems modulates swimming motility by controlling expression of *flhDC* and *fliA*. *Mol Microbiol* **69**, 137-151.

Attila, C., Ueda, A. & Wood, T. K. (2008). PA2663 (PpyR) increases biofilm formation in *Pseudomonas aeruginosa* PAO1 through the *psl* operon and stimulates virulence and quorum-sensing phenotypes. *Appl Microbiol Biotechnol* **78**, 293-307.

Ausmees, N., Jacobsson, K. & Lindberg, M. (2001). A unipolarly located, cell-surface-associated agglutinin, RapA, belongs to a family of *Rhizobium*-adhering proteins (Rap) in *Rhizobium leguminosarum* bv. *trifolii*. *Microbiology* **147**, 549-559.

Bagdasarian, M., Lurz, R., Ruckert, B., Franklin, F. C. H., Bagdasarian, M., Frey, J. & Timmis, K. N. (1981). Specific-purpose plasmid cloning vectors: 2 broad host range, high copy mumber, RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas.* . *Gene* **16**, 237-247.

Bahlawane, C., McIntosh, M., Krol, E. & Becker, A. (2008). *Sinorhizobium meliloti* regulator MucR couples exopolysaccharide synthesis and motility. *Mol Plant Microbe Interact* **21**, 1498-1509.

Barken, K. B., Pamp, S. J., Yang, L. & other authors (2008). Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in *Pseudomonas aeruginosa* biofilms. *Environ Microbiol* **10**, 2331-2343.

Barnard, A. M. & Salmond, G. P. (2007). Quorum sensing in *Erwinia* species. *Anal Bioanal Chem* **387**, 415-423.

Barra-Bily, L., Fontenelle, C., Jan, G., Flechard, M., Trautwetter, A., Pandey, S. P., Walker, G. C. & Blanco, C. (2010a). Proteomic alterations explain phenotypic changes in *Sinorhizobium meliloti* lacking the RNA chaperone Hfq. *J Bacteriol* **192**, 1719-1729.

Barra-Bily, L., Pandey, S. P., Trautwetter, A., Blanco, C. & Walker, G. C. (2010b). The *Sinorhizobium meliloti* RNA chaperone Hfq mediates symbiosis of *S. meliloti* and alfalfa. *J Bacteriol* **192**, 1710-1718.

Bartels, F. W., McIntosh, M., Fuhrmann, A., Metzendorf, C., Plattner, P., Sewald, N., Anselmetti, D., Ros, R. & Becker, A. (2007). Effector-stimulated single molecule protein-DNA interactions of a quorum-sensing system in *Sinorhizobium meliloti*. *Biophys J* **92**, 4391-4400.

Bassler, B. L., Wright, M., Showalter, R. E. & Silverman, M. R. (1993). Intercellular signalling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence. *Mol Microbiol* **9**, 773-786.

Bassler, B. L., Wright, M. & Silverman, M. R. (1994). Multiple signalling systems controlling expression of luminescence in *Vibrio harveyi:* sequence and function of genes encoding a second sensory pathway. *Mol Microbiol* **13**, 273-286.

Baysse, C., Cullinane, M., Denervaud, V., Burrowes, E., Dow, J. M., Morrissey, J. P., Tam, L., Trevors, J. T. & O'Gara, F. (2005). Modulation of quorum sensing in

Pseudomonas aeruginosa through alteration of membrane properties. *Microbiology* **151**, 2529-2542.

Bazire, A., Dheilly, A., Diab, F., Morin, D., Jebbar, M., Haras, D. & Dufour, A. (2005). Osmotic stress and phosphate limitation alter production of cell-to-cell signal molecules and rhamnolipid biosurfactant by *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* **253**, 125-131.

Beck von Bodman, S., Hayman, G. T. & Farrand, S. K. (1992). Opine catabolism and conjugal transfer of the nopaline Ti plasmid pTiC58 are coordinately regulated by a single repressor. *Proc Natl Acad Sci U S A* **89**, 643-647.

Beringer, J. E. (1974). R factor transfer in *Rhizobium leguminosarum*. *J Gen Microbiol* **84**, 188-198.

Bertani, I., Sevo, M., Kojic, M. & Venturi, V. (2003). Role of GacA, LasI, RhlI, Ppk, PsrA, Vfr and ClpXP in the regulation of the stationary-phase sigma factor *rpoS*/RpoS in *Pseudomonas*. *Arch Microbiol* **180**, 264-271.

Bertani, I., Rampioni, G., Leoni, L. & Venturi, V. (2007). The *Pseudomonas putida* Lon protease is involved in *N*-acyl homoserine lactone quorum sensing regulation. *BMC Microbiol* **7**, 71.

Bertram-Drogatz, P. A., Quester, I., Becker, A. & Puhler, A. (1998). The *Sinorhizobium meliloti* MucR protein, which is essential for the production of high-molecular-weight succinoglycan exopolysaccharide, binds to short DNA regions upstream of *exoH* and *exoY*. *Mol Gen Genet* **257**, 433-441.

Bittinger, M. A., Milner, J. L., Saville, B. J. & Handelsman, J. (1997). *rosR*, a determinant of nodulation competitiveness in *Rhizobium etli*. *Mol Plant Microbe Interact* **10**, 180-186.

Bittinger, M. A. & Handelsman, J. (2000). Identification of genes in the RosR regulon of *Rhizobium etli*. *J Bacteriol* **182**, 1706-1713.

Bjarnsholt, T. & Givskov, M. (2007). The role of quorum sensing in the pathogenicity of the cunning aggressor Pseudomonas aeruginosa. *Anal Bioanal Chem* **387**, 409-414.

Bodini, S. F., Manfredini, S., Epp, M., Valentini, S. & Santori, F. (2009). Quorum sensing inhibition activity of garlic extract and p-coumaric acid. *Lett Appl Microbiol* **49**, 551-555.

Bollinger, N., Hassett, D. J., Iglewski, B. H., Costerton, J. W. & McDermott, T. R. (2001). Gene expression in *Pseudomonas aeruginosa*: evidence of iron override effects on quorum sensing and biofilm-specific gene regulation. *J Bacteriol* **183**, 1990-1996.

Boncompagni, E., Osteras, M., Poggi, M. C. & le Rudulier, D. (1999). Occurrence of choline and glycine betaine uptake and metabolism in the family rhizobiaceae and their roles in osmoprotection. *Appl Environ Microbiol* **65**, 2072-2077.

Boon, C., Deng, Y., Wang, L. H., He, Y., Xu, J. L., Fan, Y., Pan, S. Q. & Zhang, L. H. (2008). A novel DSF-like signal from *Burkholderia cenocepacia* interferes with *Candida albicans* morphological transition. *Isme J* **2**, 27-36.

Bose, B., Auchtung, J. M., Lee, C. A. & Grossman, A. D. (2008). A conserved anti-repressor controls horizontal gene transfer by proteolysis. *Mol Microbiol* **70**, 570-582.

Bose, J. L., Kim, U., Bartkowski, W., Gunsalus, R. P., Overley, A. M., Lyell, N. L., Visick, K. L. & Stabb, E. V. (2007). Bioluminescence in *Vibrio fischeri* is controlled by the redox-responsive regulator ArcA. *Mol Microbiol* **65**, 538-553.

Bottomley, M. J., Muraglia, E., Bazzo, R. & Carfi, A. (2007). Molecular insights into quorum sensing in the human pathogen Pseudomonas aeruginosa from the structure of the virulence regulator LasR bound to its autoinducer. *J Biol Chem* **282**, 13592-13600.

Braeken, K., Daniels, R., Vos, K., Fauvart, M., Bachaspatimayum, D., Vanderleyden, J. & Michiels, J. (2008). Genetic determinants of swarming in *Rhizobium etli*. *Microb Ecol* **55**, 54-64.

Branny, P., Pearson, J. P., Pesci, E. C., Kohler, T., Iglewski, B. H. & Van Delden, C. (2001). Inhibition of quorum sensing by a *Pseudomonas aeruginosa dksA* homologue. *J Bacteriol* **183**, 1531-1539.

Bredenbruch, F., Nimtz, M., Wray, V., Morr, M., Muller, R. & Haussler, S. (2005). Biosynthetic pathway of *Pseudomonas aeruginosa* 4-hydroxy-2-alkylquinolines. *J Bacteriol* **187**, 3630-3635.

Bredenbruch, F., Geffers, R., Nimtz, M., Buer, J. & Haussler, S. (2006). The *Pseudomonas aeruginosa* quinolone signal (PQS) has an iron-chelating activity. *Environ Microbiol* **8**, 1318-1329.

Brelles-Marino, G. & Bedmar, E. J. (2001). Detection, purification and characterisation of quorum-sensing signal molecules in plant-associated bacteria. *J Biotechnol* **91**, 197-209.

Bruckner, R. & Titgemeyer, F. (2002). Carbon catabolite repression in bacteria: choice of the carbon source and autoregulatory limitation of sugar utilization. *FEMS Microbiol Lett* **209**, 141-148.

Buchanan-Wollaston, A. V. (1979). Generalized transduction in *Rhizobium leguminosarum*. *Journal general microbiology*.

Burrowes, E., Abbas, A., O'Neill, A., Adams, C. & O'Gara, F. (2005). Characterisation of the regulatory RNA RsmB from *Pseudomonas aeruginosa* PAO1. *Res Microbiol* **156**, 7-16.

Busso, D., Delagoutte-Busso, B. & Moras, D. (2005). Construction of a set Gateway-based destination vectors for high-throughput cloning and expression screening in *Escherichia coli*. *Anal Biochem* **343**, 313-321.

Byers, J. T., Lucas, C., Salmond, G. P. & Welch, M. (2002). Nonenzymatic turnover of an *Erwinia carotovora* quorum-sensing signaling molecule. *J Bacteriol* **184**, 1163-1171.

Caetano-Anolles, G., Crist-Estes, D. K. & Bauer, W. D. (1988a). Chemotaxis of *Rhizobium meliloti* to the plant flavone luteolin requires functional nodulation genes. *J Bacteriol* **170**, 3164-3169.

Caetano-Anolles, G., Wall, L. G., De Micheli, A. T., Macchi, E. M., Bauer, W. D. & Favelukes, G. (1988b). Role of motility and chemotaxis in efficiency of nodulation by *Rhizobium meliloti*. *Plant Physiol* **86**, 1228-1235.

Campbell, J., Lin, Q., Geske, G. D. & Blackwell, H. E. (2009). New and unexpected insights into the modulation of LuxR-type quorum sensing by cyclic dipeptides. *ACS Chem Biol* **4**, 1051-1059.

Cao, H., Krishnan, G., Goumnerov, B., Tsongalis, J., Tompkins, R. & Rahme, L. G. (2001). A quorum sensing-associated virulence gene of *Pseudomonas aeruginosa* encodes a LysR-like transcription regulator with a unique self-regulatory mechanism. *Proc Natl Acad Sci U S A* **98**, 14613-14618.

Cao, H., Yang, M., Zheng, H., Zhang, J., Zhong, Z. & Zhu, J. (2009). Complex quorum-sensing regulatory systems regulate bacterial growth and symbiotic nodulation in *Mesorhizobium tianshanense*. *Arch Microbiol* **191**, 283-289.

Cao, J. G. & Meighen, E. A. (1989). Purification and structural identification of an autoinducer for the luminescence system of *Vibrio harveyi*. *J Biol Chem* **264**, 21670-21676.

Carty, N. L., Layland, N., Colmer-Hamood, J. A., Calfee, M. W., Pesci, E. C. & Hamood, A. N. (2006). PtxR modulates the expression of QS-controlled virulence factors in the *Pseudomonas aeruginosa* strain PAO1. *Mol Microbiol* **61**, 782-794.

Chai, Y., Zhu, J. & Winans, S. C. (2001). TrlR, a defective TraR-like protein of *Agrobacterium tumefaciens*, blocks TraR function in vitro by forming inactive TrlR:TraR dimers. *Mol Microbiol* **40**, 414-421.

Chalupowicz, L., Barash, I., Panijel, M., Sessa, G. & Manulis-Sasson, S. (2009). Regulatory interactions between quorum-sensing, auxin, cytokinin, and the Hrp regulon in relation to gall formation and epiphytic fitness of *Pantoea agglomerans* pv. *gypsophilae*. *Mol Plant Microbe Interact* **22**, 849-856.

Chancey, S. T., Wood, D. W. & Pierson, L. S., 3rd (1999). Two-component transcriptional regulation of *N*-acyl-homoserine lactone production in *Pseudomonas aureofaciens*. *Appl Environ Microbiol* **65**, 2294-2299.

Chatterjee, A., Cui, Y., Hasegawa, H. & Chatterjee, A. K. (2007). PsrA, the *Pseudomonas* sigma regulator, controls regulators of epiphytic fitness, quorum-sensing signals, and plant interactions in *Pseudomonas syringae* pv. tomato strain DC3000. *Appl Environ Microbiol* **73**, 3684-3694.

Chatterjee, J., Miyamoto, C. M., Zouzoulas, A., Lang, B. F., Skouris, N. & Meighen, E. A. (2002). MetR and CRP bind to the *Vibrio harveyi lux* promoters and regulate luminescence. *Mol Microbiol* **46**, 101-111.

Chen, G., Jeffrey, P. D., Fuqua, C., Shi, Y. & Chen, L. (2007). Structural basis for antiactivation in bacterial quorum sensing. *Proc Natl Acad Sci U S A* **104**, 16474-16479.

Chen, W. P. & Kuo, T. T. (1993). A simple and rapid method for the preparation of gram-negative bacterial genomic DNA. *Nucleic Acids Res* **21**, 2260.

Chen, X., Schauder, S., Potier, N., Van Dorsselaer, A., Pelczer, I., Bassler, B. L. & Hughson, F. M. (2002). Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* **415**, 545-549.

Chevrot, R., Rosen, R., Haudecoeur, E., Cirou, A., Shelp, B. J., Ron, E. & Faure, D. (2006). GABA controls the level of quorum-sensing signal in *Agrobacterium tumefaciens*. *Proc Natl Acad Sci U S A* **103**, 7460-7464.

Chin, K. H., Lee, Y. C., Tu, Z. L. & other authors (2010). The cAMP receptor-like protein CLP is a novel c-di-GMP receptor linking cell-cell signaling to virulence gene expression in *Xanthomonas campestris*. *J Mol Biol* **396**, 646-662.

Choi, J., Shin, D. & Ryu, S. (2007). Implication of quorum sensing in *Salmonella enterica* serovar *typhimurium* virulence: the *luxS* gene is necessary for expression of genes in pathogenicity island 1. *Infect Immun* **75**, 4885-4890.

Choi, S. H. & Greenberg, E. P. (1992). Genetic dissection of DNA binding and luminescence gene activation by the *Vibrio fischeri* LuxR protein. *J Bacteriol* **174**, 4064-4069.

Chugani, S. A., Whiteley, M., Lee, K. M., D'Argenio, D., Manoil, C. & Greenberg, E. P. (2001). QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **98**, 2752-2757.

Chun, C. K., Ozer, E. A., Welsh, M. J., Zabner, J. & Greenberg, E. P. (2004). Inactivation of a *Pseudomonas aeruginosa* quorum-sensing signal by human airway epithelia. *Proc Natl Acad Sci U S A* **101**, 3587-3590.

Clarke, M. B. & Sperandio, V. (2005). Transcriptional autoregulation by quorum sensing *Escherichia col*i regulators B and C (QseBC) in enterohaemorrhagic *E. coli* (EHEC). *Mol Microbiol* **58**, 441-455.

Clarke, M. B., Hughes, D. T., Zhu, C., Boedeker, E. C. & Sperandio, V. (2006). The QseC sensor kinase: a bacterial adrenergic receptor. *Proc Natl Acad Sci U S A* **103**, 10420-10425.

Clough, S. J., Flavier, A. B., Schell, M. A. & Denny, T. P. (1997a). Differential expression of virulence genes and motility in *Ralstonia* (*Pseudomonas*) *solanacearum* during exponential growth. *Appl Environ Microbiol* **63**, 844-850.

Clough, S. J., Lee, K. E., Schell, M. A. & Denny, T. P. (1997b). A two-component system in *Ralstonia* (*Pseudomonas*) *solanacearum* modulates production of PhcA-regulated virulence factors in response to 3-hydroxypalmitic acid methyl ester. *J Bacteriol* **179**, 3639-3648.

Cornelis, P. & Aendekerk, S. (2004). A new regulator linking quorum sensing and iron uptake in *Pseudomonas aeruginosa*. *Microbiology* **150**, 752-756.

Cornelis, P., Matthijs, S. & Van Oeffelen, L. (2009). Iron uptake regulation in *Pseudomonas aeruginosa*. *Biometals* **22**, 15-22.

Coulthurst, S. J., Kurz, C. L. & Salmond, G. P. (2004). *luxS* mutants of *Serratia* defective in autoinducer-2-dependent 'quorum sensing' show strain-dependent impacts on virulence and production of carbapenem and prodigiosin. *Microbiology* **150**, 1901-1910.

Cox, A. R., Thomson, N. R., Bycroft, B., Stewart, G. S., Williams, P. & Salmond, G. P. (1998). A pheromone-independent CarR protein controls carbapenem antibiotic synthesis in the opportunistic human pathogen *Serratia marcescens*. *Microbiology* **144 (Pt 1)**, 201-209.

