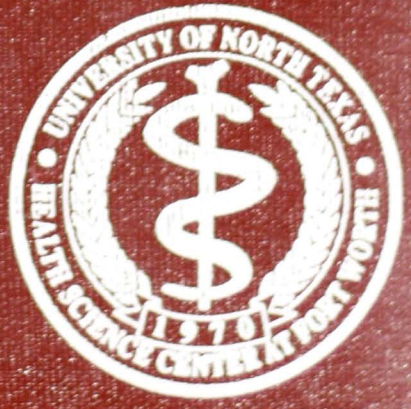
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Patankar, Arati V., Quorum Sensing in *Sinorhizobium meliloti.* Doctor of Philosophy (Microbiology and Immunology), December, 2008, 170 pp., 14 tables, 23 illustrations, bibliography, 212 titles.

The overall goal of this study was to elucidate the role of a series of transcriptional regulators and potential signal molecules in the coordination of gene regulation in *Sinorhizobium meliloti*. The agriculturally important gram-negative soil bacterium *S. meliloti*, forms a symbiotic association with its host legume, *Medicago sativa* (alfalfa); thereby serving as a good model for studying host-bacterial interactions. Often, bacteria associated with eukaryotic hosts utilize global gene regulatory systems to coordinate their behavior in order to establish pathogenic or symbiotic associations. Quorum sensing is one such form of bacterial gene regulation which is mediated by signaling molecules and regulatory proteins in a population density dependent manner.

In *S. meliloti*, the process of quorum sensing has been shown to play an important role in the relationship with its host plant. Control of essential processes such as plant nodulation and exopolysaccharide production has been attributed to the Sin/ExpR quorum-sensing system of *S. meliloti*. Interestingly, *S. meliloti* contains four additional (*SMc04032*, *SMc00658*, *SMc00878* and *SMc00877*) putative quorum-sensing response regulators whose regulatory network was not known. The predicted protein sequences of these genes contain features typical of the LuxR family of proteins i.e., an N-terminal signal binding domain and a C-terminal helix-turn-helix DNA binding domain. In order to identify their regulatory role, mutants of the response regulators were constructed and

their expression profile was determined by employing genome-wide microarray and real-time PCR expression analysis. Through these analyses, it was determined that the *SMc004032* locus controls expression of genes involved in the active methyl cycle, while the *SMc00658*, *SMc00878* and *SMc00877* loci control expression of genes from the denitrification of pathway of *S. meliloti*. Further, through phenotypic studies it was established that *SMc04032* impacts stress response adaptation, and effective competition for plant nodulation. This suggests that *SMc04032* could play a role in bacterial survival in the soil as well as within the host. The ability to denitrify is highly variable in different strains of *S. meliloti*. Through growth and enzymatic assays, it was established that the wild-type strain of this study, *S. meliloti* Rm8530, is a partial denitrifier in which, the capacity to metabolize nitrate is impaired. It was further determined that *SMc00658*, *SMc00878* and *SMc00877* modulated nitrite reductase activity under aerobic conditions, implying that these genes are involved in aerobic denitrification and therefore probably play a role in detoxification in *S. meliloti*.

Based on the sequenced-genome analysis, *S. meliloti* possess homologs of other mediators of quorum sensing, that might be responsible for the synthesis of novel signal molecules. Bioreporter strains and mass spectrometry analysis were employed to identify production of cyclic dipeptides in *S. meliloti*. These compounds have been previously reported as quorum-sensing signal molecules in several bacteria.

The results presented in this study provide a better understanding of *S. meliloti*'s metabolic and physiological properties and will be fundamental in future studies of bacterial interaction with its host and survival within its ecological niche.

# QUORUM SENSING IN *Sinorhizobium meliloti*

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Arati V. Patankar, M.S.

Fort Worth, Texas

December 2008

## ACKNOWLEDGEMENTS

I would like to thank Dr. Juan E. González, my thesis advisor, for his constant guidance, support, encouragement and interest throughout the course of this thesis. This project brought more difficulties than most and his sustained enthusiasm, patience and vision have helped me overcome many of the hurdles that I faced. I also thank the University of Texas at Dallas for allowing me to accomplish my Ph.D. work at their facilities.

I sincerely thank the members of my graduate committee, Dr. Jerry W. Simecka, Dr. Stephen O. Mathew, Dr. Harlan P. Jones, and Dr.Wolfram Siede for their guidance, patience, and encouragement throughout my work. Dr. Jerry W. Simecka has especially been invaluable in mentoring and providing timely help in different phases of my study.

I wish to thank my friends and colleagues Dr. Sarah Glenn, Dr. Audry Almengor, Dr. Neela Keshavan, Dr. Hanh Hoang, Jennifer Morris, Natalie Gurich, and Konrad Mueller who shared my experiences and provided constant motivation and support.

Finally, I am deeply grateful to my husband Nitin Chaturvedi, my parents, my parents-in-law, and my sisters, Dipali, Radhika and Darshana, for their undying encouragement and for being my pillars of strength.

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# CHAPTER I

## ORPHAN LUXR REGULATORS OF QUORUM SENSING

Bacteria modulate their behavior by releasing and responding to the accumulation of signal molecules. This population coordination, referred to as quorum sensing, is prevalent in Gram-negative and Gram-positive bacteria. The essential constituents of quorum-sensing systems include a signal producer, or synthase, and a cognate transcriptional regulator that responds to the accumulated signal molecules. With the availability of bacterial genome sequences and increased elucidation of quorum-sensing circuits, genes that encode for additional transcriptional regulators, usually in excess of the synthase, have been identified. These additional regulators are referred to as 'orphan' regulators, since they are not directly associated with a synthase. Orphan regulators have been characterized in several Gram-negative bacteria where their addition, expands the regulatory network of the bacteria.

### Introduction

Bacteria are often social organisms that form communities in their natural environments. Within these communities bacteria are subjected to constant changes in external conditions such as the availability of nutrients or the presence of toxic compounds. Therefore, it is critical that bacteria coordinate their behavior in order to adapt and survive. The regulation of genes mediated by signaling molecules and

regulatory proteins in a population density manner is referred to as quorum sensing. This phenomenon enables bacteria not only to sense members of their own species but others as well (131). Since it was first reported in marine bacteria by Nealson *et al.* in 1970, quorum sensing has been well characterized in both Gram-negative and Gram-positive microorganisms (136). Quorum sensing plays a role in human pathogenesis (*Pseudomonas aeruginosa*), symbiosis (*Sinorhizobium meliloti*), plasmid conjugation (*Agrobacterium tumefaciens*), and competence (*Bacillus subtilis*) (40, 62, 120, 142, 176, 195, 197).

**The quorum sensing paradigm in Gram-negative bacteria**

*Vibrio fischeri* is a Gram-negative marine bacterium that is non-luminescent when free-living in sea water. However, this bacterium can symbiotically colonize the light organ of certain fishes and squids where it produces light (69, 163) . Production of light by *V. fischeri* can also be observed in liquid cultures and is associated with high population densities (69). Nealson *et al.* attributed this production of light at high population densities to autoinduction; a process during which an extracellular signal induces light-producing genes in the bacterial population(136). Later, this autoinducer signal was characterized as *N*-(3-oxohexanoyl)-L-homoserine lactone (3O-$C_6$-HSL) (56).

A bacterial autoinducer synthase (LuxI) constitutively produces *N*-acyl homoserine lactones (AHL) at low levels. At low population densities, the AHLs diffuse out of the bacterial cells and into the surrounding environment (69). As the cell population increases, the signal accumulates in and around the bacteria. When the signal reaches a critical concentration corresponding to a particular cell population density, it

interacts with a specific transcriptional response regulator, LuxR (69, 197). The LuxR-AHL complex binds to the '*lux*' box, a chromosomal DNA sequence located upstream of the promoter for the bioluminescence genes (50, 58). Binding of the complex leads to activation of transcription which results in the production of light (58). One of the target promoters of the LuxR-AHL complex is the *luxI* gene itself; therefore, activation of transcription also leads to the amplification of the quorum-sensing signal (59, 60, 69) . The *V. fischeri* quorum-sensing network serves as the paradigm for most Gram-negative bacterial quorum-sensing networks (Fig. 1). Autoinducer synthases and response regulators subsequently identified in other bacteria are consequently referred to as LuxI- and LuxR-type proteins, respectively (72).

## Autoinducers

A wide array of molecules have been identified as population density-dependent signals in Gram-negative bacteria. Though they differ in their chemical and structural properties, they have a common role as autoinducers: to bind to response regulators and, in conjunction, control the expression of genes.