Crossman, L. C., Castillo-Ramirez, S., McAnnula, C. & other authors (2008). A common genomic framework for a diverse assembly of plasmids in the symbiotic nitrogen fixing bacteria. *PLoS One* **3**, e2567.

Cubo, M. T., Economou, A., Murphy, G., Johnston, A. W. & Downie, J. A. (1992). Molecular characterization and regulation of the rhizosphere-expressed genes *rhiABCR* that can influence nodulation by *Rhizobium leguminosarum* biovar *viciae*. *J Bacteriol* **174**, 4026-4035.

Czajkowski, R. & Jafra, S. (2009). Quenching of acyl-homoserine lactone-dependent quorum sensing by enzymatic disruption of signal molecules. *Acta Biochim Pol* **56**, 1-16.

D'Haeze, W. & Holsters, M. (2002). Nod factor structures, responses, and perception during initiation of nodule development. *Glycobiology* **12**, 79R-105R.

Daniels, R., De Vos, D. E., Desair, J. & other authors (2002). The *cin* quorum sensing locus of *Rhizobium etli* CNPAF512 affects growth and symbiotic nitrogen fixation. *J Biol Chem* **277**, 462-468.

Daniels, R., Vanderleyden, J. & Michiels, J. (2004). Quorum sensing and swarming migration in bacteria. *FEMS Microbiol Rev* **28**, 261-289.

Daniels, R., Reynaert, S., Hoekstra, H. & other authors (2006). Quorum signal molecules as biosurfactants affecting swarming in *Rhizobium etli*. *Proc Natl Acad Sci U S A* **103**, 14965-14970.

Danino, V. E., Wilkinson, A., Edwards, A. & Downie, J. A. (2003). Recipient-induced transfer of the symbiotic plasmid pRL1JI in *Rhizobium leguminosarum* bv. *viciae* is regulated by a quorum-sensing relay. *Mol Microbiol* **50**, 511-525.

Davis, B. M., Kimsey, H. H., Kane, A. V. & Waldor, M. K. (2002). A satellite phage-encoded antirepressor induces repressor aggregation and cholera toxin gene transfer. *Embo J* **21**, 4240-4249.

Dazzo, F. B., Napoli, C. A. & Hubbell, D. H. (1976). Adsorption of bacteria to roots as related to host specificity in the *Rhizobium*-clover symbiosis. *Appl Environ Microbiol* **32**, 166-171.

Dazzo, F. B. (1981). Bacterial attachment as related to cellular recognition in the *Rhizobium*-legume symbiosis. *J Supramol Struct Cell Biochem* **16**, 29-41.

de Kievit, T., Seed, P. C., Nezezon, J., Passador, L. & Iglewski, B. H. (1999). RsaL, a novel repressor of virulence gene expression in *Pseudomonas aeruginosa*. *J Bacteriol* **181**, 2175-2184.

de Kievit, T. R. & Iglewski, B. H. (2000). Bacterial quorum sensing in pathogenic relationships. *Infect Immun* **68**, 4839-4849.

De Lay, N. & Gottesman, S. (2009). The Crp-activated small noncoding regulatory RNA CyaR (RyeE) links nutritional status to group behavior. *J Bacteriol* **191**, 461-476.

Degrassi, G., Aguilar, C., Bosco, M., Zahariev, S., Pongor, S. & Venturi, V. (2002). Plant growth-promoting *Pseudomonas putida* WCS358 produces and secretes four cyclic dipeptides: cross-talk with quorum sensing bacterial sensors. *Curr Microbiol* **45**, 250-254.

Degrassi, G., Devescovi, G., Solis, R., Steindler, L. & Venturi, V. (2007). *Oryza sativa* rice plants contain molecules that activate different quorum-sensing *N*-acyl homoserine lactone biosensors and are sensitive to the specific AiiA lactonase. *FEMS Microbiol Lett* **269**, 213-220.

DeLisa, M. P., Valdes, J. J. & Bentley, W. E. (2001). Mapping stress-induced changes in autoinducer AI-2 production in chemostat-cultivated *Escherichia coli* K-12. *J Bacteriol* **183**, 2918-2928.

Deutscher, J., Francke, C. & Postma, P. W. (2006). How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. *Microbiol Mol Biol Rev* **70**, 939-1031.

Deziel, E., Lepine, F., Milot, S., He, J., Mindrinos, M. N., Tompkins, R. G. & Rahme, L. G. (2004). Analysis of *Pseudomonas aeruginosa* 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication. *Proc Natl Acad Sci U S A* **101**, 1339-1344.

Deziel, E., Gopalan, S., Tampakaki, A. P., Lepine, F., Padfield, K. E., Saucier, M., Xiao, G. & Rahme, L. G. (2005). The contribution of MvfR to *Pseudomonas aeruginosa* pathogenesis and quorum sensing circuitry regulation: multiple quorum sensing-regulated genes are modulated without affecting *lasRI, rhlRI* or the production of *N*-acyl-L-homoserine lactones. *Mol Microbiol* **55**, 998-1014.

Dharmatilake, A. J. & Bauer, W. D. (1992). Chemotaxis of *Rhizobium meliloti* towards nodulation gene-inducing compounds from alfalfa roots. *Appl Environ Microbiol* **58**, 1153-1158.

Diaz, C. L., Logman, T., Stam, H. C. & Kijne, J. W. (1995). Sugar-binding activity of pea lectin expressed in white cover hairy roots. *Plant Physiol* **109**, 1167-1177.

Dibb, N. J., Downie, J. A. & Brewin, N. J. (1984). Identification of a rhizosphere protein encoded by the symbiotic plasmid of *Rhizobium leguminosarum*. *J Bacteriol* **158**, 621-627.

Diggle, S. P., Winzer, K., Lazdunski, A., Williams, P. & Camara, M. (2002). Advancing the quorum in *Pseudomonas aeruginosa*: MvaT and the regulation of *N*-acylhomoserine lactone production and virulence gene expression. *J Bacteriol* **184**, 2576-2586.

Diggle, S. P., Winzer, K., Chhabra, S. R., Worrall, K. E., Camara, M. & Williams, P. (2003). The *Pseudomonas aeruginosa* quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates *rhl*-dependent genes at the onset of stationary phase and can be produced in the absence of LasR. *Mol Microbiol* **50**, 29-43.

Diggle, S. P., Lumjiaktase, P., Dipilato, F., Winzer, K., Kunakorn, M., Barrett, D. A., Chhabra, S. R., Camara, M. & Williams, P. (2006). Functional genetic analysis reveals a 2-alkyl-4-quinolone signaling system in the human pathogen *Burkholderia pseudomallei* and related bacteria. *Chem Biol* **13**, 701-710.

Diggle, S. P., Matthijs, S., Wright, V. J. & other authors (2007). The *Pseudomonas aeruginosa* 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment. *Chem Biol* **14**, 87-96.

Ditta, G., Stanfield, S., Corbin, D. & Helinski, D. R. (1980). Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proc Natl Acad Sci U S A* **77**, 7347-7351.

Dixon, R. & Kahn, D. (2004). Genetic regulation of biological nitrogen fixation. *Nat Rev Microbiol* **2**, 621-631.

Dominguez-Ferreras, A., Soto, M. J., Perez-Arnedo, R., Olivares, J. & Sanjuan, J. (2009). Importance of trehalose biosynthesis for *Sinorhizobium meliloti* osmotolerance and nodulation of alfalfa roots. *J Bacteriol*.

Dong, Y. H., Xu, J. L., Li, X. Z. & Zhang, L. H. (2000). AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. *Proc Natl Acad Sci U S A* **97**, 3526-3531.

Dong, Y. H., Wang, L. H., Xu, J. L., Zhang, H. B., Zhang, X. F. & Zhang, L. H. (2001). Quenching quorum-sensing-dependent bacterial infection by an *N*-acyl homoserine lactonase. *Nature* **411**, 813-817.

Dong, Y. H. & Zhang, L. H. (2005). Quorum sensing and quorum-quenching enzymes. *J Microbiol* **43 Spec No**, 101-109.

Dong, Y. H., Zhang, X. F., Xu, J. L., Tan, A. T. & Zhang, L. H. (2005). VqsM, a novel AraC-type global regulator of quorum-sensing signalling and virulence in *Pseudomonas aeruginosa*. *Mol Microbiol* **58**, 552-564.

Dong, Y. H., Zhang, X. F., An, S. W., Xu, J. L. & Zhang, L. H. (2008). A novel two-component system BqsS-BqsR modulates quorum sensing-dependent biofilm decay in *Pseudomonas aeruginosa*. *Commun Integr Biol* **1**, 88-96.

Downie, J. A., Ma, Q. S., Knight, C. D., Hombrecher, G. & Johnston, A. W. (1983). Cloning of the symbiotic region of *Rhizobium leguminosarum*: the nodulation genes are between the nitrogenase genes and a *nifA*-like gene. *Embo J* **2**, 947-952.

Downie, J. A. & Walker, S. A. (1999). Plant responses to nodulation factors. *Curr Opin Plant Biol* **2**, 483-489.

Downie, J. A. (2005). Legume haemoglobins: symbiotic nitrogen fixation needs bloody nodules. *Curr Biol* **15**, R196-198.

Downie, J. A. & Gonzalez, J. E. (2008). Cell-to-cell communication in rhizobia: quorum sensing and plant signalling. *Chemical communication among bacteria ASM Press*, 213-232.

Draganov, D. I., Teiber, J. F., Speelman, A., Osawa, Y., Sunahara, R. & La Du, B. N. (2005). Human paraoxonases (PON1, PON2, and PON3) are lactonases with overlapping and distinct substrate specificities. *J Lipid Res* **46**, 1239-1247.

Duan, K. & Surette, M. G. (2007). Environmental regulation of *Pseudomonas aeruginosa* PAO1 Las and Rhl quorum-sensing systems. *J Bacteriol* **189**, 4827-4836.

Dubuis, C. & Haas, D. (2007). Cross-species GacA-controlled induction of antibiosis in pseudomonads. *Appl Environ Microbiol* **73**, 650-654.

Dunlap, P. V. (1999). Quorum regulation of luminescence in *Vibrio fischeri*. *J Mol Microbiol Biotechnol* **1**, 5-12.

Durfee, T., Hansen, A. M., Zhi, H., Blattner, F. R. & Jin, D. J. (2008). Transcription profiling of the stringent response in *Escherichia coli*. *J Bacteriol* **190**, 1084-1096.

Eberhard, A., Burlingame, A. L., Eberhard, C., Kenyon, G. L., Nealson, K. H. & Oppenheimer, N. J. (1981). Structural identification of autoinducer of *Photobacterium fischeri* luciferase. *Biochemistry* **20**, 2444-2449.

Economou, A., Hawkins, F. K., Downie, J. A. & Johnston, A. W. (1989). Transcription of *rhiA*, a gene on a *Rhizobium leguminosarum* bv. *viciae* Sym plasmid, requires *rhiR* and is repressed by flavanoids that induce *nod* genes. *Mol Microbiol* **3**, 87-93.

Edwards, A., Frederix, M., Wisniewski-Dye, F., Jones, J., Zorreguieta, A. & Downie, J. A. (2009). The *cin* and *rai* quorum-sensing regulatory systems in *Rhizobium leguminosarum* are coordinated by ExpR and CinS, a small regulatory protein coexpressed with CinI. *J Bacteriol* **191**, 3059-3067.

Engebrecht, J. & Silverman, M. (1984). Identification of genes and gene products necessary for bacterial bioluminescence. *Proc Natl Acad Sci U S A* **81**, 4154-4158.

Erickson, D. L., Lines, J. L., Pesci, E. C., Venturi, V. & Storey, D. G. (2004). *Pseudomonas aeruginosa relA* contributes to virulence in *Drosophila melanogaster*. *Infect Immun* **72**, 5638-5645.

Farrow, J. M., 3rd, Sund, Z. M., Ellison, M. L., Wade, D. S., Coleman, J. P. & Pesci, E. C. (2008). PqsE functions independently of PqsR-*Pseudomonas* quinolone signal and enhances the *rhl* quorum-sensing system. *J Bacteriol* **190**, 7043-7051.

Federle, M. J. (2009). Autoinducer-2-based chemical communication in bacteria: complexities of interspecies signaling. *Contrib Microbiol* **16**, 18-32.

Feldman, M., Weiss, E. I., Ofek, I. & Steinberg, D. (2009). Interference of cranberry constituents in cell-cell signaling system of *Vibrio harveyi*. *Curr Microbiol* **59**, 469-474.

Ferluga, S. & Venturi, V. (2009). OryR is a LuxR-family protein involved in interkingdom signaling between pathogenic *Xanthomonas oryzae* pv. *oryzae* and rice. *J Bacteriol* **191**, 890-897.

Fic, E., Bonarek, P., Gorecki, A., Kedracka-Krok, S., Mikolajczak, J., Polit, A., Tworzydlo, M., Dziedzicka-Wasylewska, M. & Wasylewski, Z. (2009). cAMP

receptor protein from *Escherichia coli* as a model of signal transduction in proteins--a review. *J Mol Microbiol Biotechnol* **17**, 1-11.

Fidopiastis, P. M., Miyamoto, C. M., Jobling, M. G., Meighen, E. A. & Ruby, E. G. (2002). LitR, a new transcriptional activator in *Vibrio fischeri*, regulates luminescence and symbiotic light organ colonization. *Mol Microbiol* **45**, 131-143.

Figurski, D. H. & Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. *Proc Natl Acad Sci U S A* **76**, 1648-1652.

Finnie, C., Zorreguieta, A., Hartley, N. M. & Downie, J. A. (1998). Characterization of *Rhizobium leguminosarum* exopolysaccharide glycanases that are secreted via a type I exporter and have a novel heptapeptide repeat motif. *J Bacteriol* **180**, 1691-1699.

Flavier, A. B., Ganova-Raeva, L. M., Schell, M. A. & Denny, T. P. (1997). Hierarchical autoinduction in *Ralstonia solanacearum*: control of acyl-homoserine lactone production by a novel autoregulatory system responsive to 3-hydroxypalmitic acid methyl ester. *J Bacteriol* **179**, 7089-7097.

Flavier, A. B., Schell, M. A. & Denny, T. P. (1998). An RpoS (sigmaS) homologue regulates acylhomoserine lactone-dependent autoinduction in *Ralstonia solanacearum*. *Mol Microbiol* **28**, 475-486.

Fouhy, Y., Lucey, J. F., Ryan, R. P. & Dow, J. M. (2006). Cell-cell signaling, cyclic di-GMP turnover and regulation of virulence in *Xanthomonas campestris*. *Res Microbiol* **157**, 899-904.

Fraiberg, M., Borovok, I., Weiner, R. M. & Lamed, R. Discovery and characterization of cadherin domains in *Saccharophagus degradans* 2-40. *J Bacteriol* **192**, 1066-1074.

Fraley, C. D., Rashid, M. H., Lee, S. S., Gottschalk, R., Harrison, J., Wood, P. J., Brown, M. R. & Kornberg, A. (2007). A polyphosphate kinase 1 (*ppk1*) mutant of *Pseudomonas aeruginosa* exhibits multiple ultrastructural and functional defects. *Proc Natl Acad Sci U S A* **104**, 3526-3531.

Franssen, H. J., Vijn, I., Yang, W. C. & Bisseling, T. (1992). Developmental aspects of the *Rhizobium*-legume symbiosis. *Plant Mol Biol* **19**, 89-107.

Freeman, J. A. & Bassler, B. L. (1999a). Sequence and function of LuxU: a two-component phosphorelay protein that regulates quorum sensing in *Vibrio harveyi*. *J Bacteriol* **181**, 899-906.

Freeman, J. A. & Bassler, B. L. (1999b). A genetic analysis of the function of LuxO, a two-component response regulator involved in quorum sensing in *Vibrio harveyi*. *Mol Microbiol* **31**, 665-677.

Freeman, J. A., Lilley, B. N. & Bassler, B. L. (2000). A genetic analysis of the functions of LuxN: a two-component hybrid sensor kinase that regulates quorum sensing in *Vibrio harveyi*. *Mol Microbiol* **35**, 139-149.

Friedman, A. M., Long, S. R., Brown, S. E., Buikema, W. J. & Ausubel, F. M. (1982). Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. *Gene* **18**, 289-296.

Fry, J., Wood, M. & Poole, P. S. (2001). Investigation of myo-inositol catabolism in *Rhizobium leguminosarum* bv. *viciae* and its effect on nodulation competitiveness. *Mol Plant Microbe Interact* **14**, 1016-1025.

Fujishige, N. A., Kapadia, N. N., De Hoff, P. L. & Hirsch, A. M. (2006). Investigations of *Rhizobium* biofilm formation. *FEMS Microbiol Ecol* **56**, 195-206.

Fuqua, W. C. & Winans, S. C. (1994). A LuxR-LuxI type regulatory system activates *Agrobacterium* Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. *J Bacteriol* **176**, 2796-2806.

Gage, D. J. (2004). Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiol Mol Biol Rev* **68**, 280-300.

Gambello, M. J. & Iglewski, B. H. (1991). Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase expression. *J Bacteriol* **173**, 3000-3009.

Gao, M., Teplitski, M., Robinson, J. B. & Bauer, W. D. (2003). Production of substances by *Medicago truncatula* that affect bacterial quorum sensing. *Mol Plant Microbe Interact* **16**, 827-834.

Gao, M., Chen, H., Eberhard, A., Gronquist, M. R., Robinson, J. B., Rolfe, B. G. & Bauer, W. D. (2005). *sinI*- and *expR*-dependent quorum sensing in *Sinorhizobium meliloti*. *J Bacteriol* **187**, 7931-7944.

Gao, M., Barnett, M. J., Long, S. R. & Teplitski, M. (2010). Role of the *Sinorhizobium meliloti* global regulator Hfq in gene regulation and symbiosis. *Mol Plant Microbe Interact* **23**, 355-365.

Gao, Y. J., Zhong, Z. T., Sun, K. J., Wang, H. & Zhu, J. (2006a). The quorum-sensing system in a plant bacterium *Mesorhizobium huakuii* affects growth rate and symbiotic nodulation. *Plant Soil* **286**, 53-60.

Gao, Y. J., Zhong, Z. T., Sun, K. J., Wang, H. & Zhu, J. (2006b). The quorum-sensing system in a plant bacterium *Mesorhizobium huakuii* affects growth rate and symbiotic nodulation. *Plant and Soil* **286**, 53-60.

Garcia-Rodriguez, F. M. & Toro, N. (2000). *Sinorhizobium meliloti nfe* (nodulation formation efficiency) genes exhibit temporal and spatial expression patterns similar to those of genes involved in symbiotic nitrogen fixation. *Mol Plant Microbe Interact* **13**, 583-591.

Garg, R. P., Yindeeyoungyeon, W., Gilis, A., Denny, T. P., Van Der Lelie, D. & Schell, M. A. (2000). Evidence that *Ralstonia eutropha* (*Alcaligenes eutrophus*) contains a functional homologue of the *Ralstonia solanacearum* Phc cell density sensing system. *Mol Microbiol* **38**, 359-367.

Genetello, C., Van Larebeke, N., Holsters, M., De Picker, A., Van Montagu, M. & Schell, J. (1977). Ti plasmids of *Agrobacterium* as conjugative plasmids. *Nature* **265**, 561-563.

Genin, S., Brito, B., Denny, T. P. & Boucher, C. (2005). Control of the *Ralstonia solanacearum* Type III secretion system (Hrp) genes by the global virulence regulator PhcA. *FEBS Lett* **579**, 2077-2081.

Georgellis, D., Lynch, A. S. & Lin, E. C. (1997). *In vitro* phosphorylation study of the *arc* two-component signal transduction system of *Escherichia coli*. *J Bacteriol* **179**, 5429-5435.

Geurts, R., Fedorova, E. & Bisseling, T. (2005). Nod factor signaling genes and their function in the early stages of *Rhizobium* infection. *Curr Opin Plant Biol* **8**, 346-352.

Gilson, L., Kuo, A. & Dunlap, P. V. (1995). AinS and a new family of autoinducer synthesis proteins. *J Bacteriol* **177**, 6946-6951.

Girard, G., van Rij, E. T., Lugtenberg, B. J. & Bloemberg, G. V. (2006). Regulatory roles of *psrA* and *rpoS* in phenazine-1-carboxamide synthesis by *Pseudomonas chlororaphis* PCL1391. *Microbiology* **152**, 43-58.

Givskov, M., de Nys, R., Manefield, M., Gram, L., Maximilien, R., Eberl, L., Molin, S., Steinberg, P. D. & Kjelleberg, S. (1996). Eukaryotic interference with homoserine lactone-mediated prokaryotic signalling. *J Bacteriol* **178**, 6618-6622.

Glenn, S. A., Gurich, N., Feeney, M. A. & Gonzalez, J. E. (2007). The ExpR/Sin quorum-sensing system controls succinoglycan production in *Sinorhizobium meliloti*. *J Bacteriol* **189**, 7077-7088.

Gong, W., Hao, B., Mansy, S. S., Gonzalez, G., Gilles-Gonzalez, M. A. & Chan, M. K. (1998). Structure of a biological oxygen sensor: a new mechanism for heme-driven signal transduction. *Proc Natl Acad Sci U S A* **95**, 15177-15182.

Gonzalez, J. E., Reuhs, B. L. & Walker, G. C. (1996). Low molecular weight EPS II of *Rhizobium meliloti* allows nodule invasion in *Medicago sativa*. *Proc Natl Acad Sci U S A* **93**, 8636-8641.

Gonzalez, J. E. & Marketon, M. M. (2003). Quorum sensing in nitrogen-fixing rhizobia. *Microbiol Mol Biol Rev* **67**, 574-592.

Gordon, D. M., Ryder, M. H., Heinrich, K. & Murphy, P. J. (1996). An experimental test of the rhizopine concept in *Rhizobium meliloti*. *Appl Environ Microbiol* **62**, 3991-3996.

Gray, K. M., Pearson, J. P., Downie, J. A., Boboye, B. E. & Greenberg, E. P. (1996). Cell-to-cell signaling in the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum*: autoinduction of a stationary phase and rhizosphere-expressed genes. *J Bacteriol* **178**, 372-376.

Gray, K. M. & Garey, J. R. (2001). The evolution of bacterial LuxI and LuxR quorum sensing regulators. *Microbiology* **147**, 2379-2387.

Gristwood, T., Fineran, P. C., Everson, L., Williamson, N. R. & Salmond, G. P. (2009). The PhoBR two-component system regulates antibiotic biosynthesis in *Serratia* in response to phosphate. *BMC Microbiol* **9**, 112.

Groisman, E. A. (2001). The pleiotropic two-component regulatory system PhoP-PhoQ. *J Bacteriol* **183**, 1835-1842.

Guina, T., Wu, M., Miller, S. I. & other authors (2003). Proteomic analysis of *Pseudomonas aeruginosa* grown under magnesium limitation. *J Am Soc Mass Spectrom* **14**, 742-751.

Gulash, M., Ames, P., Larosiliere, R. C. & Bergman, K. (1984). Rhizobia are attracted to localized sites on legume roots. *Appl Environ Microbiol* **48**, 149-152.

Gupta, R., Gobble, T. R. & Schuster, M. (2009). GidA posttranscriptionally regulates rhl quorum sensing in Pseudomonas aeruginosa. *J Bacteriol* **191**, 5785-5792.

Gurich, N. & Gonzalez, J. E. (2009). Role of quorum sensing in *Sinorhizobium meliloti*-Alfalfa symbiosis. *J Bacteriol* **191**, 4372-4382.

Gust, B., Chandra, G., Jakimowicz, D., Yuqing, T., Bruton, C. J. & Chater, K. F. (2004). Lambda red-mediated genetic manipulation of antibiotic-producing *Streptomyces*. *Adv Appl Microbiol* **54**, 107-128.

Habeeb, L. F., Wang, L. & Winans, S. C. (1991). Transcription of the octopine catabolism operon of the *Agrobacterium* tumor-inducing plasmid pTiA6 is activated by a LysR-type regulatory protein. *Mol Plant Microbe Interact* **4**, 379-385.

Hammer, B. K. & Bassler, B. L. (2003). Quorum sensing controls biofilm formation in *Vibrio cholerae*. *Mol Microbiol* **50**, 101-104.

Hammer, B. K. & Bassler, B. L. (2007). Regulatory small RNAs circumvent the conventional quorum sensing pathway in pandemic *Vibrio cholerae*. *Proc Natl Acad Sci U S A* **104**, 11145-11149.

Hanzelka, B. L. & Greenberg, E. P. (1995). Evidence that the *N*-terminal region of the *Vibrio fischeri* LuxR protein constitutes an autoinducer-binding domain. *J Bacteriol* **177**, 815-817.

Hanzelka, B. L., Parsek, M. R., Val, D. L., Dunlap, P. V., Cronan, J. E., Jr. & Greenberg, E. P. (1999). Acylhomoserine lactone synthase activity of the *Vibrio fischeri* AinS protein. *J Bacteriol* **181**, 5766-5770.

Hasegawa, H., Chatterjee, A., Cui, Y. & Chatterjee, A. K. (2005). Elevated temperature enhances virulence of *Erwinia carotovora* subsp. *carotovora* strain EC153 to plants and stimulates production of the quorum sensing signal, *N*-acyl homoserine lactone, and extracellular proteins. *Appl Environ Microbiol* **71**, 4655-4663.

Haudecoeur, E., Planamente, S., Cirou, A., Tannieres, M., Shelp, B. J., Morera, S. & Faure, D. (2009a). Proline antagonizes GABA-induced quenching of quorum-sensing in *Agrobacterium tumefaciens*. *Proc Natl Acad Sci U S A* **106**, 14587-14592.

Haudecoeur, E., Tannieres, M., Cirou, A., Raffoux, A., Dessaux, Y. & Faure, D. (2009b). Different regulation and roles of lactonases AiiB and AttM in *Agrobacterium tumefaciens* C58. *Mol Plant Microbe Interact* **22**, 529-537.

He, X., Chang, W., Pierce, D. L., Seib, L. O., Wagner, J. & Fuqua, C. (2003). Quorum sensing in *Rhizobium* sp. strain NGR234 regulates conjugal transfer (*tra*) gene expression and influences growth rate. *J Bacteriol* **185**, 809-822.

He, Y. W., Ng, A. Y., Xu, M., Lin, K., Wang, L. H., Dong, Y. H. & Zhang, L. H. (2007). *Xanthomonas campestris* cell-cell communication involves a putative nucleotide receptor protein Clp and a hierarchical signalling network. *Mol Microbiol* **64**, 281-292.

He, Y. W. & Zhang, L. H. (2008). Quorum sensing and virulence regulation in *Xanthomonas campestris*. *FEMS Microbiol Rev* **32**, 842-857.

He, Y. W., Boon, C., Zhou, L. & Zhang, L. H. (2009). Co-regulation of *Xanthomonas campestris* virulence by quorum sensing and a novel two-component regulatory system RavS/RavR. *Mol Microbiol* **71**, 1464-1476.

Helmann, J. D. (2002). The extracytoplasmic function (ECF) sigma factors. *Adv Microb Physiol* **46**, 47-110.

Hendrickson, E. L., Plotnikova, J., Mahajan-Miklos, S., Rahme, L. G. & Ausubel, F. M. (2001). Differential roles of the *Pseudomonas aeruginosa* PA14 *rpoN* gene in pathogenicity in plants, nematodes, insects, and mice. *J Bacteriol* **183**, 7126-7134.

Henke, J. M. & Bassler, B. L. (2004a). Three parallel quorum-sensing systems regulate gene expression in *Vibrio harveyi*. *J Bacteriol* **186**, 6902-6914.

Henke, J. M. & Bassler, B. L. (2004b). Quorum sensing regulates type III secretion in *Vibrio harveyi* and *Vibrio parahaemolyticus*. *J Bacteriol* **186**, 3794-3805.

Hense, B. A., Kuttler, C., Muller, J., Rothballer, M., Hartmann, A. & Kreft, J. U. (2007). Does efficiency sensing unify diffusion and quorum sensing? *Nat Rev Microbiol* **5**, 230-239.

Heurlier, K., Denervaud, V., Pessi, G., Reimmann, C. & Haas, D. (2003). Negative control of quorum sensing by RpoN (sigma54) in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **185**, 2227-2235.

Heurlier, K., Williams, F., Heeb, S., Dormond, C., Pessi, G., Singer, D., Camara, M., Williams, P. & Haas, D. (2004). Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **186**, 2936-2945.

Higgins, D. A., Pomianek, M. E., Kraml, C. M., Taylor, R. K., Semmelhack, M. F. & Bassler, B. L. (2007). The major *Vibrio cholerae* autoinducer and its role in virulence factor production. *Nature* **450**, 883-886.

Hirsch, A. M. (1999). Role of lectins (and rhizobial exopolysaccharides) in legume nodulation. *Curr Opin Plant Biol* **2**, 320-326.

Hirsch, P. R. (1979). Plasmid-determined bacteriocin production by *Rhizobium leguminosarum*. *J Gen Microbiol* **113**, 219-228.

Ho, S. C., Schindler, M. & Wang, J. L. (1990a). Carbohydrate binding activities of *Bradyrhizobium japonicum*. II. Isolation and characterization of a galactose-specific lectin. *J Cell Biol* **111**, 1639-1643.

Ho, S. C., Wang, J. L. & Schindler, M. (1990b). Carbohydrate binding activities of *Bradyrhizobium japonicum.* I. Saccharide-specific inhibition of homotypic and heterotypic adhesion. *J Cell Biol* **111**, 1631-1638.

Ho, S. C., Wang, J. L., Schindler, M. & Loh, J. T. (1994). Carbohydrate binding activities of *Bradyrhizobium japonicum*. III. Lectin expression, bacterial binding, and nodulation efficiency. *Plant J* **5**, 873-884.

Hoang, H. H., Becker, A. & Gonzalez, J. E. (2004). The LuxR homolog ExpR, in combination with the Sin quorum sensing system, plays a central role in *Sinorhizobium meliloti* gene expression. *J Bacteriol* **186**, 5460-5472.

Hoang, H. H., Gurich, N. & Gonzalez, J. E. (2008). Regulation of motility by the ExpR/Sin quorum-sensing system in *Sinorhizobium meliloti*. *J Bacteriol* **190**, 861-871.

Holden, M. T., Ram Chhabra, S., de Nys, R. & other authors (1999). Quorum-sensing cross talk: isolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other gram-negative bacteria. *Mol Microbiol* **33**, 1254-1266.

Holmes, K., Tavender, T. J., Winzer, K., Wells, J. M. & Hardie, K. R. (2009). AI-2 does not function as a quorum sensing molecule in *Campylobacter jejuni* during exponential growth *in vitro*. *BMC Microbiol* **9**, 214.

Horng, Y. T., Deng, S. C., Daykin, M. & other authors (2002). The LuxR family protein SpnR functions as a negative regulator of *N*-acylhomoserine lactone-dependent quorum sensing in *Serratia marcescens*. *Mol Microbiol* **45**, 1655-1671.

Hwang, I., Cook, D. M. & Farrand, S. K. (1995). A new regulatory element modulates homoserine lactone-mediated autoinduction of Ti plasmid conjugal transfer. *J Bacteriol* **177**, 449-458.

Ishihama, A. (1999). Modulation of the nucleoid, the transcription apparatus, and the translation machinery in bacteria for stationary phase survival. *Genes Cells* **4**, 135-143.

Ivanov, D. B., Philippova, M. P. & Tkachuk, V. A. (2001). Structure and functions of classical cadherins. *Biochemistry (Mosc)* **66**, 1174-1186.

Jain, V., Kumar, M. & Chatterji, D. (2006). ppGpp: stringent response and survival. *J Microbiol* **44**, 1-10.

Janczarek, M. & Skorupska, A. (2007). The *Rhizobium leguminosarum* bv. *trifolii* RosR: transcriptional regulator involved in exopolysaccharide production. *Mol Plant Microbe Interact* **20**, 867-881.

Janczarek, M., Jaroszuk-Scisel, J. & Skorupska, A. (2009). Multiple copies of *rosR* and *pssA* genes enhance exopolysaccharide production, symbiotic competitiveness and clover nodulation in *Rhizobium leguminosarum* bv. *trifolii*. *Antonie Van Leeuwenhoek* **96**, 471-486.

Jensen, V., Lons, D., Zaoui, C., Bredenbruch, F., Meissner, A., Dieterich, G., Munch, R. & Haussler, S. (2006). RhlR expression in *Pseudomonas aeruginosa* is modulated by the *Pseudomonas quinolone* signal via PhoB-dependent and -independent pathways. *J Bacteriol* **188**, 8601-8606.

Jiang, G., Krishnan, A. H., Kim, Y. W., Wacek, T. J. & Krishnan, H. B. (2001). A functional myo-inositol dehydrogenase gene is required for efficient nitrogen fixation and competitiveness of *Sinorhizobium fredii* USDA191 to nodulate soybean (Glycine max [*L.*] Merr.). *J Bacteriol* **183**, 2595-2604.

Jimenez-Zurdo, J. I., van Dillewijn, P., Soto, M. J., de Felipe, M. R., Olivares, J. & Toro, N. (1995). Characterization of a *Rhizobium meliloti* proline dehydrogenase mutant altered in nodulation efficiency and competitiveness on alfalfa roots. *Mol Plant Microbe Interact* **8**, 492-498.

Johnston, A. W. & Beringer, J. E. (1975). Identification of the rhizobium strains in pea root nodules using genetic markers. *J Gen Microbiol* **87**, 343-350.

Jones, K. M., Kobayashi, H., Davies, B. W., Taga, M. E. & Walker, G. C. (2007). How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. *Nat Rev Microbiol* **5**, 619-633.

Jones, K. M., Sharopova, N., Lohar, D. P., Zhang, J. Q., VandenBosch, K. A. & Walker, G. C. (2008). Differential response of the plant *Medicago truncatula* to its symbiont *Sinorhizobium meliloti* or an exopolysaccharide-deficient mutant. *Proc Natl Acad Sci U S A* **105**, 704-709.

Jude, F., Kohler, T., Branny, P., Perron, K., Mayer, M. P., Comte, R. & van Delden, C. (2003). Posttranscriptional control of quorum-sensing-dependent virulence genes by DksA in *Pseudomonas aeruginosa*. *J Bacteriol* **185**, 3558-3566.

Juhas, M., Wiehlmann, L., Huber, B. & other authors (2004). Global regulation of quorum sensing and virulence by VqsR in *Pseudomonas aeruginosa*. *Microbiology* **150**, 831-841.