## Autoinducer-1

In Gram-negative bacteria, the autoinducer molecule is usually an *N*-acyl homoserine lactone (AHL) or AI-1. AHLs are the predominant signals for communication between bacteria of the same species (195). Its structure consists of a hydrophilic homoserine lactone ring attached to a fatty acid side chain of variable length, which can range from $C_4$ to $C_{18}$ carbon atoms usually in increments of two-carbons (122, 167). The acyl chain can also contain double bonds or have an oxo or hydroxyl

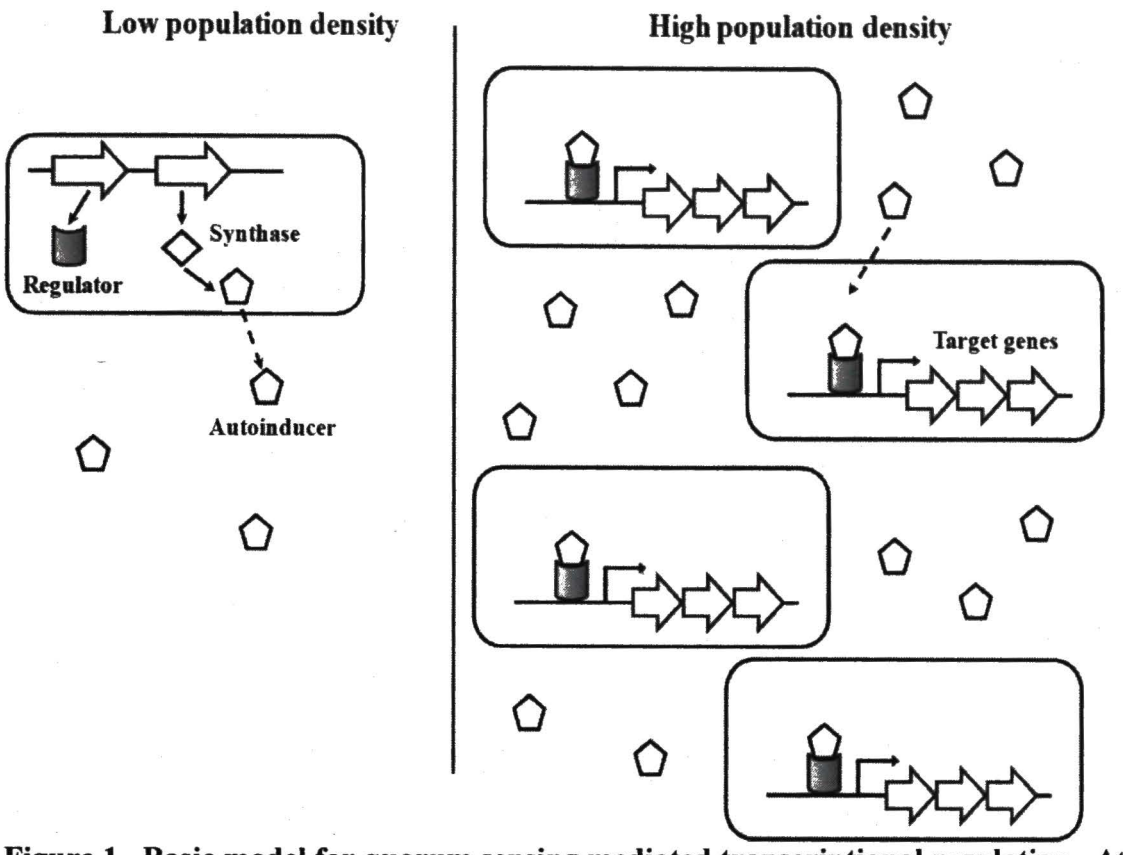



**Figure 1. Basic model for quorum sensing mediated transcriptional regulation.** At low population densities, basal level of the signal molecule or autoinducer is generated by a synthase. The autoinducer diffuses out of the bacterial cells and gets diluted in the surrounding environment. As the bacterial population density increases, the autoinducer signal accumulates, and when the signal reaches a threshold concentration it is able to interact with the response regulator protein. The autoinducer-response regulator complex binds to DNA upstream of target genes and affects transcription. The synthase and response regulator proteins were first discovered in the marine bacterium *Vibrio fischeri*, where they were labeled LuxI and LuxR respectively. Similar proteins subsequently identified in other bacteria were therefore called LuxI- or LuxR-type proteins (69, 72, 197)

substitution at the 3rd carbon (167). The modifications and the length of the side chain provide specificity to the autoinducer signal. The overall amphipathic nature of the AHL allows it to transverse the phospholipid bilayer of the cell membrane. Short-chain AHL molecules are capable of freely diffusing across bacterial membranes (148). Long-chain AHLs (> $C_{12}$) on the other hand appear to be actively transported via efflux pumps across the membrane (148). These autoinducers can be detected and characterized by several bioreporters and structurally identified with a combination of HPLC purification, NMR, and mass spectrometry (56, 167, 177).

**Autoinducer-2**

Gram-negative bacteria also appear to be capable of interspecies communication, for which they utilize the autoinducer AI-2 (131, 195). This autoinducer is produced by several bacteria to control bioluminescence, virulence, and motility (159, 179). AI-2 is produced from 4, 5-dihydroxy-2, 3-pentanedione (DPD) which undergoes various rearrangements to generate species specific signal molecules. For example, in *Vibrio harveyi*, AI-2 is in the form of 2-methyl-2,3,3,4-tetrahydroxytetrarofuran-borate, whereas in *Salmonella typhimurium*, AI-2 is a 2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (37, 132). The rearrangement of the parent precursor DPD to generate multiple forms of AI-2 molecules is believed to help bacteria respond to their own AI-2 and those produced by other species (202).

**PQS**

In addition to the AHL signaling molecules ($C_4$-HSL and 3O-$C_{12}$-HSL), *P. aeruginosa* produces a third distinct signal molecule, 3,4-dihydroxy-2-heptylquinoline,

referred to as PQS (151). Along with AHLs, PQS serves as a quorum sensing signaling molecule that controls the expression of virulence genes and biofilm formation (51, 52). Chemically, PQS is a hydrophobic quinolone and, unlike AHLs, is transported out of the cells via membrane vesicles (123). This mechanism of transport plays a role in preventing the membrane encased signal from degradation, and is proposed to be crucial in establishing efficient communication within a bacterial population (123).

**Cyclic Dipeptides**

Diketopiperazines (DKPs) are the smallest form of cyclic peptides found in nature (157). DKPs are endogenous to different members of the animal and plant kingdoms (157). They appear to be by-products of larger proteins and can have antifungal, antibacterial and antitumor activities (157). Several species of *Pseudomonas* are known to produce bioactive DKPs that serve as quorum-sensing signals as they accumulate in a density dependent manner and activate the response regulators of various biosensors (49, 95). DKPs have also been shown to control quorum sensing phenotypes such as swarming motility in *Serratia liquefaciens* and pathogenicity in *Vibrio* species (95, 143).

**3-OH Palmitic acid**

In the plant pathogen *Ralstonia solanacearum*, a volatile fatty acid derivative is involved in production of virulence factors like exopolysaccharides. The volatile factor was identified as 3-hydroxypalmitic acid methyl ester (3-OH PAME) produced by the *phcB* gene (66). 3-OH PAME accumulates in response to population density and is thought to be involved in intracellular communication. 3-OH PAME is a species specific signal

identified in a diverse set of *R. solanacearum*, but missing in bacteria of other species or genera (66).

### Other signals

Xanthomonads are Gram-negative plant pathogens that mediate virulence through the secretion of exoenzymes and exopolysaccharides (32). Virulence is regulated by two distinct autoinducers. The diffusible signal factor, DSF (cis-11-methyl-2-dodecenoic acid), is produced by the *rpf* operon and controls production of exopolysaccharides, extracellular enzymes, and biofilm dispersal (91, 193). DSF-like activity was identified in Gram-positive and fungal pathogens, indicating a possible role in inter-kingdom communication (193). The second signal, an extracellular diffusible factor, DF (butyrolactone), is controlled by the *pigB* gene product, and this signal is required for pigment production and exopolysaccharide biosynthesis (156).

The range of signal molecules acting as autoinducers showcases the diversity of quorum sensing signaling molecules. Some of these molecules (AHLs, DKPs) seem to serve exclusively for intercellular communication, whilst, others like AI-2 are universally recognized across several species. Additionally, some signals seem to be specific to certain species. For example 3-OH PAME is mainly detected in the *Ralstonia* species whereas DSF and DF are produced by the *Xanthomonas* species. The array of recognized autoinducer molecules keeps increasing as newer mechanisms of quorum sensing are identified.

## LuxI-type AHL synthase

LuxI-type proteins catalyze the synthesis of AHL molecules in the presence of appropriate substrates (60). These substrates include S-adenosylmethionine (SAM) and cellular pools of acylated acyl carrier proteins (ACP) from fatty acid metabolism (69, 85, 189, 197). The catalytic model proposes that SAM binds to the active site of the LuxI-type homologs, and the appropriate acyl side chain is transferred to this complex from an ACP. The acyl group forms an amide bond with the amino group of SAM. The ligated intermediate lactonizes to form the AHL, and the byproduct, 5-methylthioadenosine is released. LuxI homologs are usually 200 amino acids in size (69, 145, 197). Random and site-specific mutations have indicated that the enzymatic functions of these proteins reside at the amino terminus (69, 87, 144). The carboxyl-terminal residues are divergent in most strains and are probably critical in recognizing the different acyl side-chains of the AHLs (87, 144). A single bacterial strain can encode for multiple synthases (e.g., *lasI* and *rhlI* of *P. aeruginosa*), while in several bacteria (e.g., *S. meliloti* and *P. aeruginosa*), a single synthase can produce multiple AHLs (39, 122, 200).