Juhas, M., Wiehlmann, L., Salunkhe, P., Lauber, J., Buer, J. & Tummler, B. (2005). GeneChip expression analysis of the VqsR regulon of *Pseudomonas aeruginosa* TB. *FEMS Microbiol Lett* **242**, 287-295.

Kanamaru, K., Kanamaru, K., Tatsuno, I., Tobe, T. & Sasakawa, C. (2000). SdiA, an *Escherichia coli* homologue of quorum-sensing regulators, controls the expression of virulence factors in enterohaemorrhagic *Escherichia coli* O157:H7. *Mol Microbiol* **38**, 805-816.

Kang, Y., Lunin, V. V., Skarina, T., Savchenko, A., Schurr, M. J. & Hoang, T. T. (2009). The long-chain fatty acid sensor, PsrA, modulates the expression of *rpoS* and the type III secretion *exsCEBA* operon in *Pseudomonas aeruginosa*. *Mol Microbiol* **73**, 120-136.

Kannenberg, E. L., Rathbun, E. A. & Brewin, N. J. (1992). Molecular dissection of structure and function in the lipopolysaccharide of *Rhizobium leguminosarum strain* 3841 using monoclonal antibodies and genetic analysis. *Mol Microbiol* **6**, 2477-2487.

Kaplan, F., Badri, D. V., Zachariah, C. & other authors (2009). Bacterial attraction and quorum sensing inhibition in *Caenorhabditis elegans* exudates. *J Chem Ecol* **35**, 878-892.

Karimova, G., Pidoux, J., Ullmann, A. & Ladant, D. (1998). A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proc Natl Acad Sci U S A* **95**, 5752-5756.

Karunakaran, R., Mauchline, T. H., Hosie, A. H. & Poole, P. S. (2005). A family of promoter probe vectors incorporating autofluorescent and chromogenic reporter proteins for studying gene expression in Gram-negative bacteria. *Microbiology* **151**, 3249-3256.

Kay, E., Humair, B., Denervaud, V., Riedel, K., Spahr, S., Eberl, L., Valverde, C. & Haas, D. (2006). Two GacA-dependent small RNAs modulate the quorum-sensing response in *Pseudomonas aeruginosa*. *J Bacteriol* **188**, 6026-6033.

Kelly, R. C., Bolitho, M. E., Higgins, D. A. & other authors (2009). The *Vibrio cholerae* quorum-sensing autoinducer CAI-1: analysis of the biosynthetic enzyme CqsA. *Nat Chem Biol*.

Kerr, A., Manigault, P. & Tempe, J. (1977). Transfer of virulence *in vivo* and *in vitro* in *Agrobacterium*. *Nature* **265**, 560-561.

Keshavan, N. D., Chowdhary, P. K., Haines, D. C. & Gonzalez, J. E. (2005). L-Canavanine made by *Medicago sativa* interferes with quorum sensing in *Sinorhizobium meliloti*. *J Bacteriol* **187**, 8427-8436.

Key, J. & Moffat, K. (2005). Crystal structures of deoxy and CO-bound bjFixLH reveal details of ligand recognition and signaling. *Biochemistry* **44**, 4627-4635.

Khan, M. S., Zahin, M., Hasan, S., Husain, F. M. & Ahmad, I. (2009). Inhibition of quorum sensing regulated bacterial functions by plant essential oils with special reference to clove oil. *Lett Appl Microbiol* **49**, 354-360.

Kier, L. D., Weppelman, R. M. & Ames, B. N. (1979). Regulation of nonspecific acid phosphatase in *Salmonella*: *phoN* and *phoP* genes. *J Bacteriol* **138**, 155-161.

Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F. & Hopwood, D. A. (2000). Practical *Streptomyces* genetics. *The John Innes Foundation, Norfolk, UK.*

Kijne, J. W., Smit, G., Diaz, C. L. & Lugtenberg, B. J. (1988). Lectin-enhanced accumulation of manganese-limited *Rhizobium leguminosarum* cells on pea root hair tips. *J Bacteriol* **170**, 2994-3000.

Kim, E. J., Wang, W., Deckwer, W. D. & Zeng, A. P. (2005). Expression of the quorum-sensing regulatory protein LasR is strongly affected by iron and oxygen concentrations in cultures of *Pseudomonas aeruginosa* irrespective of cell density. *Microbiology* **151**, 1127-1138.

Kim, S. K., Wilmes-Riesenberg, M. R. & Wanner, B. L. (1996). Involvement of the sensor kinase EnvZ in the *in vivo* activation of the response-regulator PhoB by acetyl phosphate. *Mol Microbiol* **22**, 135-147.

Kim, T. J., Young, B. M. & Young, G. M. (2008). Effect of flagellar mutations on *Yersinia enterocolitica* biofilm formation. *Appl Environ Microbiol* **74**, 5466-5474.

Kint, G., Sonck, K. A., Schoofs, G., De Coster, D., Vanderleyden, J. & De Keersmaecker, S. C. (2009). 2D proteome analysis initiates new insights on the *Salmonella Typhimurium* LuxS protein. *BMC Microbiol* **9**, 198.

Kitten, T., Kinscherf, T. G., McEvoy, J. L. & Willis, D. K. (1998). A newly identified regulator is required for virulence and toxin production in *Pseudomonas syringae*. *Mol Microbiol* **28**, 917-929.

Klauck, E., Typas, A. & Hengge, R. (2007). The sigmaS subunit of RNA polymerase as a signal integrator and network master regulator in the general stress response in *Escherichia coli*. *Sci Prog* **90**, 103-127.

Klose, K. E., Novik, V. & Mekalanos, J. J. (1998). Identification of multiple sigma54-dependent transcriptional activators in *Vibrio cholerae*. *J Bacteriol* **180**, 5256-5259.

Kobayashi, K. (2007). Gradual activation of the response regulator DegU controls serial expression of genes for flagellum formation and biofilm formation in *Bacillus subtilis*. *Mol Microbiol* **66**, 395-409.

Kojic, M. & Venturi, V. (2001). Regulation of *rpoS* gene expression in *Pseudomonas*: involvement of a TetR family regulator. *J Bacteriol* **183**, 3712-3720.

Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., 2nd & Peterson, K. M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**, 175-176.

Kovacikova, G. & Skorupski, K. (2002). Regulation of virulence gene expression in *Vibrio cholerae* by quorum sensing: HapR functions at the *aphA* promoter. *Mol Microbiol* **46**, 1135-1147.

Krehenbrink, M. & Downie, J. A. (2008). Identification of protein secretion systems and novel secreted proteins in *Rhizobium leguminosarum* bv. *viciae*. *BMC Genomics* **9**, 55.

Krol, E. & Becker, A. (2004). Global transcriptional analysis of the phosphate starvation response in *Sinorhizobium meliloti* strains 1021 and 2011. *Mol Genet Genomics* **272**, 1-17.

Kuo, A., Blough, N. V. & Dunlap, P. V. (1994). Multiple *N*-acyl-L-homoserine lactone autoinducers of luminescence in the marine symbiotic bacterium *Vibrio fischeri*. *J Bacteriol* **176**, 7558-7565.

Kwasigroch, J. M. & Rooman, M. (2006). Prelude and Fugue, predicting local protein structure, early folding regions and structural weaknesses. *Bioinformatics* **22**, 1800-1802.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lamarche, M. G., Wanner, B. L., Crepin, S. & Harel, J. (2008). The phosphate regulon and bacterial virulence: a regulatory network connecting phosphate homeostasis and pathogenesis. *FEMS Microbiol Rev* **32**, 461-473.

Lamb, J. W., Downie, J. A. & Johnston, A. W. (1982). Plasmid-determined nodulation and nitrogen-fixation abilities in *Rhizobium phaseoli*. . *Mol Gen Genet* **186**, 449-452.

Lange, R. & Hengge-Aronis, R. (1991). Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. *Mol Microbiol* **5**, 49-59.

Latifi, A., Foglino, M., Tanaka, K., Williams, P. & Lazdunski, A. (1996). A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. *Mol Microbiol* **21**, 1137-1146.

Laub, M. T. & Goulian, M. (2007). Specificity in two-component signal transduction pathways. *Annu Rev Genet* **41**, 121-145.

Laue, B. E., Jiang, Y., Chhabra, S. R., Jacob, S., Stewart, G. S., Hardman, A., Downie, J. A., O'Gara, F. & Williams, P. (2000). The biocontrol strain *Pseudomonas fluorescens* F113 produces the *Rhizobium* small bacteriocin, *N*-(3-hydroxy-7-cis-tetradecenoyl)homoserine lactone, via HdtS, a putative novel *N*-acylhomoserine lactone synthase. *Microbiology* **146 (Pt 10)**, 2469-2480.

Laus, M. C., van Brussel, A. A. & Kijne, J. W. (2005). Role of cellulose fibrils and exopolysaccharides of *Rhizobium leguminosarum* in attachment to and infection of *Vicia sativa* root hairs. *Mol Plant Microbe Interact* **18**, 533-538.

Laus, M. C., Logman, T. J., Lamers, G. E., Van Brussel, A. A., Carlson, R. W. & Kijne, J. W. (2006). A novel polar surface polysaccharide from *Rhizobium leguminosarum* binds host plant lectin. *Mol Microbiol* **59**, 1704-1713.

Le, T. B., Fiedler, H. P., den Hengst, C. D., Ahn, S. K., Maxwell, A. & Buttner, M. J. (2009). Coupling of the biosynthesis and export of the DNA gyrase inhibitor simocyclinone in *Streptomyces antibioticus*. *Mol Microbiol* **72**, 1462-1474.

Ledgham, F., Soscia, C., Chakrabarty, A., Lazdunski, A. & Foglino, M. (2003a). Global regulation in *Pseudomonas aeruginosa*: the regulatory protein AlgR2 (AlgQ) acts as a modulator of quorum sensing. *Res Microbiol* **154**, 207-213.

Ledgham, F., Ventre, I., Soscia, C., Foglino, M., Sturgis, J. N. & Lazdunski, A. (2003b). Interactions of the quorum sensing regulator QscR: interaction with itself and the other regulators of *Pseudomonas aeruginosa* LasR and RhlR. *Mol Microbiol* **48**, 199-210.

Lelong, C., Aguiluz, K., Luche, S., Kuhn, L., Garin, J., Rabilloud, T. & Geiselmann, J. (2007). The Crl-RpoS regulon of *Escherichia coli*. *Mol Cell Proteomics* **6**, 648-659.

Lemon, K. P., Higgins, D. E. & Kolter, R. (2007). Flagellar motility is critical for *Listeria monocytogenes* biofilm formation. *J Bacteriol* **189**, 4418-4424.

Lenz, D. H., Mok, K. C., Lilley, B. N., Kulkarni, R. V., Wingreen, N. S. & Bassler, B. L. (2004). The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae*. *Cell* **118**, 69-82.

Lenz, D. H., Miller, M. B., Zhu, J., Kulkarni, R. V. & Bassler, B. L. (2005). CsrA and three redundant small RNAs regulate quorum sensing in *Vibrio cholerae*. *Mol Microbiol* **58**, 1186-1202.

Lenz, D. H. & Bassler, B. L. (2007). The small nucleoid protein Fis is involved in *Vibrio cholerae* quorum sensing. *Mol Microbiol* **63**, 859-871.

Leon, E., Navarro-Aviles, G., Santiveri, C. M. & other authors (2010). A bacterial antirepressor with SH3 domain topology mimics operator DNA in sequestering the repressor DNA recognition helix. *Nucleic Acids Res*.

Lewenza, S., Conway, B., Greenberg, E. P. & Sokol, P. A. (1999). Quorum sensing in *Burkholderia cepacia*: identification of the LuxRI homologs CepRI. *J Bacteriol* **181**, 748-756.

Lewenza, S. & Sokol, P. A. (2001). Regulation of ornibactin biosynthesis and *N*-acyl-L-homoserine lactone production by CepR in *Burkholderia cepacia*. *J Bacteriol* **183**, 2212-2218.

Lewis, R. J., Brannigan, J. A., Smith, I. & Wilkinson, A. J. (1996). Crystallisation of the *Bacillus subtilis* sporulation inhibitor SinR, complexed with its antagonist, SinI. *FEBS Lett* **378**, 98-100.

Li, L. L., Malone, J. E. & Iglewski, B. H. (2007). Regulation of the *Pseudomonas aeruginosa* quorum-sensing regulator VqsR. *J Bacteriol* **189**, 4367-4374.

Liang, H., Li, L., Dong, Z., Surette, M. G. & Duan, K. (2008a). The YebC family protein PA0964 negatively regulates the Pseudomonas aeruginosa quinolone signal system and pyocyanin production. *J Bacteriol* **190**, 6217-6227.

Liang, H., Li, L., Kong, W., Shen, L. & Duan, K. (2009). Identification of a novel regulator of the quorum-sensing systems in Pseudomonas aeruginosa. *FEMS Microbiol Lett* **293**, 196-204.

Liang, W., Pascual-Montano, A., Silva, A. J. & Benitez, J. A. (2007). The cyclic AMP receptor protein modulates quorum sensing, motility and multiple genes that affect intestinal colonization in *Vibrio cholerae*. *Microbiology* **153**, 2964-2975.

Liang, W., Sultan, S. Z., Silva, A. J. & Benitez, J. A. (2008b). Cyclic AMP post-transcriptionally regulates the biosynthesis of a major bacterial autoinducer to modulate the cell density required to activate quorum sensing. *FEBS Lett* **582**, 3744-3750.

Lilley, B. N. & Bassler, B. L. (2000). Regulation of quorum sensing in *Vibrio harveyi* by LuxO and sigma-54. *Mol Microbiol* **36**, 940-954.

Linares, J. F., Moreno, R., Fajardo, A., Martinez-Solano, L., Escalante, R., Rojo, F. & Martinez, J. L. (2010). The global regulator Crc modulates metabolism, susceptibility to antibiotics and virulence in *Pseudomonas aeruginosa*. *Environ Microbiol*.

Lithgow, J. K., Wilkinson, A., Hardman, A., Rodelas, B., Wisniewski-Dye, F., Williams, P. & Downie, J. A. (2000). The regulatory locus *cinRI* in *Rhizobium leguminosarum* controls a network of quorum-sensing loci. *Mol Microbiol* **37**, 81-97.

Liu, R., Tran, V. M. & Schmidt, E. L. (1989). Nodulating competitiveness of a nonmotile Tn*7* Mutant of *Bradyrhizobium japonicum* in nonsterile soil. *Appl Environ Microbiol* **55**, 1895-1900.

Liu, Z., Miyashiro, T., Tsou, A., Hsiao, A., Goulian, M. & Zhu, J. (2008). Mucosal penetration primes *Vibrio cholerae* for host colonization by repressing quorum sensing. *Proc Natl Acad Sci U S A* **105**, 9769-9774.

Loh, J., Carlson, R. W., York, W. S. & Stacey, G. (2002a). Bradyoxetin, a unique chemical signal involved in symbiotic gene regulation. *Proc Natl Acad Sci U S A* **99**, 14446-14451.

Loh, J., Lohar, D. P., Andersen, B. & Stacey, G. (2002b). A two-component regulator mediates population-density-dependent expression of the *Bradyrhizobium japonicum* nodulation genes. *J Bacteriol* **184**, 1759-1766.

Loh, J., Pierson, E. A., Pierson, L. S., 3rd, Stacey, G. & Chatterjee, A. (2002c). Quorum sensing in plant-associated bacteria. *Curr Opin Plant Biol* **5**, 285-290.

Loh, J. & Stacey, G. (2003). Nodulation gene regulation in *Bradyrhizobium japonicum*: a unique integration of global regulatory circuits. *Appl Environ Microbiol* **69**, 10-17.

Loh, J. T., Ho, S. C., de Feijter, A. W., Wang, J. L. & Schindler, M. (1993). Carbohydrate binding activities of *Bradyrhizobium japonicum*: unipolar localization of the lectin BJ38 on the bacterial cell surface. *Proc Natl Acad Sci U S A* **90**, 3033-3037.

Loh, J. T., Yuen-Tsai, J. P., Stacey, M. G., Lohar, D., Welborn, A. & Stacey, G. (2001). Population density-dependent regulation of the *Bradyrhizobium japonicum* nodulation genes. *Mol Microbiol* **42**, 37-46.

Lopez-Rubio, J. J., Elias-Arnanz, M., Padmanabhan, S. & Murillo, F. J. (2002). A repressor-antirepressor pair links two loci controlling light-induced carotenogenesis in *Myxococcus xanthus*. *J Biol Chem* **277**, 7262-7270.

Luo, Z. Q., Qin, Y. & Farrand, S. K. (2000). The antiactivator TraM interferes with the autoinducer-dependent binding of TraR to DNA by interacting with the C-terminal region of the quorum-sensing activator. *J Biol Chem* **275**, 7713-7722.

Lupp, C., Urbanowski, M., Greenberg, E. P. & Ruby, E. G. (2003). The *Vibrio fischeri* quorum-sensing systems *ain* and *lux* sequentially induce luminescence gene expression and are important for persistence in the squid host. *Mol Microbiol* **50**, 319-331.

Lupp, C. & Ruby, E. G. (2004). *Vibrio fischeri* LuxS and AinS: comparative study of two signal synthases. *J Bacteriol* **186**, 3873-3881.