## Non-LuxI-type AHL synthases

Some Gram-negative bacteria have AHL synthases that are unrelated in sequence to the LuxI-type synthases, although they are proposed to catalyze AHL synthesis by a similar reaction. One such group is present in *Vibrio* species and consists of the LuxM/AinS/VanM-type synthases that are homologous to each other. The LuxM from *Vibrio harveyi* directs the synthesis of 3OH-$C_4$-HSL, the AinS from *V. fisheri* directs the synthesis of $C_8$-HSL, and VanM from *V. angillarum* directs the synthesis of 3OH-$C_6$-

HSL and 3OH-$C_6$-HSL (13, 86, 133). Unlike LuxI-type proteins that use acyl-acyl carrier proteins, the synthases from this group catalyzes AHL synthesis from either an octanoyl-acyl carrier protein or octanoyl coenzyme A (86). Finally, AHLs can be produced by the HdtS-type synthase. In *Pseudomonas fluorescens* F113 the *hdt*S gene directs the production of $C_6$-HSL, $C_{10}$-HSL, and 3O-$C_{14:1}$-HSL when introduced into *Escherichia coli*. The HdtS protein sequence does not show homology to either the LuxI or LuxM-type of AHL synthases; however HdtS is most closely related to the lysophosphatidic acid acyltransferase, a PlsC protein from *E. coli*. The PlsC protein is responsible for the transfer of an acyl chain from either an acyl-acyl carrier protein or an acyl-coenzyme A to lysophosphatidic acid, resulting in the production of phosphatidic acid. It has been proposed that HdtS could similarly transfer acyl chains to SAM to generate AHLs (108).

**LuxR-type regulators**

The autoinducers produced by quorum-sensing systems are detected by LuxR-type regulators which relay the information into a cellular response. Genetic and biochemical evidence identified the LuxR protein of *V. fischeri* and its homologs as the autoinducer receptors (60). Overall protein sequence comparisons reveal that LuxR proteins share 18-25% identity, and they contain domains which share higher sequence conservation (197). These conserved domains include an AHL-binding domain and a DNA binding domain. The amino-terminal region of the LuxR-type protein binds its corresponding AHL, and the carboxy-terminal region containing the helix-turn-helix domain interacts with the DNA (38, 69). Binding of the LuxR-type protein to its cognate

autoinducer causes a conformational change which results in multimerization of the proteins (191, 207) . The nature of the acyl side chain of the autoinducer (e.g., chain length, modification at the 3rd carbon), and the amino acid composition of the AHL-binding domain, imparts specificity to the regulator for a particular signal (170, 209). A domain in the middle of the protein is critical for multimerization and stabilization of the complex (191, 207). The LuxR-AHL complex thus formed binds upstream of the transcriptional start site and recruits RNA polymerase by making direct contact. Transcriptional activation of target genes occurs by recognizing and binding to specific DNA sequences of dyad symmetry called *lux* boxes at quorum-sensing regulated promoters. The *lux* box is an 18-22 base pair inverted repeat sequence centered at about -40 from the transcriptional start site (81, 197). Though the presence of a *lux* box is necessary for DNA binding in the case of LuxR and TraR (*tra* box) of *A. tumefaciens*, others, like LasR of *P. aeruginosa*, do not have well defined sequences upstream of their target genes.

Most transcriptional regulators consist of functional regions called domains which can determine the regulatory profile of the proteins (64). For example, the DNA binding domain of the protein can function as an activator or repressor of transcription (28). Through phylogenetic analysis, LuxR-type proteins have been divided into two groups, A and B (113). Interestingly, members of group A (e.g., LuxR, TraR, and LasR) are transcriptional activators whereas those from group B are repressors and include EsaR (*Pantoea stewartii*) and ExpR (*Erwinia* species) (16, 134). In the absence of AHLs, repressor proteins bind the promoters of target genes thereby preventing transcription by

blocking access to the RNA polymerase. Unlike activators, repressors do not directly associate with RNA polymerase (16). Binding to an AHL causes conformational changes that release the repressor from DNA and relieve repression. Therefore, either via activation or repression, the LuxR-type response regulators, along with their specific signals, serves to modulate the behavior of the entire bacterial population.

## Orphan LuxR homologs

Characterization of quorum sensing in bacteria has led to the identification of several LuxR and LuxI homologs. Typically the genes for these proteins lie in close proximity to each other on their genome and are referred to as the cognate *luxR/I* pair (Table 1). As more bacterial genomes are sequenced, the presence of additional LuxR homologs has become evident. Many of these do not have an associated synthase on their genome and are therefore referred to as orphan LuxR homologs (Table 1) (68). Their predicted protein sequences have the amino receiver binding domain and carboxyl DNA binding domain, typical of the LuxR family of proteins. Orphan LuxR homologs usually do not directly affect the synthesis of autoinducers, but interact with them to expand the existing regulatory network of the bacterium. This review discusses the characterization of various orphan LuxR homologs identified in Gram-negative bacteria and their contributions to the regulatory circuit in those bacteria (Table 1).

## TrlR of *Agrobacterium tumefaciens*

*A. tumefaciens* is a plant pathogen that causes crown gall tumors mediated by the virulence genes from its Ti (tumor-inducing) plasmid. Transfer of the Ti plasmid by

**Table 1. Orphan LuxR homologs in gram-negative bacteria.**

| Organism | Orphan LuxR homolog | % Identity to LuxR* | Function | Cognate pair | Function | Ref. |
|---|---|---|---|---|---|---|
| *A. tumefaciens* | TrlR | 16 | Inhibit conjugation of Ti plasmid | TraR/TraI | Conjugation of Ti plasmid | (70, 140) |
| *P. aeruginosa* | QscR | 24 | Inhibit premature activation of *las*/*rhl* regulon, virulence factor production | LasR/I | Virulence, biofilm | (39) |
| | | | | RhlR/I | Virulence | |
| *Erwinia* species | VirR/ExpR2 | 24 | Production of plant cell wall degrading enzymes | ExpR/I | Enzyme production | (9) |
| | CarR | 25 | Antibiotic production | | | |
| *Serratia* sp. ATCC39006 | CarR | 27 | Antibiotic production | SmaR/I | Antibiotic and pigment production | (41) |
| *R. leguminosarum* | BisR | 18 | Symbiotic plasmid conjugation | CinR/I | Symbiotic plasmid acquisition | (116, 162, 199) |
| | | | | TraR/I | Symbiotic plasmid conjugation | |
| | | | | RhiR/I | Rhizosphere genes | |
| | | | | RaiR/I | Function unknown | |
| *S. meliloti* | ExpR | 19 | Exopolysaccharide production, motility | SinR/I | Timing of plant nodulation | (75, 122, 149) |
| | SMc04032 | 19 | Stress adaptation, competition for plant nodulation | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | SMc00658, SMc00878, SMc00877 | 19 20 22 | Putative role in denititrification pathway | | | |
| *A. vitis* | AviR | 20 | Necrosis in grape, hypersensitive response in tobacco | AvsR/I | Necrosis in grape, hypersensitive response in tobacco | (88, 89, 208) |
| | AvhR | 20 | Necrosis in grape, hypersensitive response in tobacco | | | |
| *S. enterica* | SdiA | 24 | Resistance to host defenses | - | - | (2) |
| *E. coli* | SdiA | 25 | Transcription of cell divisions genes, resistance to antibiotics | - | - | (2) |
| *X. campestris* | XccR | 18 | Plant pathogenesis | - | - | (206) |
| *X. oryzae* | OryR | 18 | Plant pathogenesis | - | - | (63) |
| *B. mallei* | BmaR4, BmaR5 | 30, 25 | Virulence | BmaR/I1, BmaR/I3 | Virulence | (187) |
| *B. pseudomallei* | BpmR4, BpmR5 | 31, 25 | Virulence | BmlR/I, BpmR/I2, BpmR/I3 | Virulence | (186) |
| *B. thilandensis* | BtaR4, BtaR5 | 29, 26 | Virulence, metabolism | BtaR/I1, BtaR/I2, BtaR/I3 | Virulence | (188) |

*Amino acid identity compared to the LuxR protein of *V. fischeri*.

conjugation to other agrobacteria is critical for increasing the overall number of pathogenic bacteria. Conjugation of the Ti plasmid is controlled by quorum sensing in *A. tumefaciens*. Genes required for both, plasmid-transfer and for quorum sensing resides on the Ti plasmid. Conjugation is initiated by the plant-tumor-produced compounds called octopines which activate the transcriptional regulator, OccR, on the Ti plasmid. OccR, in turn, activates transcription of the quorum-sensing response regulator *traR* (71). Moreover, the cognate autoinducer synthase, TraI, produces the signal 3O-$C_8$-HSL. This signal accumulates as the bacterial population increases and activates the transcription of genes required for conjugation (the *tra* and *trb* operons) in conjunction with TraR (Fig. 2) (70, 72) (62) . In addition to octopines, the plant tumor also produces mannopines which serve mostly as a source of nutrition. Octopine-type Ti plasmids code for genes involved in the catabolism of mannopines in the *mot* (mannityl opine catabolism) operon. A gene of the *mot* operon called *trlR* (traR like regulator) highly resembles the *traR* gene (140). The first of 181 amino acids of TrlR and TraR show 88% identity, whereas the remaining region of 31 residues of TrlR lacks homology (140). DNA sequence analysis of *trlR* identified a frameshift mutation after the 542$^{nd}$ residue, and restoration of the mutation increased homology with *traR* to 90 % (140). Due to the location of the mutation in the carboxy terminus of the protein, TrlR cannot bind to DNA, but its amino-terminus can still bind AHLs. TrlR forms heterodimers with TraR and prevents its activity as a regulator, which in turn inhibits conjugation (31). Mannopines activate expression of the *mot* operon, and though they were not initially considered to play a role in conjugation, it is now evident that mannopines inhibit conjugation indirectly by controlling the