Lupp, C. & Ruby, E. G. (2005). *Vibrio fischeri* uses two quorum-sensing systems for the regulation of early and late colonization factors. *J Bacteriol* **187**, 3620-3629.

Lyon, G. J. & Novick, R. P. (2004). Peptide signaling in *Staphylococcus aureus* and other Gram-positive bacteria. *Peptides* **25**, 1389-1403.

Maj, D., Wielbo, J., Marek-Kozaczuk, M. & Skorupska, A. (2008). Response to flavonoids as a factor influencing competitiveness and symbiotic activity of *Rhizobium leguminosarum*. *Microbiol Res*.

Malott, R. J., Baldwin, A., Mahenthiralingam, E. & Sokol, P. A. (2005). Characterization of the *cciIR* quorum-sensing system in *Burkholderia cenocepacia*. *Infect Immun* **73**, 4982-4992.

Malott, R. J. & Sokol, P. A. (2007). Expression of the *bviIR* and *cepIR* quorum-sensing systems of *Burkholderia vietnamiensis*. *J Bacteriol* **189**, 3006-3016.

Manefield, M., Rasmussen, T. B., Henzter, M., Andersen, J. B., Steinberg, P., Kjelleberg, S. & Givskov, M. (2002). Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover. *Microbiology* **148**, 1119-1127.

Marketon, M. M. & Gonzalez, J. E. (2002). Identification of two quorum-sensing systems in *Sinorhizobium meliloti*. *J Bacteriol* **184**, 3466-3475.

Marketon, M. M., Gronquist, M. R., Eberhard, A. & Gonzalez, J. E. (2002). Characterization of the *Sinorhizobium meliloti sinR/sinI* locus and the production of novel *N*-acyl homoserine lactones. *J Bacteriol* **184**, 5686-5695.

Marketon, M. M., Glenn, S. A., Eberhard, A. & Gonzalez, J. E. (2003). Quorum sensing controls exopolysaccharide production in *Sinorhizobium meliloti*. *J Bacteriol* **185**, 325-331.

Masuda, S. & Bauer, C. E. (2002). AppA is a blue light photoreceptor that antirepresses photosynthesis gene expression in *Rhodobacter sphaeroides*. *Cell* **110**, 613-623.

Mathesius, U., Mulders, S., Gao, M., Teplitski, M., Caetano-Anolles, G., Rolfe, B. G. & Bauer, W. D. (2003). Extensive and specific responses of a eukaryote to bacterial quorum-sensing signals. *Proc Natl Acad Sci U S A* **100**, 1444-1449.

Mavrodi, D. V., Ksenzenko, V. N., Bonsall, R. F., Cook, R. J., Boronin, A. M. & Thomashow, L. S. (1998). A seven-gene locus for synthesis of phenazine-1-carboxylic acid by *Pseudomonas fluorescens* 2-79. *J Bacteriol* **180**, 2541-2548.

McAnulla, C., Edwards, A., Sanchez-Contreras, M., Sawers, R. G. & Downie, J. A. (2007). Quorum-sensing-regulated transcriptional initiation of plasmid transfer and replication genes in *Rhizobium leguminosarum* biovar *viciae*. *Microbiology* **153**, 2074-2082.

McClean, K. H., Winson, M. K., Fish, L. & other authors (1997). Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. *Microbiology* **143 (Pt 12)**, 3703-3711.

McGowan, S. J., Barnard, A. M., Bosgelmez, G. & other authors (2005). Carbapenem antibiotic biosynthesis in *Erwinia carotovora* is regulated by physiological and genetic factors modulating the quorum sensing-dependent control pathway. *Mol Microbiol* **55**, 526-545.

McGuffin, L. J., Bryson, K. & Jones, D. T. (2000). The PSIPRED protein structure prediction server. *Bioinformatics* **16**, 404-405.

McIntosh, M., Krol, E. & Becker, A. (2008). Competitive and cooperative effects in quorum-sensing-regulated galactoglucan biosynthesis in *Sinorhizobium meliloti*. *J Bacteriol* **190**, 5308-5317.

McIntosh, M., Meyer, S. & Becker, A. (2009). Novel *Sinorhizobium meliloti* quorum sensing positive and negative regulatory feedback mechanisms respond to phosphate availability. *Mol Microbiol*.

McKnight, S. L., Iglewski, B. H. & Pesci, E. C. (2000). The *Pseudomonas* quinolone signal regulates *rhl* quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* **182**, 2702-2708.

Medina, G., Juarez, K., Diaz, R. & Soberon-Chavez, G. (2003a). Transcriptional regulation of *Pseudomonas aeruginosa rhlR*, encoding a quorum-sensing regulatory protein. *Microbiology* **149**, 3073-3081.

Medina, G., Juarez, K. & Soberon-Chavez, G. (2003b). The *Pseudomonas aeruginosa rhlAB* operon is not expressed during the logarithmic phase of growth even in the presence of its activator RhlR and the autoinducer *N*-butyryl-homoserine lactone. *J Bacteriol* **185**, 377-380.

Merritt, P. M., Danhorn, T. & Fuqua, C. (2007). Motility and chemotaxis in *Agrobacterium tumefaciens* surface attachment and biofilm formation. *J Bacteriol* **189**, 8005-8014.

Michael, B., Smith, J. N., Swift, S., Heffron, F. & Ahmer, B. M. (2001). SdiA of *Salmonella enterica* is a LuxR homolog that detects mixed microbial communities. *J Bacteriol* **183**, 5733-5742.

Miller, J. H. (1972). Experiments in molecular genetics. *New York: Cold Spring Harbor Laboratory Press*.

Miller, L. D., Yost, C. K., Hynes, M. F. & Alexandre, G. (2007). The major chemotaxis gene cluster of *Rhizobium leguminosarum* bv. *viciae* is essential for competitive nodulation. *Mol Microbiol* **63**, 348-362.

Miller, M. B., Skorupski, K., Lenz, D. H., Taylor, R. K. & Bassler, B. L. (2002). Parallel quorum sensing systems converge to regulate virulence in *Vibrio cholerae*. *Cell* **110**, 303-314.

Miller, S. I., Kukral, A. M. & Mekalanos, J. J. (1989). A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. *Proc Natl Acad Sci U S A* **86**, 5054-5058.

Miller, S. T., Xavier, K. B., Campagna, S. R., Taga, M. E., Semmelhack, M. F., Bassler, B. L. & Hughson, F. M. (2004). *Salmonella typhimurium* recognizes a chemically distinct form of the bacterial quorum-sensing signal AI-2. *Mol Cell* **15**, 677-687.

Milton, D. L., Chalker, V. J., Kirke, D., Hardman, A., Camara, M. & Williams, P. (2001). The LuxM homologue VanM from *Vibrio anguillarum* directs the synthesis

of *N*-(3-hydroxyhexanoyl)homoserine lactone and *N*-hexanoylhomoserine lactone. *J Bacteriol* **183**, 3537-3547.

Minogue, T. D., Wehland-von Trebra, M., Bernhard, F. & von Bodman, S. B. (2002). The autoregulatory role of EsaR, a quorum-sensing regulator in *Pantoea stewartii* ssp. *stewartii*: evidence for a repressor function. *Mol Microbiol* **44**, 1625-1635.

Miyamoto, C. M., Dunlap, P. V., Ruby, E. G. & Meighen, E. A. (2003). LuxO controls *luxR* expression in *Vibrio harveyi*: evidence for a common regulatory mechanism in *Vibrio*. *Mol Microbiol* **48**, 537-548.

Miyashiro, T., Wollenberg, M. S., Cao, X., Oehlert, D. & Ruby, E. G. (2010). A single *qrr* gene is necessary and sufficient for LuxO-mediated regulation in *Vibrio fischeri*. *Mol Microbiol*.

Mongiardini, E. J., Ausmees, N., Perez-Gimenez, J., Julia Althabegoiti, M., Ignacio Quelas, J., Lopez-Garcia, S. L. & Lodeiro, A. R. (2008). The rhizobial adhesion protein RapA1 is involved in adsorption of rhizobia to plant roots but not in nodulation. *FEMS Microbiol Ecol* **65**, 279-288.

Mongiardini, E. J., Perez-gimenez, J., Althabegoiti, M. J., Covelli, J., Quelas, J. I., Lopez-garcia, S. L. & Lodeiro, A. (2009). Overproduction of the rhizobial adhesin RapA1 increases competitiveness for nodulation. *Soil Biology and Biochemistry* **41**, 2017-2020.

Morici, L. A., Carterson, A. J., Wagner, V. E. & other authors (2007). *Pseudomonas aeruginosa* AlgR represses the Rhl quorum-sensing system in a biofilm-specific manner. *J Bacteriol* **189**, 7752-7764.

Moris, M., Braeken, K., Schoeters, E., Verreth, C., Beullens, S., Vanderleyden, J. & Michiels, J. (2005). Effective symbiosis between *Rhizobium etli* and *Phaseolus vulgaris* requires the alarmone ppGpp. *J Bacteriol* **187**, 5460-5469.

Nasser, W., Bouillant, M. L., Salmond, G. & Reverchon, S. (1998). Characterization of the *Erwinia chrysanthemi expI-expR* locus directing the synthesis of two *N*-acyl-homoserine lactone signal molecules. *Mol Microbiol* **29**, 1391-1405.

Nasser, W. & Reverchon, S. (2007). New insights into the regulatory mechanisms of the LuxR family of quorum sensing regulators. *Anal Bioanal Chem* **387**, 381-390.

Nealson, K. H., Platt, T. & Hastings, J. W. (1970). Cellular control of the synthesis and activity of the bacterial luminscent system. *J Bacteriol* **104**, 313-322.

Neiditch, M. B., Federle, M. J., Miller, S. T., Bassler, B. L. & Hughson, F. M. (2005). Regulation of LuxPQ receptor activity by the quorum-sensing signal autoinducer-2. *Mol Cell* **18**, 507-518.

Niehaus, K., Kapp, D. & puhler, A. (1993). Plant defense and delayed infection of alfalfa pseudonodules induced by an exopolysaccharide (EPSI)-deficient *Rhizobium meliloti* mutant. *Planta* **190**, 415-425.

Novick, R. P. & Geisinger, E. (2008). Quorum sensing in staphylococci. *Annu Rev Genet* **42**, 541-564.

O'Grady, E. P., Viteri, D. F., Malott, R. J. & Sokol, P. A. (2009). Reciprocal regulation by the CepIR and CciIR quorum sensing systems in *Burkholderia cenocepacia*. *BMC Genomics* **10**, 441.

Ochsner, U. A., Wilderman, P. J., Vasil, A. I. & Vasil, M. L. (2002). GeneChip expression analysis of the iron starvation response in *Pseudomonas aeruginosa*: identification of novel pyoverdine biosynthesis genes. *Mol Microbiol* **45**, 1277-1287.

Oger, P., Kim, K. S., Sackett, R. L., Piper, K. R. & Farrand, S. K. (1998). Octopine-type Ti plasmids code for a mannopine-inducible dominant-negative allele of *traR*, the quorum-sensing activator that regulates Ti plasmid conjugal transfer. *Mol Microbiol* **27**, 277-288.

Oglesby, A. G., Farrow, J. M., 3rd, Lee, J. H., Tomaras, A. P., Greenberg, E. P., Pesci, E. C. & Vasil, M. L. (2008). The influence of iron on *Pseudomonas aeruginosa* physiology: a regulatory link between iron and quorum sensing. *J Biol Chem* **283**, 15558-15567.

Oldroyd, G. E. & Downie, J. A. (2006). Nuclear calcium changes at the core of symbiosis signalling. *Curr Opin Plant Biol* **9**, 351-357.

Oliphant, A. R., Brandl, C. J. & Struhl, K. (1989). Defining the sequence specificity of DNA-binding proteins by selecting binding sites from random-sequence oligonucleotides: analysis of yeast GCN4 protein. *Mol Cell Biol* **9**, 2944-2949.

Oppenheim, A. B., Neubauer, Z. & Calef, E. (1970). The antirepressor: a new element in the regulation of protein synthesis. *Nature* **226**, 31-32.

Oppenheim, A. B., Kobiler, O., Stavans, J., Court, D. L. & Adhya, S. (2005). Switches in bacteriophage lambda development. *Annu Rev Genet* **39**, 409-429.

Oresnik, I. (1998). Plasmid-encoded catabolic genes in *Rhizobium leguminosarum* bv. *trfolii:* evidence for a plant-inducible rhamnose locus involved in competition for nodulation. *Molecular Plant Microbe Interactions* **11**, 1175-1185.

Ozer, E. A., Pezzulo, A., Shih, D. M., Chun, C., Furlong, C., Lusis, A. J., Greenberg, E. P. & Zabner, J. (2005). Human and murine paraoxonase 1 are host modulators of *Pseudomonas aeruginosa* quorum-sensing. *FEMS Microbiol Lett* **253**, 29-37.

Parsek, M. R., Val, D. L., Hanzelka, B. L., Cronan, J. E., Jr. & Greenberg, E. P. (1999). Acyl homoserine-lactone quorum-sensing signal generation. *Proc Natl Acad Sci U S A* **96**, 4360-4365.

Parsek, M. R. & Greenberg, E. P. (2000). Acyl-homoserine lactone quorum sensing in gram-negative bacteria: a signaling mechanism involved in associations with higher organisms. *Proc Natl Acad Sci U S A* **97**, 8789-8793.

Passador, L., Cook, J. M., Gambello, M. J., Rust, L. & Iglewski, B. H. (1993). Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science* **260**, 1127-1130.

Patankar, A. V. & Gonzalez, J. E. (2009). An orphan LuxR homolog of *Sinorhizobium meliloti* affects stress adaptation and competition for nodulation. *Appl Environ Microbiol* **75**, 946-955.

Paul, B. J., Barker, M. M., Ross, W., Schneider, D. A., Webb, C., Foster, J. W. & Gourse, R. L. (2004). DksA: a critical component of the transcription initiation machinery that potentiates the regulation of rRNA promoters by ppGpp and the initiating NTP. *Cell* **118**, 311-322.

Pearson, J. P., Gray, K. M., Passador, L., Tucker, K. D., Eberhard, A., Iglewski, B. H. & Greenberg, E. P. (1994). Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc Natl Acad Sci U S A* **91**, 197-201.

Pearson, J. P., Van Delden, C. & Iglewski, B. H. (1999). Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. *J Bacteriol* **181**, 1203-1210.

Pearson, J. P. (2002). Early activation of quorum sensing. *J Bacteriol* **184**, 2569-2571.

Pellock, B. J., Teplitski, M., Boinay, R. P., Bauer, W. D. & Walker, G. C. (2002). A LuxR homolog controls production of symbiotically active extracellular polysaccharide II by *Sinorhizobium meliloti*. *J Bacteriol* **184**, 5067-5076.

Perron, K., Comte, R. & van Delden, C. (2005). DksA represses ribosomal gene transcription in *Pseudomonas aeruginosa* by interacting with RNA polymerase on ribosomal promoters. *Mol Microbiol* **56**, 1087-1102.

Pesci, E. C., Pearson, J. P., Seed, P. C. & Iglewski, B. H. (1997). Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* **179**, 3127-3132.

Pesci, E. C., Milbank, J. B., Pearson, J. P., McKnight, S., Kende, A. S., Greenberg, E. P. & Iglewski, B. H. (1999). Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **96**, 11229-11234.

Pessi, G. & Haas, D. (2000). Transcriptional control of the hydrogen cyanide biosynthetic genes *hcnABC* by the anaerobic regulator ANR and the quorum-sensing regulators LasR and RhlR in *Pseudomonas aeruginosa*. *J Bacteriol* **182**, 6940-6949.

Pessi, G. & Haas, D. (2001). Dual control of hydrogen cyanide biosynthesis by the global activator GacA in *Pseudomonas aeruginosa* PAO1. *FEMS Microbiol Lett* **200**, 73-78.

Pessi, G., Williams, F., Hindle, Z., Heurlier, K., Holden, M. T., Camara, M., Haas, D. & Williams, P. (2001). The global posttranscriptional regulator RsmA modulates production of virulence determinants and *N*-acylhomoserine lactones in *Pseudomonas aeruginosa*. *J Bacteriol* **183**, 6676-6683.

Piper, K. R., Beck von Bodman, S. & Farrand, S. K. (1993). Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. *Nature* **362**, 448-450.

Podbielski, A. & Kreikemeyer, B. (2004). Cell density--dependent regulation: basic principles and effects on the virulence of Gram-positive cocci. *Int J Infect Dis* **8**, 81-95.

Pompeani, A. J., Irgon, J. J., Berger, M. F., Bulyk, M. L., Wingreen, N. S. & Bassler, B. L. (2008). The *Vibrio harveyi* master quorum-sensing regulator, LuxR, a TetR-type protein is both an activator and a repressor: DNA recognition and binding specificity at target promoters. *Mol Microbiol* **70**, 76-88.

Pongsilp, N., Triplett, E. W. & Sadowsky, M. J. (2005). Detection of homoserine lactone-like quorum sensing molecules in *Bradyrhizobium* strains. *Curr Microbiol* **51**, 250-254.

Poole, P. S., Schofield, N. A., Reid, C. J., Drew, E. M. & Walshaw, D. L. (1994). Identification of chromosomal genes located downstream of *dctD* that affect the requirement for calcium and the lipopolysaccharide layer of *Rhizobium leguminosarum*. *Microbiology* **140 (Pt 10)**, 2797-2809.