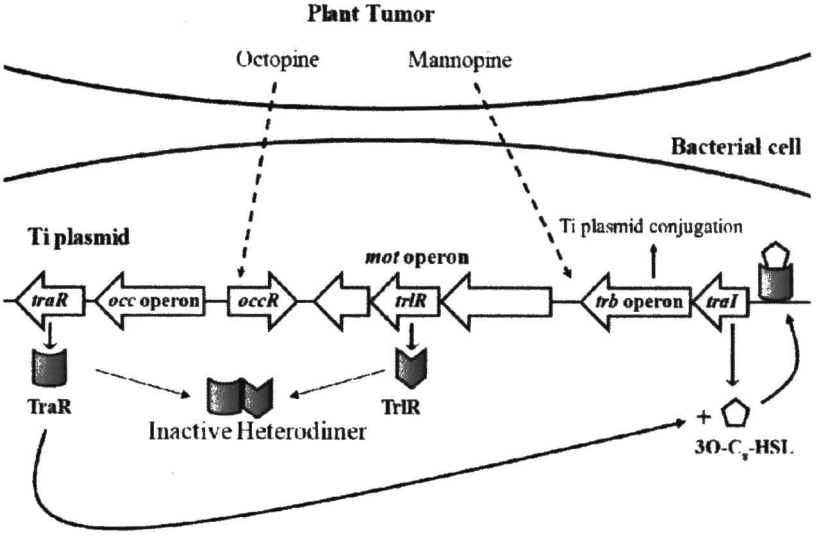



**Figure 2. TrlR of *A .tumefaciens*.** Conjugation of the Ti plasmid in *A. tumefaciens* is controlled by quorum sensing, where TraR and TraI serve as the cognate response regulator and synthase, respectively (62, 73). Quorum sensing is activated by a plant tumor-produced compound called octopine, which initiates transcription of *traR* via OccR (71). Mannopine, another compound secreted by the plant tumor, initiates transcription of the mannopine catabolism operon (*mot* operon), including *trlR* of this operon (140). In the absence of better sources of carbon other than mannopines, formation of heterodimers between TrlR and TraR prevent initiation of the energy intensive process of conjugation (31).

expression of the TrlR inactivator (31). When richer sources of carbon and energy are available, (e.g., succinate), mannopine catabolism is repressed, leading to reduced accumulation of TrlR, ensuring that the energy expensive process of conjugation occurs only during nutritionally conducive conditions (31).

## QscR of *Pseudomonas aeruginosa*

In the opportunistic pathogen *P. aeruginosa,* two LuxI-type proteins, LasI and RhlI, synthesize 3O-$C_{12}$-HSL and $C_4$-HSL, respectively. These AHLs bind to their cognate response regulators, LasR and RhlR, and regulate a number of virulence factors including the production of elastases, rhamnolipids, and biofilm formation (39, 109). In addition, *P. aeruginosa* has a third LuxR-type quorum-sensing regulator, QscR (quorum sensing control repressor) for which no cognate LuxI-type gene has been identified (Fig. 3). Mutants of *qscR* are hypervirulent, express quorum-sensing-controlled genes early, and form blue colonies due to overproduction of the phenazine pigment (39). QscR has been shown to delay the expression of several quorum-sensing-controlled virulence factors like phenazine and hydrogen peroxide by forming inactive heterodimers with LasR and RhlR (109, 112). Furthermore, QscR responds to the 3O-$C_{12}$-HSL produced by LasI and controls expression of genes independent of the LasR/I or RhlR/I systems (112). In the *P. aeruginosa* genome, *qscR* is flanked by the phenazine operon (*phz*) and the gene *PA1897* which encodes for a hypothetical protein. QscR activates transcription of *PA1897* specifically in response to 3O-$C_{12}$- HSL and represses the *phz* operon due to formation of inactive heterodimers with LasR.

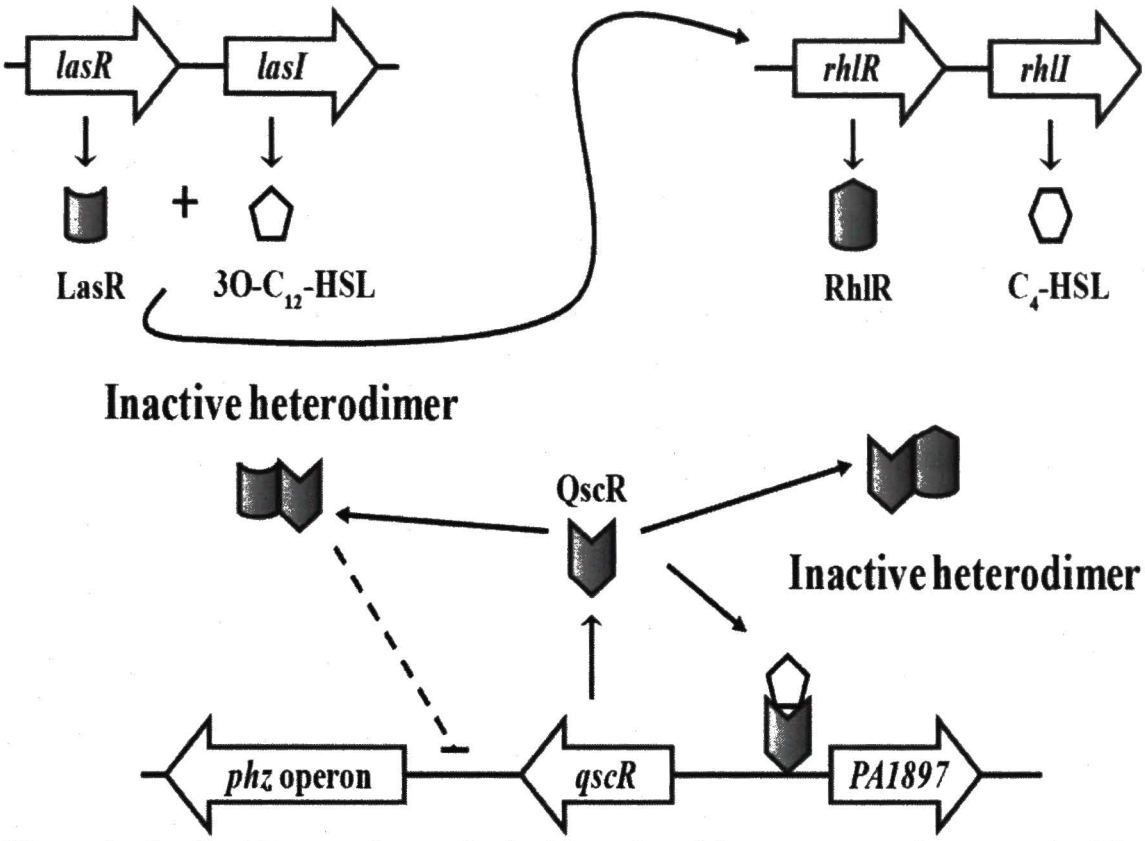



**Figure 3. QscR of *P. aeruginosa*.** In the hierarchy of the quorum sensing systems of *P. aeruginosa*, the *las* system controls the *rhl* and *qscR* systems. At low population densities, in the absence of accumulated *lasI* produced autoinducer (3O-$C_{12}$-HSL), QscR forms heterodimers with LasR and RhlR to prevent the premature activation of their regulons (39, 109). For example, the phenazine biosynthetic operon (*phz)* is a part of the *las* regulon but is inhibited by QscR heterodimers at low population densities. As the population density increases, inhibition is relieved as QscR is released from the heterodimers, making LasR available for activation. Concurrently, QscR binds to 3O-$C_{12}$-HSL from the *las* system and regulates its independent regulon (*PA1897*) (112)

The quorum-sensing systems of *P. aeruginosa* are hierarchical, where the *las* system controls the expression of the *rhl* system at the level of transcription. The *las* system also indirectly controls QscR, since the 3O-$C_{12}$-HSL produced by the *las* system is required by QscR to control gene expression (112). In addition to 3O-$C_{12}$-HSL, QscR can respond to other long chain AHLs such as 3O-$C_{10}$- HSL, $C_{10}$-HSL and $C_{12}$-HSL, suggesting it may respond to signals produced by other bacteria (110). Thus QscR serves to expand the regulatory network of *P. aeruginosa* by utilizing the existing components of the resident quorum-sensing systems.