Poplawsky, A. R. & Chun, W. (1997). *pigB* determines a diffusible factor needed for extracellular polysaccharide slime and xanthomonadin production in *Xanthomonas campestris* pv. *campestris*. *J Bacteriol* **179**, 439-444.

Poplawsky, A. R., Walters, D. M., Rouviere, P. E. & Chun, W. (2005). A gene for a dioxygenase-like protein determines the production of the DF signal in *Xanthomonas campestris* pv. *campestris*. *Mol Plant Pathol* **6**, 653-657.

Quandt, J., Clark, R. G., Venter, A. P., Clark, S. R., Twelker, S. & Hynes, M. F. (2004). Modified RP4 and Tn5-Mob derivatives for facilitated manipulation of large plasmids in Gram-negative bacteria. *Plasmid* **52**, 1-12.

Quinones, B., Pujol, C. J. & Lindow, S. E. (2004). Regulation of AHL production and its contribution to epiphytic fitness in *Pseudomonas syringae*. *Mol Plant Microbe Interact* **17**, 521-531.

Rajamani, S., Bauer, W. D., Robinson, J. B., Farrow, J. M., 3rd, Pesci, E. C., Teplitski, M., Gao, M., Sayre, R. T. & Phillips, D. A. (2008). The vitamin riboflavin and its derivative lumichrome activate the LasR bacterial quorum-sensing receptor. *Mol Plant Microbe Interact* **21**, 1184-1192.

Rampioni, G., Bertani, I., Zennaro, E., Polticelli, F., Venturi, V. & Leoni, L. (2006). The quorum-sensing negative regulator RsaL of *Pseudomonas aeruginosa* binds to the *lasI* promoter. *J Bacteriol* **188**, 815-819.

Rampioni, G., Schuster, M., Greenberg, E. P., Bertani, I., Grasso, M., Venturi, V., Zennaro, E. & Leoni, L. (2007). RsaL provides quorum sensing homeostasis and functions as a global regulator of gene expression in *Pseudomonas aeruginosa*. *Mol Microbiol* **66**, 1557-1565.

Ramsay, J. P., Sullivan, J. T., Jambari, N., Ortori, C. A., Heeb, S., Williams, P., Barrett, D. A., Lamont, I. L. & Ronson, C. W. (2009). A LuxRI-family regulatory system controls excision and transfer of the *Mesorhizobium loti* strain R7A symbiosis island by activating expression of two conserved hypothetical genes. *Mol Microbiol* **73**, 1141-1155.

Recourt, K., van Brussel, A. A., Driessen, A. J. & Lugtenberg, B. J. (1989). Accumulation of a nod gene inducer, the flavonoid naringenin, in the cytoplasmic membrane of *Rhizobium leguminosarum* biovar *viciae* is caused by the pH-dependent hydrophobicity of naringenin. *J Bacteriol* **171**, 4370-4377.

Redfield, R. J. (2002). Is quorum sensing a side effect of diffusion sensing? *Trends Microbiol* **10**, 365-370.

Reeve, W. G., Tiwari, R. P., Wong, C. M., Dilworth, M. J. & Glenn, A. R. (1998). The transcriptional regulator gene *phrR* in *Sinorhizobium meliloti* WSM419 is regulated by low pH and other stresses. *Microbiology* **144 (Pt 12)**, 3335-3342.

Reimmann, C., Beyeler, M., Latifi, A., Winteler, H., Foglino, M., Lazdunski, A. & Haas, D. (1997). The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer *N*-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. *Mol Microbiol* **24**, 309-319.

Reverchon, S., Bouillant, M. L., Salmond, G. & Nasser, W. (1998). Integration of the quorum-sensing system in the regulatory networks controlling virulence factor synthesis in *Erwinia chrysanthemi*. *Mol Microbiol* **29**, 1407-1418.

Rezzonico, F. & Duffy, B. (2008). Lack of genomic evidence of AI-2 receptors suggests a non-quorum sensing role for *luxS* in most bacteria. *BMC Microbiol* **8**, 154.

Rinaudi, L. V. & Gonzalez, J. E. (2009). The low-molecular-weight fraction of exopolysaccharide II from *Sinorhizobium meliloti* is a crucial determinant of biofilm formation. *J Bacteriol* **191**, 7216-7224.

Rivas, M., Seeger, M., Jedlicki, E. & Holmes, D. S. (2007). Second acyl homoserine lactone production system in the extreme acidophile *Acidithiobacillus ferrooxidans*. *Appl Environ Microbiol* **73**, 3225-3231.

Robleto, E. A., Kmiecik, K., Oplinger, E. S., Nienhuis, J. & Triplett, E. W. (1998). Trifolitoxin production increases nodulation competitiveness of *Rhizobium etli* CE3 under agricultural conditions. *Appl Environ Microbiol* **64**, 2630-2633.

Rodelas, B., Lithgow, J. K., Wisniewski-Dye, F., Hardman, A., Wilkinson, A., Economou, A., Williams, P. & Downie, J. A. (1999). Analysis of quorum-sensing-dependent control of rhizosphere-expressed (*rhi*) genes in *Rhizobium leguminosarum* bv. *viciae*. *J Bacteriol* **181**, 3816-3823.

Rosemeyer, V., Michiels, J., Verreth, C. & Vanderleyden, J. (1998). *luxI*- and *luxR*-homologous genes of *Rhizobium etli* CNPAF512 contribute to synthesis of autoinducer molecules and nodulation of *Phaseolus vulgaris*. *J Bacteriol* **180**, 815-821.

Rubio, L. M. & Ludden, P. W. (2005). Maturation of nitrogenase: a biochemical puzzle. *J Bacteriol* **187**, 405-414.

Russo, D. M., Williams, A., Edwards, A., Posadas, D. M., Finnie, C., Dankert, M., Downie, J. A. & Zorreguieta, A. (2006). Proteins exported via the PrsD-PrsE type I secretion system and the acidic exopolysaccharide are involved in biofilm formation by *Rhizobium leguminosarum*. *J Bacteriol* **188**, 4474-4486.

Ruvkun, G. B. & Ausubel, F. M. (1981). A general method for site-directed mutagenesis in prokaryotes. *Nature* **289**, 85-88.

Ryan, R. P., Fouhy, Y., Lucey, J. F. & other authors (2006). Cell-cell signaling in *Xanthomonas campestris* involves an HD-GYP domain protein that functions in cyclic di-GMP turnover. *Proc Natl Acad Sci U S A* **103**, 6712-6717.

Ryan, R. P., McCarthy, Y., Watt, S. A., Niehaus, K. & Dow, J. M. (2009). Intraspecies signaling involving the diffusible signal factor BDSF (*cis*-2-dodecenoic acid) influences virulence in *Burkholderia cenocepacia*. *J Bacteriol* **191**, 5013-5019.

Ryan, R. P., McCarthy, Y., Andrade, M., Farah, C. S., Armitage, J. P. & Dow, J. M. (2010). Cell-cell signal-dependent dynamic interactions between HD-GYP and GGDEF domain proteins mediate virulence in *Xanthomonas campestris*. *Proc Natl Acad Sci U S A* **107**, 5989-5994.

Salahuddin, A. (1992). Legume lectins: homologous proteins with similar structure but distinct carbohydrate binding specificity. *Indian J Biochem Biophys* **29**, 388-393.

Sambrook, J., Fritch, E. & Maniatis, T. (2001). Molecular Cloning: A Laboratory Manual. *(Cold Spring Harbor Lab Press, Cold Spring Harbor, NY), 3rd Ed.*

Sanchez-Contreras, M., Bauer, W. D., Gao, M., Robinson, J. B. & Allan Downie, J. (2007). Quorum-sensing regulation in rhizobia and its role in symbiotic interactions with legumes. *Philos Trans R Soc Lond B Biol Sci* **362**, 1149-1163.

Schachtman, D. P., Reid, R. J. & Ayling, S. M. (1998). Phosphorus uptake by plants: from soil to cell. *Plant Physiol* **116**, 447-453.

Schaefer, A. L., Hanzelka, B. L., Eberhard, A. & Greenberg, E. P. (1996a). Quorum sensing in *Vibrio fischeri*: probing autoinducer-LuxR interactions with autoinducer analogs. *J Bacteriol* **178**, 2897-2901.

Schaefer, A. L., Val, D. L., Hanzelka, B. L., Cronan, J. E., Jr. & Greenberg, E. P. (1996b). Generation of cell-to-cell signals in quorum sensing: acyl homoserine lactone synthase activity of a purified *Vibrio fischeri* LuxI protein. *Proc Natl Acad Sci U S A* **93**, 9505-9509.

Schaefer, A. L., Greenberg, E. P., Oliver, C. M. & other authors (2008). A new class of homoserine lactone quorum-sensing signals. *Nature* **454**, 595-599.

Schafer, A., Tauch, A., Jager, W., Kalinowski, J., Thierbach, G. & Puhler, A. (1994). Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **145**, 69-73.

Schauder, S., Shokat, K., Surette, M. G. & Bassler, B. L. (2001). The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. *Mol Microbiol* **41**, 463-476.

Schirmer, T. & Jenal, U. (2009). Structural and mechanistic determinants of c-di-GMP signalling. *Nat Rev Microbiol* **7**, 724-735.

Schripsema, J., de Rudder, K. E., van Vliet, T. B., Lankhorst, P. P., de Vroom, E., Kijne, J. W. & van Brussel, A. A. (1996). Bacteriocin *small* of *Rhizobium leguminosarum* belongs to the class of *N*-acyl-L-homoserine lactone molecules, known as autoinducers and as quorum sensing co-transcription factors. *J Bacteriol* **178**, 366-371.

Schuster, M., Lostroh, C. P., Ogi, T. & Greenberg, E. P. (2003). Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J Bacteriol* **185**, 2066-2079.

Schuster, M., Hawkins, A. C., Harwood, C. S. & Greenberg, E. P. (2004). The *Pseudomonas aeruginosa* RpoS regulon and its relationship to quorum sensing. *Mol Microbiol* **51**, 973-985.

Sherwood, M. T. (1970). Improved synthetic medium for the growth of *Rhizobium*. *J Appl Bacteriol* **33**, 708-713.

Shikuma, N. J., Fong, J. C., Odell, L. S., Perchuk, B. S., Laub, M. T. & Yildiz, F. H. (2009). Overexpression of VpsS, a hybrid sensor kinase, enhances biofilm formation in *Vibrio cholerae*. *J Bacteriol* **191**, 5147-5158.

Showalter, R. E., Martin, M. O. & Silverman, M. R. (1990). Cloning and nucleotide sequence of *luxR*, a regulatory gene controlling bioluminescence in *Vibrio harveyi*. *J Bacteriol* **172**, 2946-2954.

Siehnel, R., Traxler, B., An, D. D., Parsek, M. R., Schaefer, A. L. & Singh, P. K. (2010). A unique regulator controls the activation threshold of quorum-regulated genes in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **107**, 7916-7921.

Sircili, M. P., Walters, M., Trabulsi, L. R. & Sperandio, V. (2004). Modulation of enteropathogenic *Escherichia coli* virulence by quorum sensing. *Infect Immun* **72**, 2329-2337.

Skorupska, A., Janczarek, M., Marczak, M., Mazur, A. & Krol, J. (2006). Rhizobial exopolysaccharides: genetic control and symbiotic functions. *Microb Cell Fact* **5**, 7.

Smit, G., Kijne, J. W. & Lugtenberg, B. J. (1987). Involvement of both cellulose fibrils and a Ca^{2+}-dependent adhesin in the attachment of *Rhizobium leguminosarum* to pea root hair tips. *J Bacteriol* **169**, 4294-4301.

Smit, G., Kijne, J. W. & Lugtenberg, B. J. (1989a). Roles of flagella, lipopolysaccharide, and a Ca^{2+}-dependent cell surface protein in attachment of *Rhizobium leguminosarum* biovar *viciae* to pea root hair tips. *J Bacteriol* **171**, 569-572.

Smit, G., Logman, T. J., Boerrigter, M. E., Kijne, J. W. & Lugtenberg, B. J. (1989b). Purification and partial characterization of the *Rhizobium leguminosarum* biovar *viciae* Ca^{2+}-dependent adhesin, which mediates the first step in attachment of cells of the family Rhizobiaceae to plant root hair tips. *J Bacteriol* **171**, 4054-4062.

Smit, G., Swart, S., Lugtenberg, B. J. & Kijne, J. W. (1992). Molecular mechanisms of attachment of *Rhizobium* bacteria to plant roots. *Mol Microbiol* **6**, 2897-2903.

Sonck, K. A., Kint, G., Schoofs, G., Vander Wauven, C., Vanderleyden, J. & De Keersmaecker, S. C. (2009). The proteome of *Salmonella Typhimurium* grown under *in vivo*-mimicking conditions. *Proteomics* **9**, 565-579.

Sonnleitner, E., Schuster, M., Sorger-Domenigg, T., Greenberg, E. P. & Blasi, U. (2006). Hfq-dependent alterations of the transcriptome profile and effects on quorum sensing in *Pseudomonas aeruginosa*. *Mol Microbiol* **59**, 1542-1558.

Soto, M. J., Zorzano, A., Mercado-Blanco, J., Lepek, V., Olivares, J. & Toro, N. (1993). Nucleotide sequence and characterization of *Rhizobium meliloti* nodulation competitiveness genes *nfe*. *J Mol Biol* **229**, 570-576.

Soto, M. J., Zorzano, A., Garcia-Rodriguez, F. M., Mercado-Blanco, J., Lopez-Lara, I. M., Olivares, J. & Toro, N. (1994). Identification of a novel *Rhizobium meliloti* nodulation efficiency *nfe* gene homolog of *Agrobacterium* ornithine cyclodeaminase. *Mol Plant Microbe Interact* **7**, 703-707.

Sourjik, V., Muschler, P., Scharf, B. & Schmitt, R. (2000). VisN and VisR are global regulators of chemotaxis, flagellar, and motility genes in *Sinorhizobium* (*Rhizobium*) *meliloti*. *J Bacteriol* **182**, 782-788.

Spaink, H., Okker, R. J., Wijffelman, C. A., Pees, E. & Lugtenberg, B. (1987). Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1JI. . *Plant Mol Biol* **9**.

Spaink, H. P. (2000). Root nodulation and infection factors produced by rhizobial bacteria. *Annu Rev Microbiol* **54**, 257-288.

Sperandio, V., Torres, A. G., Jarvis, B., Nataro, J. P. & Kaper, J. B. (2003). Bacteria-host communication: the language of hormones. *Proc Natl Acad Sci U S A* **100**, 8951-8956.

Spiro, S. (1994). The FNR family of transcriptional regulators. *Antonie Van Leeuwenhoek* **66**, 23-36.

Steindler, L., Bertani, I., De Sordi, L., Schwager, S., Eberl, L. & Venturi, V. (2009). LasI/R and RhlI/R quorum sensing in a strain of *Pseudomonas aeruginosa* beneficial to plants. *Appl Environ Microbiol* **75**, 5131-5140.

Stulke, J. & Hillen, W. (1999). Carbon catabolite repression in bacteria. *Curr Opin Microbiol* **2**, 195-201.

Sturme, M. H., Kleerebezem, M., Nakayama, J., Akkermans, A. D., Vaugha, E. E. & de Vos, W. M. (2002). Cell to cell communication by autoinducing peptides in gram-positive bacteria. *Antonie Van Leeuwenhoek* **81**, 233-243.

Suh, S. J., Runyen-Janecky, L. J., Maleniak, T. C., Hager, P., MacGregor, C. H., Zielinski-Mozny, N. A., Phibbs, P. V., Jr. & West, S. E. (2002). Effect of *vfr* mutation on global gene expression and catabolite repression control of *Pseudomonas aeruginosa*. *Microbiology* **148**, 1561-1569.

Sun, J., Daniel, R., Wagner-Dobler, I. & Zeng, A. P. (2004). Is autoinducer-2 a universal signal for interspecies communication: a comparative genomic and phylogenetic analysis of the synthesis and signal transduction pathways. *BMC Evol Biol* **4**, 36.

Surette, M. G. & Bassler, B. L. (1999). Regulation of autoinducer production in *Salmonella typhimurium*. *Mol Microbiol* **31**, 585-595.

Svenningsen, S. L., Waters, C. M. & Bassler, B. L. (2008). A negative feedback loop involving small RNAs accelerates *Vibrio cholerae*'s transition out of quorum-sensing mode. *Genes Dev* **22**, 226-238.

Swartzman, E., Silverman, M. & Meighen, E. A. (1992). The *luxR* gene product of *Vibrio harveyi* is a transcriptional activator of the *lux* promoter. *J Bacteriol* **174**, 7490-7493.

Taga, M. E., Semmelhack, J. L. & Bassler, B. L. (2001). The LuxS-dependent autoinducer AI-2 controls the expression of an ABC transporter that functions in AI-2 uptake in *Salmonella typhimurium*. *Mol Microbiol* **42**, 777-793.

Taga, M. E., Miller, S. T. & Bassler, B. L. (2003). Lsr-mediated transport and processing of AI-2 in *Salmonella typhimurium*. *Mol Microbiol* **50**, 1411-1427.