**VirR/ExpR2 of *Erwinia* species**

Bacteria of the *Erwinia* species are Gram-negative plant pathogens that cause soft rot disease in their hosts (9). Quorum-sensing-based regulation of virulence has been identified in several species of *Erwinia,* but only strains which encode for orphan LuxR homologs are discussed below. A detailed review of quorum sensing in *Erwinia* is described elsewhere (9, 15) . The strains that encode for cognate autoinducer-response regulator pairs include *Erwinia carotovora* subspecies *carotovora* (*Ecc*) strain SCC3193 (*expI*/*expR1*), *Ecc* strain ATCC39048 (*carI*/*expR*), *Ecc* strain SCR1193 (*expI*/*expR*), *Ecc* strain 71 (*ahlI*/*expR*) and *Erwinia carotovora* subspecies *atroseptica* (*Eca*) strain SCR11043 (*expI*/*expR*) (15, 24, 36). All five strains carry an additional LuxR homolog termed VirR in *Ecc, Ecc* strain ATCC39048 and *Eca* strain SCR11043 or ExpR2 in *Ecc* strain SCC3193 and *Ecc* strain 71 (Fig. 4) (24, 36, 173). Strains of *Erwinia* also differ in the type of AHLs they produce. *Ecc* strain SCC3193 produces predominately 3O-$C_8$-HSL and minor amounts of 3O-$C_6$-HSL, whereas *Ecc* ATCC39048, *Ecc* strain SCR1193,

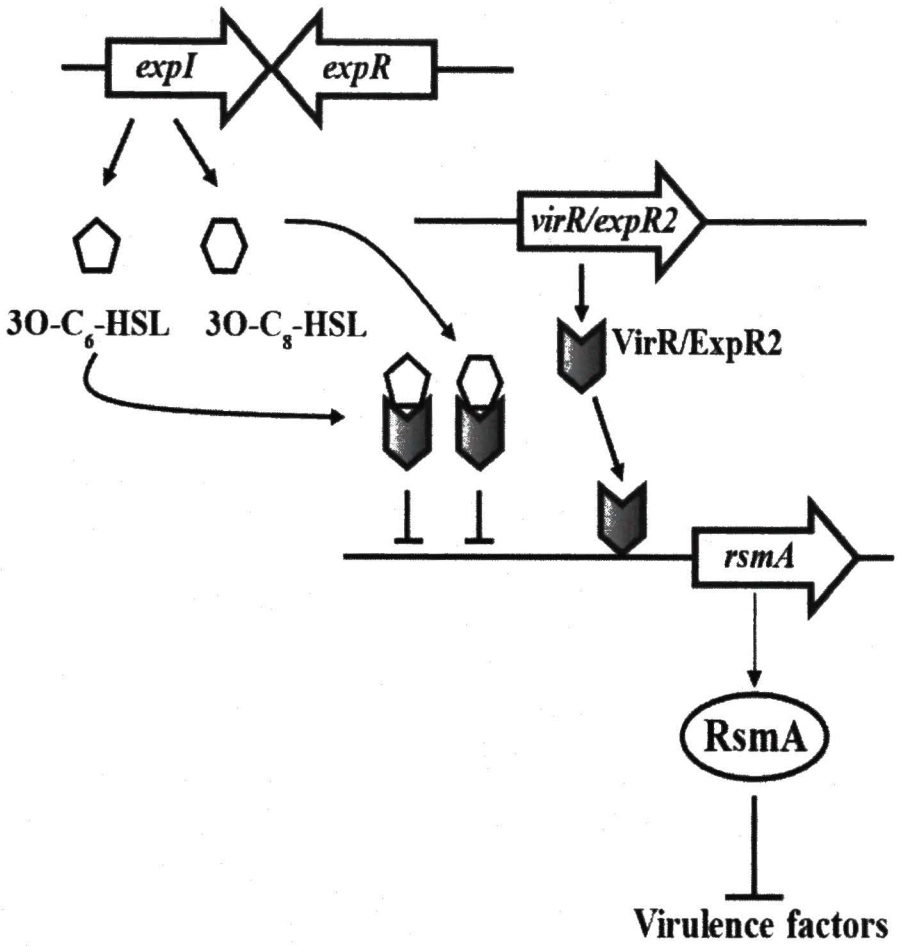



**Figure 4. VirR/ExpR2 of *Erwinia* species.** In the absence of autoinducers, VirR/ExpR2 is able to bind upstream of and activate transcription of *rsmA*. The global gene regulator, RsmA inhibits transcription of virulence factors. Quorum sensing activates virulence factor production in *Erwinia* species. The cognate pair *expI/R* produces 3O-$C_6$-HSL and 3O-$C_8$-HSL. VirR/ExpR2 is responsive to both autoinducers which sequester the regulator away from the *rsmA* promoter thereby relieving repression of virulence factors (9)

*Ecc* strain71, and *Eca* SCR11043 make only 3O-$C_6$-HSL (36). Mutants of the AHL synthase exhibit reduced expression of virulence factors such as the plant-wall-degrading exoenzymes, and increasing evidence indicates that regulation of the virulence factors is mediated by the orphan LuxR homologs in conjunction with the AHLs (9). Unlike most quorum-sensing systems where binding of an autoinducer to a response regulator leads to activation of transcription, in the *Erwinia* species, binding of the 3O-$C_8$-HSL or 3O-$C_6$-HSL autoinducers to the VirR/ExpR2 regulators relieves repression of transcription. At low population densities, (i.e., in the absence of AHLs), VirR/ExpR2 activates the transcription of the global repressor *rsmA* (36, 173). RsmA in turn represses exoenzyme production. As the population density increases and as AHLs accumulate, the binding of VirR/ExpR2 to the *rsmA* promoter is reduced (36). Production of virulence factors in *Erwinia* is also responsive to plant cell wall products; therefore, in order to mount an effective attack, synthesis of exoenzymes is coordinated with the presence of a suitable host and a sufficient number of bacteria (9).

**CarR of *Erwinia* and *Serratia* species**

Carbapenems belong to the β-lactam family of antibiotics (41). They inhibit the cross-linking of peptidoglycan and are active against both Gram-positive and Gram-negative bacteria. Strains of the Gram-negative plant pathogen, *Erwinia carotovora* subspecies *carotovora* (*Ecc*) ATCC39048 and the opportunistic pathogen *Serratia* sp. strain ATCC39006, produce the carbapenem antibiotic 'Car' (1-carbapen-2-em-3-carboxylic acid) (41). In both strains, antibiotic production is controlled by an orphan LuxR homolog, CarR, which lies upstream of the *car*A-H operon and activates the

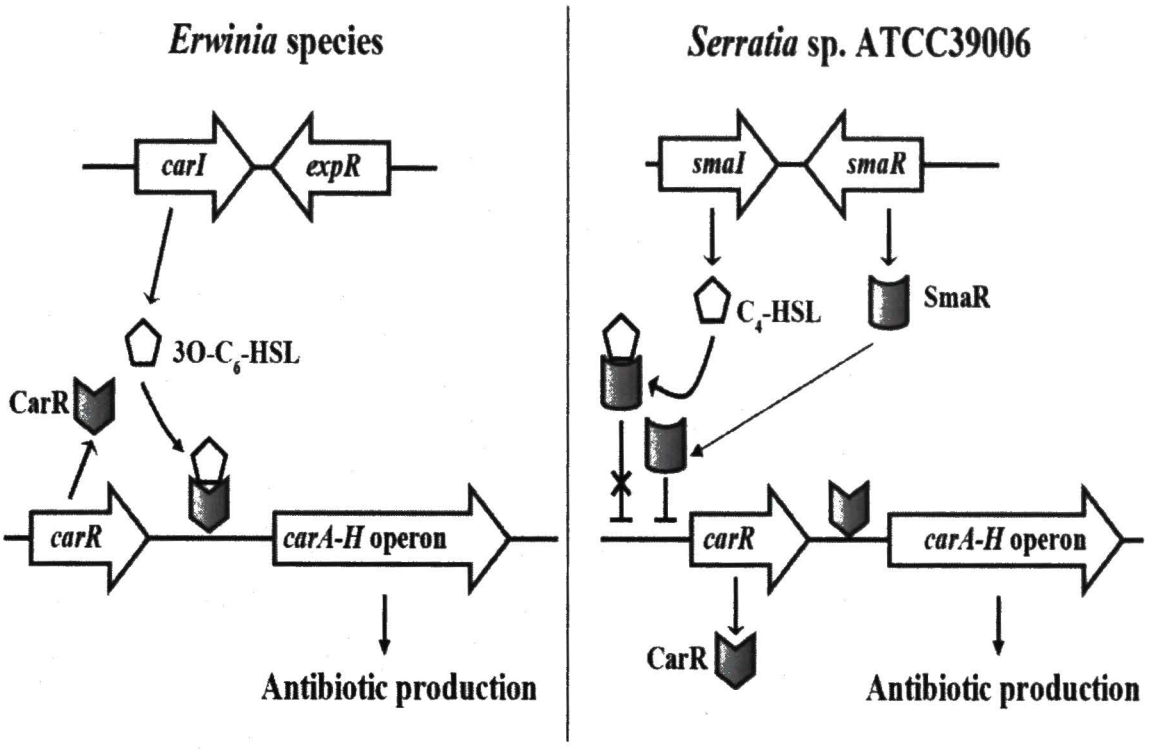



**Figure 5. CarR of *Erwinia* and *Serratia* species.** Carbapenem-antibiotic production is under the control of CarR that regulates the *carA-H* antibiotic biosynthetic operon. In the *Erwinia* species, CarR interacts with 3O-$C_6$-HSL produced by the cognate pair of *carI/expR* and binds upstream of the *car* operon to activate expression. However, in the *Serratia* species, CarR binds to DNA independent of autoinducers. At low population densities SmaR from the cognate SmaI/R pair binds upstream of *carR* to repress its transcription. As population density increases, $C_4$-HSL produced by SmaI disassociates SmaR, releasing *carR* expression (9, 41).

operon in a population density dependent manner (Fig. 5). CarA-E produces the antibiotic, whereas CarF and CarG are required for generating intrinsic resistance to the antibiotic, and the function of CarH is unknown (41). In *Ecc* ATCC39048, CarR activates transcription of the *car* operon by binding to 3O-$C_6$-HSL and activating carbapenem production. The product of *carI* results in the synthesis of the AHL, but its cognate regulator ExpR (see preceding section) is not involved in the production of the antibiotic (9, 41).

In *Serratia* sp. ATCC39006, although the *carR* and *car* operon are highly homologous to that of *Ecc* its mode of regulation differs. Here, both genes of the *smaI/R* locus are involved in antibiotic production. SmaR inhibits the transcription of *carR* in the absence of AHLs (Fig. 5). As the population density increases, accumulation of $C_4$-HSL produced by the *smaI* gene relieves this repression by sequestering SmaR. Transcription of liberated *carR* thus allows for antibiotic production (184). Uniquely, CarR in *Serratia* activates transcription in an autoinducer independent manner. The role of the AHL in this case is to remove the repression exerted by SmaR on the expression of CarR (42, 174).

Antibiotic production in both organisms is responsive to environmental nutritional cues. Carbon sources like glycerol inhibit expression of *carI* in *Ecc*, and phosphate limiting conditions activate transcription of *smaI* (41, 174). The role of the Car antibiotic production in bacteria is not well understood, but it probably serves to defend against neighboring organisms. It is speculated that since quorum sensing in *Ecc* also controls production of plant-cell-wall-degrading enzymes which leads to the creation of a

nutrition rich environment, synchronizing antibiotic production at the same time could help stave off other competing bacteria (41).

### BisR of *Rhizobium leguminosarum*

*R. leguminosarium* bv. *viciae* forms a symbiotic association with pea, lentils and field bean plants, and most of the genes required for this association reside on the plasmid pRL1JI (53). In *R. leguminosarum*, conjugation of the symbiotic plasmid is dependent on its multiple quorum-sensing systems. The chromosomally located *cinR/I* locus is at the top of an intricate regulatory cascade, where CinI synthesizes 3OH-$C_{14:1}$-HSL, and CinR, in response to this AHL, positively regulates *cinI* (53, 116). The pRL1JI plasmid carries two quorum sensing systems. RhiI is involved in the production of $C_6$, $C_7$, and $C_8$-HSL which in conjunction with RhiR regulates genes involved in the rhizosphere interaction of *R. leguminosarum* with pea plants (162). The second system on the plasmid, the Tra system, resembles the conjugation controlling system found in several plant-associated Rhizobia (Fig. 6) (44). The Tra locus consists of *traI*, which encodes for the autoinducer 3O-$C_8$-HSL, the *trb* operon, which is required for plasmid transfer, and two LuxR homologs, BisR and TraR (199). The cognate regulator of TraI is TraR, which, along with 3O-$C_8$-HSL, activates the transcription of *traI* and the *trb* operon, thereby increasing plasmid transfer frequencies (44, 199). BisR is an orphan LuxR homolog which is highly homologous to CinR (59%) (199). BisR regulates the expression of *traR* upon sensing 3OH-$C_{14:1}$-HSL, and mutants of *bisR* or *traR* have reduced plasmid transfer frequencies (Fig. 6) (199). Paradoxically, BisR also represses production of 3OH-$C_{14:1}$-HSL by binding to the promoter of *cinI* (126). BisR serves a unique role in *R. leguminosarum* in

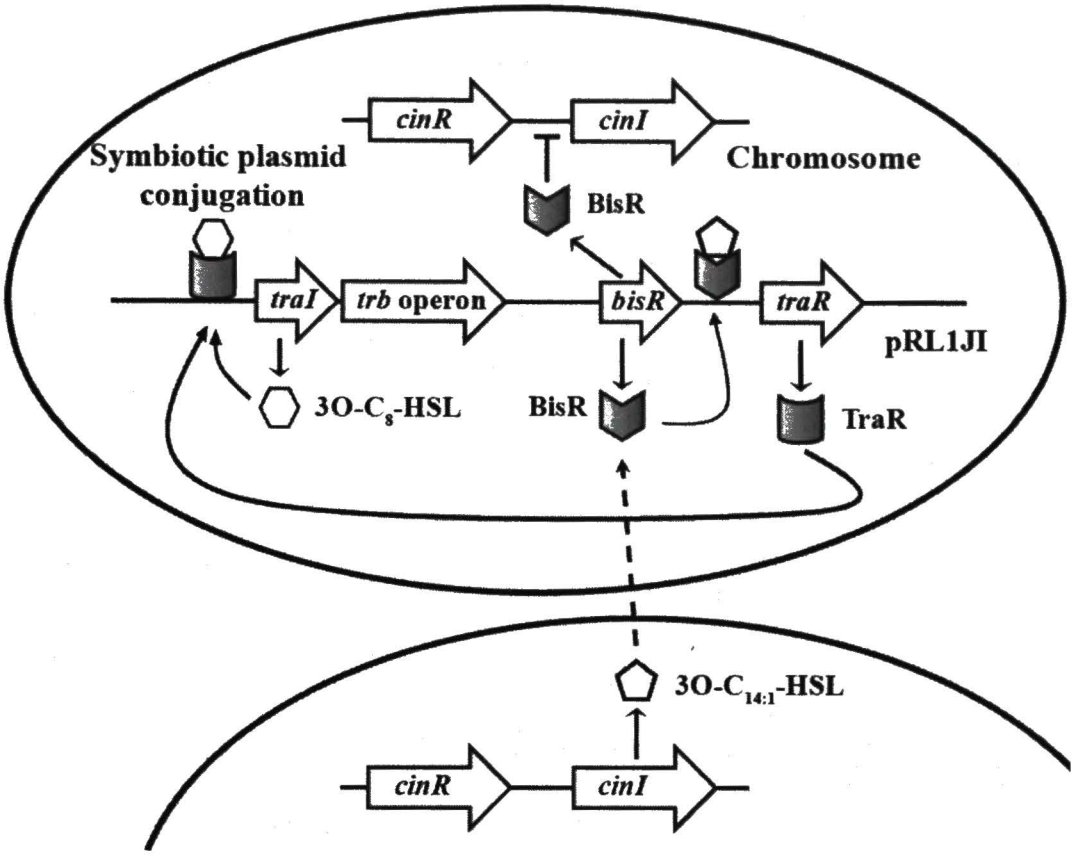



**Figure 6. BisR of *R. leguminosarum*.** In *R. leguminosarum* transfer of the symbiotic plasmid pRL1JI is controlled by quorum sensing. In cells that have the plasmid, BisR represses expression of the chromosomal *cinI*. In cells that lack the plasmid, the functional *cinR/I* produce and regulate 3O-$C_{14:1}$-HSL. BisR is responsive to 3O-$C_{14:1}$ produced by other cells. Binding of the autoinducer-BisR complex to the *traR* promoter activates production of TraR, which, in conjunction with 3O-$C_8$-HSL, activates the *trb* operon required for conjugation (44, 53, 126)

that it controls recipient-induced plasmid transfer. In recipient strains that lack the pRL1JI plasmid, the chromosomal *cinR/I* locus produces and accumulates 3OH-$C_{14:1}$-HSL. Donor strains containing the plasmid do not produce 3OH-$C_{14:1}$-HSL since plasmid encoded BisR represses *cinI*. In response to the 3OH-$C_{14:1}$-HSL produced by the recipient strains, BisR activates transcription of *traR* which eventually induces conjugation by activating the *traI-trb* operon in response to population density (44). Therefore, in the absence of 3OH-$C_{14:1}$-HSL, BisR acts as a repressor of *cinI*, and in its presence acts as an activator of *traR*.

### SdiA of *Salmonella enterica* and *Escherichia coli*

*S. enterica* sv. Typhimurium and *E. coli* encode for only one LuxR-type response regulator, SdiA, and have no known autoinducer synthase (2). The role of SdiA in *S. enterica* is better characterized, where it has been shown to regulate the *rck* (resistance to complement killing) operon and *srgE* (*sdiA*-regulated gene), a gene of unknown function (3). The *rck* operon resides on a virulence plasmid and has been shown to play a role in bacterial binding to extracellular matrix proteins and epithelial cells, and also in the avoidance of the host complement responses (2, 3). Testing for the presence of autoinducers by using supernatants of cultures to activate biosensors or SdiA-regulated genes have so far yielded no candidates (2). However, in response to either synthetic AHLs or AHLs from other bacteria, SdiA can regulate both *rck* and *srgE,* suggesting that SdiA probably responds to external stimuli (2, 129). SdiA recognizes 3O-$C_8$-HSL and 3O-$C_6$-HSL in the physiologically relevant concentrations of 1-5 nM (129).