Takaya, A., Tabuchi, F., Tsuchiya, H., Isogai, E. & Yamamoto, T. (2008). Negative regulation of quorum-sensing systems in Pseudomonas aeruginosa by ATP-dependent Lon protease. *J Bacteriol* **190**, 4181-4188.

Teiber, J. F., Horke, S., Haines, D. C., Chowdhary, P. K., Xiao, J., Kramer, G. L., Haley, R. W. & Draganov, D. I. (2008). Dominant role of paraoxonases in inactivation of the *Pseudomonas aeruginosa* quorum-sensing signal *N*-(3-oxododecanoyl)-L-homoserine lactone. *Infect Immun* **76**, 2512-2519.

Teplitski, M., Robinson, J. B. & Bauer, W. D. (2000). Plants secrete substances that mimic bacterial *N*-acyl homoserine lactone signal activities and affect population density-dependent behaviors in associated bacteria. *Mol Plant Microbe Interact* **13**, 637-648.

Teplitski, M., Eberhard, A., Gronquist, M. R., Gao, M., Robinson, J. B. & Bauer, W. D. (2003). Chemical identification of *N*-acyl homoserine lactone quorum-sensing signals produced by *Sinorhizobium meliloti* strains in defined medium. *Arch Microbiol* **180**, 494-497.

Teplitski, M., Chen, H., Rajamani, S., Gao, M., Merighi, M., Sayre, R. T., Robinson, J. B., Rolfe, B. G. & Bauer, W. D. (2004). *Chlamydomonas reinhardtii* secretes compounds that mimic bacterial signals and interfere with quorum sensing regulation in bacteria. *Plant Physiol* **134**, 137-146.

Thompson, L. S., Webb, J. S., Rice, S. A. & Kjelleberg, S. (2003). The alternative sigma factor RpoN regulates the quorum sensing gene *rhlI* in *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* **220**, 187-195.

Thorne, S. H. & Williams, H. D. (1999). Cell density-dependent starvation survival of *Rhizobium leguminosarum* bv. *phaseoli*: identification of the role of an *N*-acyl homoserine lactone in adaptation to stationary-phase survival. *J Bacteriol* **181**, 981-990.

Timmers, A. C., Soupene, E., Auriac, M. C., de Billy, F., Vasse, J., Boistard, P. & Truchet, G. (2000). Saprophytic intracellular rhizobia in alfalfa nodules. *Mol Plant Microbe Interact* **13**, 1204-1213.

Timmins, G. S., Jackson, S. K. & Swartz, H. M. (2001). The evolution of bioluminescent oxygen consumption as an ancient oxygen detoxification mechanism. *J Mol Evol* **52**, 321-332.

Tiwari, R. P., Reeve, W. G., Fenner, B. J., Dilworth, M. J., Glenn, A. R. & Howieson, J. G. (2004). Probing for pH-regulated genes in *Sinorhizobium medicae* using transcriptional analysis. *J Mol Microbiol Biotechnol* **7**, 133-139.

Torres-Quesada, O., Oruezabal, R. I., Peregrina, A., Jofre, E., Lloret, J., Rivilla, R., Toro, N. & Jimenez-Zurdo, J. I. (2010). The *Sinorhizobium meliloti* RNA chaperone Hfq influences central carbon metabolism and the symbiotic interaction with alfalfa. *BMC Microbiol* **10**, 71.

Torres, P. S., Malamud, F., Rigano, L. A. & other authors (2007). Controlled synthesis of the DSF cell-cell signal is required for biofilm formation and virulence in *Xanthomonas campestris*. *Environ Microbiol* **9**, 2101-2109.

Truchado, P., Gil-Izquierdo, A., Tomas-Barberan, F. & Allende, A. (2009). Inhibition by chestnut honey of *N*-Acyl-L-homoserine lactones and biofilm formation in *Erwinia carotovora, Yersinia enterocolitica*, and *Aeromonas hydrophila*. *J Agric Food Chem* **57**, 11186-11193.

Tsou, A. M., Frey, E. M., Hsiao, A., Liu, Z. & Zhu, J. (2008). Coordinated regulation of virulence by quorum sensing and motility pathways during the initial stages of *Vibrio cholerae* infection. *Commun Integr Biol* **1**, 42-44.

Tu, K. C. & Bassler, B. L. (2007). Multiple small RNAs act additively to integrate sensory information and control quorum sensing in *Vibrio harveyi*. *Genes Dev* **21**, 221-233.

Tun-Garrido, C., Bustos, P., Gonzalez, V. & Brom, S. (2003). Conjugative transfer of p42a from *Rhizobium etli* CFN42, which is required for mobilization of the symbiotic plasmid, is regulated by quorum sensing. *J Bacteriol* **185**, 1681-1692.

Urbanowski, M. L., Lostroh, C. P. & Greenberg, E. P. (2004). Reversible acyl-homoserine lactone binding to purified *Vibrio fischeri* LuxR protein. *J Bacteriol* **186**, 631-637.

Uroz, S., Dessaux, Y. & Oger, P. (2009). Quorum sensing and quorum quenching: the yin and yang of bacterial communication. *Chembiochem* **10**, 205-216.

Val, D. L. & Cronan, J. E., Jr. (1998). *In vivo* evidence that *S*-adenosylmethionine and fatty acid synthesis intermediates are the substrates for the LuxI family of autoinducer synthases. *J Bacteriol* **180**, 2644-2651.

Valentin-Hansen, P., Eriksen, M. & Udesen, C. (2004). The bacterial Sm-like protein Hfq: a key player in RNA transactions. *Mol Microbiol* **51**, 1525-1533.

van Delden, C., Comte, R. & Bally, A. M. (2001). Stringent response activates quorum sensing and modulates cell density-dependent gene expression in *Pseudomonas aeruginosa*. *J Bacteriol* **183**, 5376-5384.

van Rhijn, P., Goldberg, R. B. & Hirsch, A. M. (1998). *Lotus corniculatus* nodulation specificity is changed by the presence of a soybean lectin gene. *Plant Cell* **10**, 1233-1250.

van Rhijn, P., Fujishige, N. A., Lim, P. O. & Hirsch, A. M. (2001). Sugar-binding activity of pea lectin enhances heterologous infection of transgenic alfalfa plants by *Rhizobium leguminosarum* biovar *viciae*. *Plant Physiol* **126**, 133-144.

Vannini, A., Volpari, C., Gargioli, C., Muraglia, E., Cortese, R., De Francesco, R., Neddermann, P. & Marco, S. D. (2002). The crystal structure of the quorum sensing protein TraR bound to its autoinducer and target DNA. *Embo J* **21**, 4393-4401.

Vannini, A., Volpari, C. & Di Marco, S. (2004). Crystal structure of the quorum-sensing protein TraM and its interaction with the transcriptional regulator TraR. *J Biol Chem* **279**, 24291-24296.

Vendeville, A., Winzer, K., Heurlier, K., Tang, C. M. & Hardie, K. R. (2005). Making 'sense' of metabolism: autoinducer-2, LuxS and pathogenic bacteria. *Nat Rev Microbiol* **3**, 383-396.

Ventre, I., Goodman, A. L., Vallet-Gely, I. & other authors (2006). Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. *Proc Natl Acad Sci U S A* **103**, 171-176.

Vesper, S. J. & Bauer, W. D. (1986). Role of pili (fimbriae) in attachment of *Bradyrhizobium japonicum* to soybean roots. *Appl Environ Microbiol* **52**, 134-141.

Visick, K. L., Foster, J., Doino, J., McFall-Ngai, M. & Ruby, E. G. (2000). *Vibrio fischeri lux* genes play an important role in colonization and development of the host light organ. *J Bacteriol* **182**, 4578-4586.

Voss, B., Holscher, M., Baumgarth, B., Kalbfleisch, A., Kaya, C., Hess, W. R., Becker, A. & Evguenieva-Hackenberg, E. (2009). Expression of small RNAs in Rhizobiales and protection of a small RNA and its degradation products by Hfq in *Sinorhizobium meliloti*. *Biochem Biophys Res Commun* **390**, 331-336.

Wade, D. S., Calfee, M. W., Rocha, E. R., Ling, E. A., Engstrom, E., Coleman, J. P. & Pesci, E. C. (2005). Regulation of *Pseudomonas* quinolone signal synthesis in *Pseudomonas aeruginosa*. *J Bacteriol* **187**, 4372-4380.

Wagner, V. E., Bushnell, D., Passador, L., Brooks, A. I. & Iglewski, B. H. (2003). Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *J Bacteriol* **185**, 2080-2095.

Walters, M., Sircili, M. P. & Sperandio, V. (2006). AI-3 synthesis is not dependent on *luxS* in *Escherichia coli*. *J Bacteriol* **188**, 5668-5681.

Wang, H., Zhong, Z., Cai, T., Li, S. & Zhu, J. (2004a). Heterologous overexpression of quorum-sensing regulators to study cell-density-dependent phenotypes in a symbiotic plant bacterium *Mesorhizobium huakuii*. *Arch Microbiol* **182**, 520-525.

Wang, J. H., Quan, C. S., Qi, X. H., Li, X. & Fan, S. D. (2010). Determination of diketopiperazines of *Burkholderia cepacia* CF-66 by gas chromatography-mass spectrometry. *Anal Bioanal Chem*.

Wang, L., Hashimoto, Y., Tsao, C. Y., Valdes, J. & Bentley, W. E. (2005a). Cyclic AMP (cAMP) and cAMP receptor protein influence both synthesis and uptake of extracellular autoinducer 2 in *Escherichia coli*. *J Bacteriol* **187**, 2066-2076.

Wang, L., Li, J., March, J. C., Valdes, J. J. & Bentley, W. E. (2005b). *luxS*-dependent gene regulation in *Escherichia coli* K-12 revealed by genomic expression profiling. *J Bacteriol* **187**, 8350-8360.

Wang, L. H., He, Y., Gao, Y. & other authors (2004b). A bacterial cell-cell communication signal with cross-kingdom structural analogues. *Mol Microbiol* **51**, 903-912.

Wang, Y., Huang, X., Hu, H., Zhang, X. & Xu, Y. (2008). QscR acts as an intermediate in *gacA*-dependent regulation of PCA biosynthesis in *Pseudomonas* sp. M-18. *Curr Microbiol* **56**, 339-345.

Waters, C. M. & Bassler, B. L. (2005). Quorum sensing: cell-to-cell communication in bacteria. *Annu Rev Cell Dev Biol* **21**, 319-346.

Waters, C. M. & Bassler, B. L. (2006). The *Vibrio harveyi* quorum-sensing system uses shared regulatory components to discriminate between multiple autoinducers. *Genes Dev* **20**, 2754-2767.

Weber, B., Croxatto, A., Chen, C. & Milton, D. L. (2008). RpoS induces expression of the Vibrio anguillarum quorum-sensing regulator VanT. *Microbiology* **154**, 767-780.

Weber, B., Hasic, M., Chen, C., Wai, S. N. & Milton, D. L. (2009). Type VI secretion modulates quorum sensing and stress response in *Vibrio anguillarum*. *Environ Microbiol*.

Weingart, C. L., White, C. E., Liu, S. & other authors (2005). Direct binding of the quorum sensing regulator CepR of *Burkholderia cenocepacia* to two target promoters *in vitro*. *Mol Microbiol* **57**, 452-467.

Welch, M., Todd, D. E., Whitehead, N. A., McGowan, S. J., Bycroft, B. W. & Salmond, G. P. (2000). *N*-acyl homoserine lactone binding to the CarR receptor determines quorum-sensing specificity in *Erwinia*. *Embo J* **19**, 631-641.

West, S. E., Sample, A. K. & Runyen-Janecky, L. J. (1994). The *vfr* gene product, required for *Pseudomonas aeruginosa* exotoxin A and protease production, belongs to the cyclic AMP receptor protein family. *J Bacteriol* **176**, 7532-7542.

Whistler, C. A. & Ruby, E. G. (2003). GacA regulates symbiotic colonization traits of *Vibrio fischeri* and facilitates a beneficial association with an animal host. *J Bacteriol* **185**, 7202-7212.

White, C. E. & Winans, S. C. (2007). Cell-cell communication in the plant pathogen *Agrobacterium tumefaciens*. *Philos Trans R Soc Lond B Biol Sci* **362**, 1135-1148.

Whitehead, N. A., Barnard, A. M., Slater, H., Simpson, N. J. & Salmond, G. P. (2001). Quorum-sensing in Gram-negative bacteria. *FEMS Microbiol Rev* **25**, 365-404.

Whiteley, M., Parsek, M. R. & Greenberg, E. P. (2000). Regulation of quorum sensing by RpoS in *Pseudomonas aeruginosa*. *J Bacteriol* **182**, 4356-4360.

Wijffelman, C. A., Pees, E., Van Brussel, A. & Hooykaas, P. (1983). Repression of small bacteriocin excretion in *Rhizobium leguminosarum* and *Rhizobium trifolii* by transmissible plasmids. *Mol Gen Genet* **192**, 171-176.

Wilke, M. S., Heller, M., Creagh, A. L., Haynes, C. A., McIntosh, L. P., Poole, K. & Strynadka, N. C. (2008). The crystal structure of MexR from *Pseudomonas aeruginosa* in complex with its antirepressor ArmR. *Proc Natl Acad Sci U S A* **105**, 14832-14837.

Wilkinson, A., Danino, V., Wisniewski-Dye, F., Lithgow, J. K. & Downie, J. A. (2002). *N*-acyl-homoserine lactone inhibition of rhizobial growth is mediated by two quorum-sensing genes that regulate plasmid transfer. *J Bacteriol* **184**, 4510-4519.

Willcox, M. D., Zhu, H., Conibear, T. C., Hume, E. B., Givskov, M., Kjelleberg, S. & Rice, S. A. (2008). Role of quorum sensing by *Pseudomonas aeruginosa* in microbial keratitis and cystic fibrosis. *Microbiology* **154**, 2184-2194.

Williams, A. (2006). Effect of legume root exudate on attachment and biofilm formation by *Rhizobium leguminosarum*. *PhD thesis, University of East Anglia, Norwich (UK).*

Williams, A., Wilkinson, A., Krehenbrink, M., Russo, D. M., Zorreguieta, A. & Downie, J. A. (2008). Glucomannan-mediated attachment of *Rhizobium leguminosarum* to pea root hairs is required for competitive nodule infection. *J Bacteriol* **190**, 4706-4715.

Williams, P., Camara, M., Hardman, A. & other authors (2000). Quorum sensing and the population-dependent control of virulence. *Philos Trans R Soc Lond B Biol Sci* **355**, 667-680.

Williams, P. (2007). Quorum sensing, communication and cross-kingdom signalling in the bacterial world. *Microbiology* **153**, 3923-3938.

Winstanley, C. & Fothergill, J. L. (2009). The role of quorum sensing in chronic cystic fibrosis *Pseudomonas aeruginosa* infections. *FEMS Microbiol Lett* **290**, 1-9.

Winzer, K. & Williams, P. (2001). Quorum sensing and the regulation of virulence gene expression in pathogenic bacteria. *Int J Med Microbiol* **291**, 131-143.

Winzer, K., Hardie, K. R. & Williams, P. (2002). Bacterial cell-to-cell communication: sorry, can't talk now - gone to lunch! *Curr Opin Microbiol* **5**, 216-222.

Winzer, K., Hardie, K. R. & Williams, P. (2003). LuxS and autoinducer-2: their contribution to quorum sensing and metabolism in bacteria. *Adv Appl Microbiol* **53**, 291-396.

Wisniewski-Dye, F. & Downie, J. A. (2002). Quorum-sensing in *Rhizobium*. *Antonie Van Leeuwenhoek* **81**, 397-407.

Wisniewski-Dye, F., Jones, J., Chhabra, S. R. & Downie, J. A. (2002). *railR* genes are part of a quorum-sensing network controlled by *cinI* and *cinR* in *Rhizobium leguminosarum*. *J Bacteriol* **184**, 1597-1606.

Wolff, J. A., MacGregor, C. H., Eisenberg, R. C. & Phibbs, P. V., Jr. (1991). Isolation and characterization of catabolite repression control mutants of *Pseudomonas aeruginosa* PAO. *J Bacteriol* **173**, 4700-4706.

Xavier, K. B. & Bassler, B. L. (2005). Regulation of uptake and processing of the quorum-sensing autoinducer AI-2 in *Escherichia coli*. *J Bacteriol* **187**, 238-248.

Xavier, K. B., Miller, S. T., Lu, W., Kim, J. H., Rabinowitz, J., Pelczer, I., Semmelhack, M. F. & Bassler, B. L. (2007). Phosphorylation and processing of the quorum-sensing molecule autoinducer-2 in enteric bacteria. *ACS Chem Biol* **2**, 128-136.

Xiao, G., Deziel, E., He, J. & other authors (2006a). MvfR, a key *Pseudomonas aeruginosa* pathogenicity LTTR-class regulatory protein, has dual ligands. *Mol Microbiol* **62**, 1689-1699.

Xiao, G., He, J. & Rahme, L. G. (2006b). Mutation analysis of the *Pseudomonas aeruginosa mvfR* and *pqsABCDE* gene promoters demonstrates complex quorum-sensing circuitry. *Microbiology* **152**, 1679-1686.

Xiao, J., Wang, Q., Liu, Q., Xu, L., Wang, X., Wu, H. & Zhang, Y. (2009). Characterization of *Edwardsiella tarda rpoS*: effect on serum resistance, chondroitinase activity, biofilm formation, and autoinducer synthetases expression. *Appl Microbiol Biotechnol* **83**, 151-160.