The role of SdiA in *E. coli* is not well understood, especially since *E. coli* does not possess the *rck* operon or the *srgE* gene. In *E. coli*, *sdiA* was identified in screens for genes which, when expressed on plasmids, could bypass inhibition of cell division (194). SdiA stands for suppression of division of inhibition, and in response to AHLs activates transcription of the *ftsQAZ* operon involved in cell division (172, 194). SdiA in *E. coli* has also been shown to confer resistance to antibiotics like mitomycin C and quinolones (160, 196). *S. enterica* and *E. coli* are enteropathogens, therefore their perception of autoinducers from surrounding bacteria could serve to signal their arrival to the appropriate host environment (2, 98). AHLs have been detected in the rumen content of cattle, though they have not yet been characterized from human gut bacteria (61).

**XccR of *Xanthomonas campestris* and OryR of *Xanthomonas oryzae***

The plant pathogen *Xanthomonas* controls virulence-factor production by quorum sensing. *Xanthomonas* species do not produce any AHLs as autoinducers and instead use the signal factors DSF and DF (see above) for gene regulation (193). Though their genome does not encode for any AHL synthases, different *Xanthomonas* species encode for LuxR homologs. Recently two such LuxR homologs, XccR and OryR, were characterized in *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae*, respectively (63, 206). Both XccR and OryR are homologous to each other and are required for virulence in their respective host plants. Fusions of *xccR* did not respond to synthetic AHLs, and over-expressed OryR protein did not solubilize in the presence of synthetic AHLs, indicating that the genes are not activated by AHLs and that their functional proteins do not bind AHLs. Interestingly, host plant exudates activate expression of *xccR* and *oryR*,

indicating that these genes could serve in modulating interkingdom communication. The *xccR* locus in *X. campestris* is flanked by a *pip* (proline iminopeptidase) gene. Pip is widely distributed in bacteria and catalyzes the removal of N-terminal proline residues from peptides, but its biological function is largely unclear (128, 165). In *X. campestris* it is now evident that XccR in conjunction with plant exudates controls expression of *pip*, and that along with *xccR* the *pip* locus is also required for virulence (206). The pattern of a *luxR*-type gene flanking a *pip* gene is observed in several plant associated species of *Rhizobia* and *Pseudomonas* (206).

**Orphan LuxR homologs of *Sinorhizobium meliloti***

*S. meliloti* exists as a free-living soil bacterium or in a symbiotic association with leguminous plants such as alfalfa. In *S. meliloti*, the *sinR/I* locus is involved in the production and regulation of a range of AHLs ($C_{12}$-HSL, $C_{14}$-HSL, 3O-$C_{14}$-HSL, $C_{16:1}$-HSL, 3O-$C_{16:1}$-HSL and $C_{18}$-HSL) (Fig. 7) (81, 122, 183). Mutations in the Sin system result in impaired nodulation of host plants. Though the mutants infect plants and establish symbiosis, there is a delay in the nodulation process and a decrease in the overall number of nodules (122). In addition to the *sinR/I* genes, *S. meliloti* strain 8530 has an orphan LuxR-type response regulator called ExpR (149). The ExpR regulator was shown to control genes in the production of the symbiotically important exopolysaccharide EPS II (120, 149). Furthermore, microarray studies have shown that together, SinR/I and ExpR control a myriad of genes involved in motility, chemotaxis, and low-molecular weight succinoglycan, another symbiotically relevant exopolysaccharide produced by *S. meliloti* (80, 92, 93).

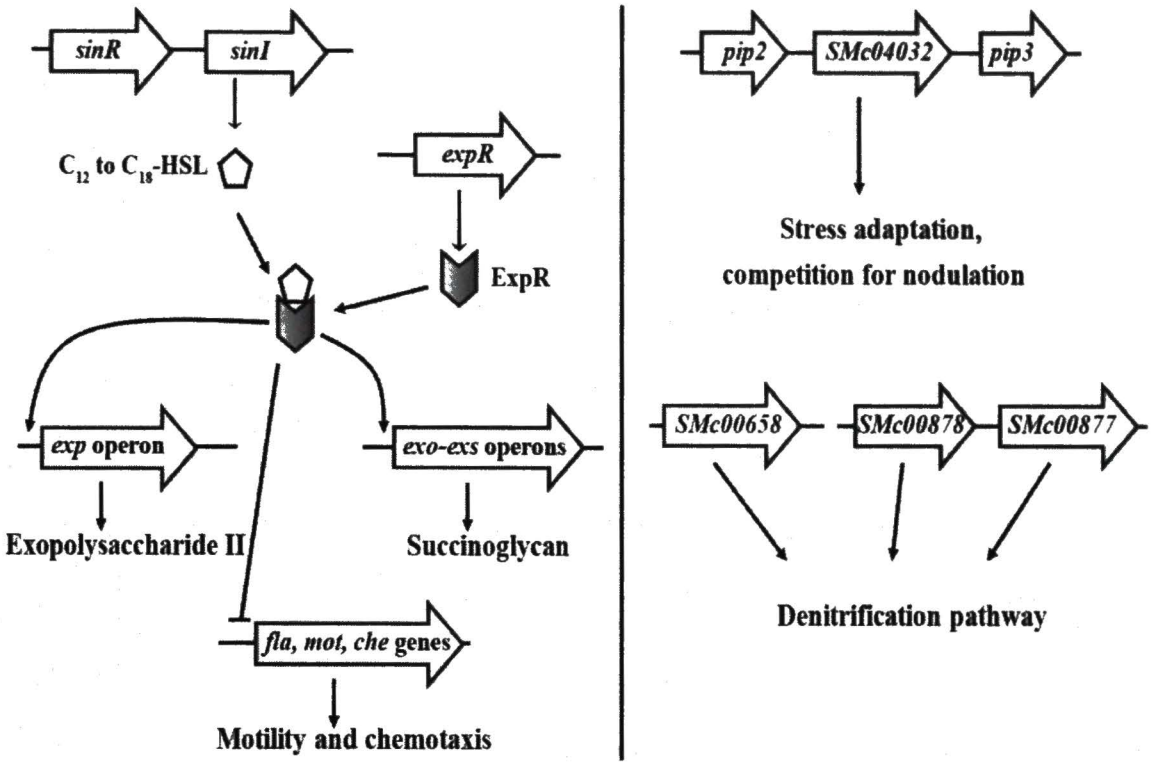



**Figure 7. Orphan LuxR homologs of *S. meliloti*.** The SinR/I system of *S. meliloti* generates long-chain AHLs ($C_{12}$ to $C_{18}$-HSL) that bind ExpR to activate the expression of symbiotically important exopolysaccharides (succcinoglycan and EPS II) and repress genes involved in motility and chemotaxis (80, 93, 120, 149). In addition to SinR and ExpR, *S. meliloti* has genes that code for other LuxR-type proteins. The regulatory role of *SMc04032* was determined to affect stress adaptation and competition for nodulation, whereas *SMc00658*, *SMc00878*, and *SMc00877* control the transcription of genes from the denitrification pathway of *S. meliloti*. The *SMc04032* gene is flanked by two proline iminopeptidase genes (*pip2* and *pip3*) (75). Association of PIP with orphan LuxR proteins is observed in several plant-related bacteria, including *Xanthomonas* species, where it has been shown to control plant virulence (206).

In addition to the SinR and ExpR response regulators, the sequenced *S. meliloti* 1021 genome indicated the presence of four additional orphan LuxR homologs (*SMc04032*, *SMc00878*, *SMc00877*, and *SMc00658*) (75). The predicted protein sequences of these regulators contain the signature response regulatory domain at the amino terminus and DNA binding helix-turn-helix domain at the carboxyl terminus. Through expression and phenotypic analysis, it was deduced that the *SMc04032* locus is involved in stress adaptation and competition for nodulation (Fig. 7) (A. Patankar and J. González, submitted for publication). The functional processes affected by *SMc00658*, *SMc00878*, and *SMc00877* mostly fall within the denitrification pathway of *S. meliloti* (Fig. 7) (A. Patankar and J. González, manuscript in preparation). Interestingly, the known AHLs of *S. meliloti* (Sin AHLs) do not serve as the effector molecules for the four additional orphan LuxR homologs. The cell-signaling mechanism involved in activating these orphan LuxR homologs in *S. meliloti* remains to be identified. Interestingly, the SMc04032 predicted protein sequence is highly homologous to plant-signal activated XccR and OryR of *Xanthomonas,* while SMc00878 and SMc00877 are highly homologous to AviR of *A. vitis* (see below) (63, 206, 208).