Yan, Q., Gao, W., Wu, X. G. & Zhang, L. Q. (2009a). Regulation of the PcoI/PcoR quorum-sensing system in *Pseudomonas fluorescens* 2P24 by the PhoP/PhoQ two-component system. *Microbiology* **155**, 124-133.

Yan, Q., Wu, X. G., Wei, H. L., Wang, H. M. & Zhang, L. Q. (2009b). Differential control of the PcoI/PcoR quorum-sensing system in *Pseudomonas fluorescens* 2P24 by sigma factor RpoS and the GacS/GacA two-component regulatory system. *Microbiol Res* **164**, 18-26.

Yang, M., Sun, K., Zhou, L., Yang, R., Zhong, Z. & Zhu, J. (2009). Functional analysis of three AHL autoinducer synthase genes in *Mesorhizobium loti* reveals the important role of quorum sensing in symbiotic nodulation. *Can J Microbiol* **55**, 210-214.

Yates, E. A., Philipp, B., Buckley, C. & other authors (2002). *N*-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. *Infect Immun* **70**, 5635-5646.

Yost, C. K., Rochepeau, P. & Hynes, M. F. (1998). *Rhizobium leguminosarum* contains a group of genes that appear to code for methyl-accepting chemotaxis proteins. *Microbiology* **144 (Pt 7)**, 1945-1956.

Young, J. P., Crossman, L. C., Johnston, A. W. & other authors (2006). The genome of *Rhizobium leguminosarum* has recognizable core and accessory components. *Genome Biol* **7**, R34.

Yu, S., Jensen, V., Seeliger, J., Feldmann, I., Weber, S., Schleicher, E., Haussler, S. & Blankenfeldt, W. (2009). Structure elucidation and preliminary assessment of hydrolase activity of PqsE, the *Pseudomonas quinolone* signal (PQS) response protein. *Biochemistry* **48**, 10298-10307.

Yuhashi, K., Ichikawa, N., Ezura, H., Akao, S., Minakawa, Y., Nukui, N., Yasuta, T. & Minamisawa, K. (2000). Rhizobitoxine production by *Bradyrhizobium elkanii* enhances nodulation and competitiveness on *Macroptilium atropurpureum*. *Appl Environ Microbiol* **66**, 2658-2663.

Zaborin, A., Romanowski, K., Gerdes, S. & other authors (2009). Red death in *Caenorhabditis elegans* caused by *Pseudomonas aeruginosa* PAO1. *Proc Natl Acad Sci U S A* **106**, 6327-6332.

Zaborina, O., Lepine, F., Xiao, G. & other authors (2007). Dynorphin activates quorum sensing quinolone signaling in *Pseudomonas aeruginosa*. *PLoS Pathog* **3**, e35.

Zhang, H. B., Wang, C. & Zhang, L. H. (2004). The quormone degradation system of *Agrobacterium tumefaciens* is regulated by starvation signal and stress alarmone (p)ppGpp. *Mol Microbiol* **52**, 1389-1401.

Zhang, L. H. (2003). Quorum quenching and proactive host defense. *Trends Plant Sci* **8**, 238-244.

Zhang, R. G., Pappas, T., Brace, J. L. & other authors (2002). Structure of a bacterial quorum-sensing transcription factor complexed with pheromone and DNA. *Nature* **417**, 971-974.

Zhang, Z. & Pierson, L. S., 3rd (2001). A second quorum-sensing system regulates cell surface properties but not phenazine antibiotic production in *Pseudomonas aureofaciens*. *Appl Environ Microbiol* **67**, 4305-4315.

Zheng, H., Zhong, Z., Lai, X., Chen, W. X., Li, S. & Zhu, J. (2006). A LuxR/LuxI-type quorum-sensing system in a plant bacterium, *Mesorhizobium tianshanense*, controls symbiotic nodulation. *J Bacteriol* **188**, 1943-1949.

Zhu, J., Beaber, J. W., More, M. I., Fuqua, C., Eberhard, A. & Winans, S. C. (1998). Analogs of the autoinducer 3-oxooctanoyl-homoserine lactone strongly inhibit activity of the TraR protein of *Agrobacterium tumefaciens*. *J Bacteriol* **180**, 5398-5405.

Zhu, J. & Winans, S. C. (1998). Activity of the quorum-sensing regulator TraR of *Agrobacterium tumefaciens* is inhibited by a truncated, dominant defective TraR-like protein. *Mol Microbiol* **27**, 289-297.

Zhu, J., Miller, M. B., Vance, R. E., Dziejman, M., Bassler, B. L. & Mekalanos, J. J. (2002). Quorum-sensing regulators control virulence gene expression in *Vibrio cholerae*. *Proc Natl Acad Sci U S A* **99**, 3129-3134.

Zorreguieta, A., Finnie, C. & Downie, J. A. (2000). Extracellular glycanases of *Rhizobium leguminosarum* are activated on the cell surface by an exopolysaccharide-related component. *J Bacteriol* **182**, 1304-1312.

Zou, Y. & Nair, S. K. (2009). Molecular basis for the recognition of structurally distinct autoinducer mimics by the *Pseudomonas aeruginosa* LasR quorum-sensing signaling receptor. *Chem Biol* **16**, 961-970.

	***cinS* mutant**				***expR* mutant**				***praR* mutant**				**function**
	WT	**mutant**	**fold**	**p**	**raw**	**mutant**	**fold**	**p**	**raw**	**mutant**	**fold**	**p**	
pRL100164	2198	861	0.30	0.11	1825	792	0.39	0.07	6708	6802	0.77	0.53	*rhiI*, autoinducer synthesis protein
pRL100169	9622	4341	0.36	0.07	6995	3332	0.39	0.12	15590	17919	0.96	0.95	rhizosphere expressed protein RhiA
pRL100170	3298	2410	0.50	0.23	1574	819	0.33	0.12	3969	3711	0.79	0.63	putative rhizosphere induced protein RhiB
pRL110060	2238	1364	0.55	0.05	1972	1340	0.70	0.13	4067	5315	1.37	0.01	putative transmembrane component of ABC transporter
pRL110096	1809	1529	0.72	0.22	1344	833	0.48	0.02	1398	1813	1.20	0.22	conserved hypothetical protein
pRL110097	991	423	0.34	0.03	1157	501	0.42	<0.01	2631	3273	1.41	0.14	conserved hypothetical protein
pRL70183	5864	3959	0.62	0.01	4318	2596	0.60	<0.01	4148	3594	0.94	0.76	conserved hypothetical exported protein
RL0561	1815	1600	0.74	0.23	1727	1088	0.63	0.01	2045	1815	0.95	0.74	putative AraC family transcriptional regulator
RL1103	1169	816	0.70	0.02	1046	655	0.62	0.01	2340	2330	1.00	0.98	conserved hypothetical protein
RL1910	2208	1891	0.92	0.29	827	590	0.63	0.05	1628	1270	0.54	0.26	conserved hypothetical protein
RL1911	1657	793	0.41	0.04	1295	560	0.34	0.13	1395	597	0.59	0.49	putative arylsulfatase
RL1933	1064	789	0.71	0.06	929	580	0.65	0.03	2017	2078	0.99	0.94	conserved hypothetical protein
RL1940	2789	1847	0.61	0.01	1895	1009	0.54	<0.01	1808	1940	1.04	0.58	hypothetical protein
RL2105A	4535	2855	0.58	0.03	3045	1646	0.55	<0.01	2941	3098	1.08	0.31	hypothetical protein
RL2107	4914	2976	0.56	0.02	4072	2283	0.57	<0.01	2993	3493	1.17	0.01	conserved hypothetical protein
RL2169	3130	2154	0.59	0.04	1389	832	0.60	<0.01	2291	2747	1.32	0.23	hypothetical exported protein
RL2262	2027	1109	0.54	<0.01	1522	730	0.50	<0.01	2509	2541	1.06	0.58	putative adenylate cyclase
RL2231	234	49	0.35	0.21	1931	1188	0.59	0.01	136	277	1.76	0.33	lipase/esterase
RL2423	3040	2203	0.62	0.04	2065	1050	0.47	0.03	2930	3049	1.05	0.65	probable peptidase/protease
RL2684	5412	4258	0.68	0.15	3522	2045	0.62	0.03	3170	2872	0.83	0.23	conserved hypothetical exported protein
RL2685	3226	2975	0.85	0.20	1276	787	0.66	0.04	1895	1972	0.94	0.74	hypothetical exported protein
RL2834	2580	1696	0.64	0.06	1770	1054	0.60	0.04	2893	2662	0.89	0.17	putative adenylate/guanylate cyclase
RL2994	2685	1949	0.73	0.07	1507	1006	0.64	0.03	1811	2008	1.23	0.14	putative transmembrane component of ABC transporter

	cinS mutant				expR mutant				praR mutant				function
	WT	mutant	fold	p	raw	mutant	fold	p	raw	mutant	fold	p	
RL3073	744	568	0.65	0.20	577	376	0.63	0.03	1505	2368	1.69	0.02	hypothetical protein
RL3074	3437	3168	0.65	0.35	1151	581	0.48	0.01	2575	4706	1.92	<0.01	*rapC, Rhizobium* adhesion protein
RL3119	2916	2445	0.69	0.15	2128	1126	0.55	0.04	2755	2926	1.00	0.98	putative two-component system response regulator transcriptional regulatory protein
RL3302	8563	4295	0.46	0.03	6874	3039	0.50	0.04	2863	3878	1.39	0.03	putative aquaporin Z
RL3382	3504	2766	0.71	0.14	2547	1723	0.63	0.03	3096	2400	0.81	0.23	putative outer membrane protein related to bp26 antigen of *Brucella*
RL4031	1020	657	0.58	0.01	1063	701	0.62	0.08	1431	1551	1.08	0.11	putative sensory transducer methyl-accepting chemotaxis protein
RL4032	1219	941	0.75	0.07	880	537	0.62	0.01	1026	1385	1.66	0.13	putative sensory transducer methyl-accepting chemotaxis protein
RL4037	2669	1336	0.46	<0.01	1678	823	0.51	<0.01	2329	2736	1.21	0.13	conserved hypothetical protein
RL4665	2974	1485	0.41	0.01	2364	974	0.41	0.01	5512	9770	1.96	0.03	conserved hypothetical protein

Supplementary table 1: Microarray results for *cinS* (A1245), *expR* (A1246) and *praR* (A1167) mutants, Group A. All numbers are the average score of three independent microarray experiments. The genes in this group are downregulated in the *cinS* and *expR* mutants. Cut-off values during the first analysis were chosen as > 500 for the WT expression level, and p > 0.1 (Genespring t-test). Figures in shaded cells did not make the cut-offs during the first analysis. The *cinS* and *expR* mutant microarrays showed the same expression pattern.

	cinS mutant				*expR* mutant				*praR* mutant				Function
	WT	mutant	fold	p	raw	mutant	fold	p	raw	mutant	fold	p	
pRL100451	4913	10372	1.94	0.01	4034	7436	1.90	<0.01	6080	20511	3.41	<0.01	putative autoaggregation protein
RL0390	4733	7543	1.89	0.11	2789	7727	2.77	0.03	3346	14140	4.34	<0.01	*praR*, HTH transcriptional regulator

Supplementary table 2: Microarray results for *cinS* (A1245), *expR* (A1246) and *praR* (A1167) mutants, Group B. All numbers are the average score of three independent microarray experiments. The genes in this group are downregulated in the *cinS* and *expR* mutants. Cut-off values during the first analysis were chosen as > 500 for the WT expression level, and p > 0.1 (Genespring t-test). Figures in shaded cells did not make the cut-offs during the first analysis.

	***cinS* mutant**				***expR* mutant**				***praR* mutant**				**function**
	WT	**mutant**	**fold**	**p**	**raw**	**mutant**	**fold**	**p**	**raw**	**mutant**	**fold**	**p**	
pRL100465	924	1125	1.37	0.22	935	1401	1.52	<0.01	1811	1509	0.93	0.71	putative AraC family transcriptional regulator
pRL120625	1702	2526	1.78	0.02	638	1220	1.96	0.07	2096	2292	1.03	0.54	putative peptidase
pRL120626	517	1231	2.18	0.01	443	1067	2.21	0.02	1094	1198	1.05	0.78	conserved hypothetical protein
pRL120627	661	1178	1.78	0.01	555	792	1.83	0.11	739	760	1.09	0.59	conserved hypothetical protein
RL1065	481	795	1.58	0.02	631	994	1.78	0.15	699	403	0.55	0.03	putative methyl-accepting chemotaxis protein
RL4370	3762	4746	1.43	0.11	1784	4673	2.68	<0.01	2472	1784	0.75	0.21	conserved hypothetical protein
RL4371	2660	4569	1.95	0.02	1863	3741	2.02	<0.01	2007	1533	0.76	0.07	conserved hypothetical protein

Supplementary table 3: Microarray results for *cinS* (A1245), *expR* (A1246) and *praR* (A1167) mutants, Group C. All numbers are the average score of three independent microarray experiments. The genes in this group are downregulated in the *cinS* and *expR* mutants. Cut-off values during the first analysis were chosen as > 500 for the WT expression level, and p > 0.1 (Genespring t-test). Figures in shaded cells did not make the cut-offs during the first analysis. The *cinS* and *expR* mutant microarrays showed the same expression pattern.

	***cinS* mutant**				***expR* mutant**				***praR* mutant**				**Function**
	WT	**mutant**	**fold**	**p**	**raw**	**mutant**	**fold**	**p**	**raw**	**mutant**	**fold**	**p**	
pRL120083	3243	3310	0.94	0.55	2470	2593	1.02	0.81	2568	1700	0.63	0.01	conserved hypothetical protein
RL1049	742	730	0.89	0.32	516	587	1.07	0.63	1148	524	0.50	0.02	putative solute-binding component of ABC transporter
RL3085	4211	3908	0.92	0.06	2361	2515	0.97	0.84	1810	932	0.52	0.02	conserved hypothetical exported protein
RL3670	55901	30023	0.62	0.17	42267	44481	0.75	0.55	44289	6679	0.13	<0.01	conserved hypothetical protein
RL4275	1876	1587	0.80	0.12	1325	1119	0.82	0.13	2663	1874	0.57	0.03	putative Acr/RND family efflux transmembrane transporter
RL0149	3217	5587	1.11	0.86	2519	1931	1.00	1.00	3314	10841	3.68	0.01	putative HTH-type transcriptional regulator
RL2811	6200	10359	1.07	0.91	4464	3252	0.81	0.65	6766	10595	1.56	0.03	conserved hypothetical protein
RL2937	987	1452	0.86	0.83	962	663	0.84	0.58	682	1273	1.93	<0.01	putative transmembrane protein
RL3634	2620	6188	1.74	0.20	1424	1329	1.20	0.75	2894	4391	1.74	0.04	hypothetical protein

Supplementary table 4: Microarray results for *cinS* (A1245), *expR* (A1246) and *praR* (A1167) mutants, Group D. All numbers are the average score of three independent microarray experiments. The genes in this group are downregulated in the *cinS* and *expR* mutants. Cut-off values during the first analysis were chosen as > 500 for the WT expression level, and p > 0.1 (Genespring t-test). Figures in shaded cells did not make the cut-offs during the first analysis.

	WT		*cinS*			*expR*			*praR*		
	Miller units	**st. dev.**	**Miller units**	**st. dev.**	**fold**	**Miller units**	**st. dev.**	**fold**	**Miller units**	**st. dev.**	**fold**
pRL110060	1752	332	192	30	0.11	152	22	0.09	971	142	0.55
pRL110096	337	131	3387	229	10.05	3524	99	10.46	1122	78	3.33
pRL110097	1420	n.d.	148	40	0.10	94	16	0.07	501	94	0.35
rhiR	1060.83	17.49	1002	178	0.94	1111	229	1.05	2048	135	1.93
RL1940	1177	13	683	112	0.58	133	19	0.11	2024	117	1.72
RL2169	259	8	3204	296	12.37	3106	618	11.99	9371	719	36.18
RL2331	2768	194	680	63	0.25	601	24	0.22	1483	79	0.54
RL2423	290	43	1917	102	6.61	1899	131	6.55	6391	285	22.04
RL3074	420	44	191	52	0.45	182	44	0.43	433	94	1.03
RL3302	5844	1524	272	47	0.05	265	69	0.05	1188	44	0.20
RL4371	2250	176	180	21	0.08	187	4	0.08	353	17	0.16
RL4665	292	27	562	n.d.	1.92	614	n.d.	2.10	3155	n.d.	10.80

Supplementary table: Promoter-lacZ expression of selected genes identified during the microarray analysis in WT, *cinS* (A1245), *expR* (A1246) and *praR* (A1167) mutants. ExpR and PraR. Cultures were grown for 3 days in AMS minimal medium or TY and the expression was measured by the b-galactosidase expression. St.dev.: standard deviations. Fold: fold change in mutant compared to expression level of WT. pRL110060 on pIJ11167, pRL110096 on pIJ11165, pRL110097 on pIJ11198, *rhiR* on pIJ9104, RL1940 on 11168, RL2169 on pIJ11175, RL2331 on pIJ11177, RL2423 on pIJ11170, RL3074 on pIJ11171, RL3302 on pIJ11169, RL4371 on pIJ11164, RL4665 on pIJ11163.