**AviR and AvhR of *Agrobacterium vitis***

*A. vitis* causes crown gall disease and necrosis in grape plants, and induces a hypersensitive-like response (HR) in non-host plants like tobacco (25, 208). *A. vitis* contains a cognate *avsR/I* locus which is involved in the production of long-chain AHLs and induction of tobacco HR and grape necrosis (88). The *avsR/I* locus is most homologous to the *sinR/I* locus of *S. meliloti*. Both loci are involved in the production

and regulation of AHLs. In addition, two orphan LuxR regulators, AviR and AvhR, were identified in *A. vitis* by Tn*5* mutagenesis. Mutants of AviR are completely defective in necrosis and HR, and produce fewer long-chain AHLs (208). Mutants of AvhR cause partial necrosis of grape plants, induce HR, and are not involved in the production of AHLs (89). Interestingly, AviR is highly homologous to the well-characterized orphan LuxR homolog ExpR of *S. meliloti* and a putative LuxR homolog (AGR-c-4942) of *A. tumefaciens* C58 (208). AviR is involved in the production of AHLs, though unlike its homolog in *S. meliloti* (ExpR), it is not involved in the production of exopolysaccharide in *A. vitis*. AvhR is also highly homologous to the LuxR homologs of *S. meliloti* (SMc00878 and SMc00877) and *A. tumefaciens* C58 (AGR-c-1279) (89).

**Orphan LuxR homologs of *Burkholderia* species**

Bacteria of the genus *Burkholderia* are Gram-negative opportunistic human pathogens (57). Quorum sensing has been implicated in pathogenesis due to its role in the production of exoenzymes (57). Strains of *B. mallei* contain two, whereas *B. pseudomallei* and *B. thailandensis* contain three pairs of cognate LuxR/I homologs that are involved in production of AHLs ranging from $C_8$ to $C_{14}$-HSL (Table 1) (186-188). Additionally, each of these strains contains two orphan response regulators, *bmaR4* and *bmaR5* in *B. mallei*, *bpmR4* and *bpmR5* in *B. pseudomallei* and *btaR4* and *btaR5* in *B. thailandensis* (186-188). The role of these orphan regulators has not been extensively characterized but it was observed that they affect different processes in different strains. In *B. mallei* and *B. pseudomallei*, mutants of *bmaR5* and *bpmR5* exhibit reduced virulence in animal models (186, 187). In *B. thailandensis*, mutations in *btaR5* affect

virulence factor (lipase) production and metabolism of various carbon sources (e.g., arabinose, glucose 6-phosphate) (188).

## Evolutionary perspectives of the orphan LuxR homologs

In natural habitats bacteria exist as members of communities that interact with each other. Communication via quorum sensing within these communities modulates the behavior of an entire population, thus imparting a multicellularity character to unicellular organisms. The coordination and regulation of gene expression is viewed as an evolutionary adaptation to survive in a changing environment (29). Bacteria exposed to variable niches, such as soil or aquatic environments, typically have larger genomes and devote more of their genes to regulation (*P. aeruginosa*, genome size= 6.3 Mb, *S. meliloti*, genome size = 6.6 Mb) (75, 201). On the other hand, intracellular pathogens which face more stable environments have much smaller genomes with only a small proportion of genes devoted to regulation (*Rickettsia* sp. genome size =1Mb) (28). The pressures exerted by a constantly changing environment select for genes that equip the bacteria with a repertoire of appropriate responses.

Two main mechanisms exist for the expansion of transcriptional networks. The first consists of internal reorganization of genes primarily by duplication and then divergence (28). The second method includes horizontal gene transfer from the large selection that is available in their diverse niches (125). Additionally, transcriptional regulatory circuits are believed to evolve independently from the gene or operons that they will ultimately control (28). A combination of these and other events probably

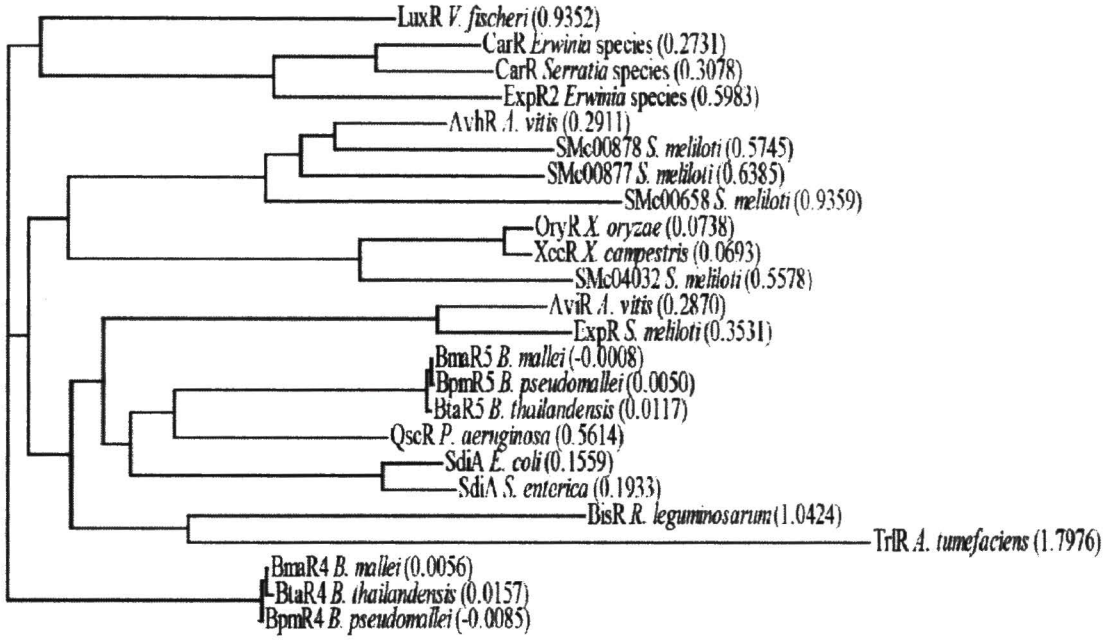



**Figure 8. Phylogenetic tree of orphan LuxR homologs.** Protein sequences were aligned using the Vector NTI AlignX program (Invitrogen). The program builds the phylogenetic tree by using the neighbor-joining method with Kimura's correction. The LuxR protein sequence from *Vibrio fischeri* was used as the reference profile. The calculated distances, related to the degree of divergence between the sequences, are indicated in parenthesis.

occurred in the evolution of orphan LuxR homologs (Fig. 8). Though *trlR* is located in the *mot* operon, it is not homologous to any of the other genes in the operon; instead, it is highly homologous to *traR* (140). If the point mutation of TrlR is rescued, its homology to TraR is 90%, indicating that *trlR* probably arose from duplication then divergence from *traR* (140). In *R. leguminosarum* bv. *viciae*, BisR is around 59% homologous to CinR and both proteins are not more than 30% homologous to other LuxR-type proteins, implying that BisR was probably a result of a duplication of CinR, after which its sequence diverged (199). Additionally, CinR and BisR both bind to 3O-$C_{14:1}$-HSL to regulate expression of genes, another factor suggestive of common ancestry (199). Phylogenetic studies also point towards the possibility that TrlR and BisR were the result of gene duplication (82). Given the high homology between the orphan LuxR homologs of *S. meliloti*, *A. vitis,* and putative LuxR homologs of *A. tumefaciens* C58, it seems likely that they came from common ancestors and then diversified in each bacteria to control different phenotypes such as exopolysaccharides production by ExpR in *S. meliloti* or necrosis and hypersensitivity induction by AviR in *A. vitis* (Fig. 8) (89, 120, 149). In *Erwinia carotovora* subspecies *carotovora* (*Ecc*), CarR is involved in antibiotic production and is linked to the antibiotic biosynthetic operon. CarR along with the antibiotic biosynthesis locus was probably acquired via horizontal gene transfer (82, 113). Though both ExpR and CarR of *Ecc* bind to 3O-$C_6$-HSL made by CarI, they both have different roles within the bacteria. The cognate regulator ExpR is maintained for its normal role in regulation whereas the ability to produce antibiotic served as a strong selective pressure to maintain the *car* genes (113). Interestingly, in phylogenetic studies,

SdiA of *S. enterica* and *E. coli* grouped with the RhlR sequences of *P. aeruginosa* instead of enterobacterial homologs, suggesting a horizontal gene transfer from *Pseudomonas* into these bacteria (82).

Though LuxI- and LuxR-type proteins are related by function they belong to distinct protein families that probably co-evolved (82). Several factors can justify the prevalence of orphan LuxR regulators in quorum-sensing systems of bacteria. These regulators can utilize the existing quorum-sensing signal in the bacteria and alleviate the cost associated with making additional signal molecules. Gain of response regulators also leads to expansion of the existing regulatory networks. Intriguingly, phylogenetic analysis performed by Zhang *et al.* grouped LuxR-type proteins into three functionally distinct clades, which consisted of LuxR-type activators, repressors, and XccR-like proteins (206). XccR-like proteins are typically orphan LuxR homologs, and some members of this clade respond to plant derived signal molecules (Fig. 8) (206). Thus, having additional LuxR homologs may increase the potential to respond to non-native signals.

## Concluding remarks

Bacteria exist as part of dynamic microbial communities within various environmental niches. Here, bacteria use quorum sensing as an effective means to translate environmental cues into global gene regulation. Using the three basic components of the signal producer, the signal itself and the signal response regulator, bacteria are able to regulate an extensive set of biological processes. The wide scope of

regulation is aided by the optimal utilization of the quorum-sensing components, such as employment of additional LuxR-type regulators called orphan LuxR regulators. Evolution and environmental pressures have selected for gain of these orphan response regulators, and their presence helps in fine tuning the existing quorum-sensing regulatory network while opening up possibilities of controlling newer independent regulons. The fact that the orphan LuxR regulators are maintained after acquisition and efficiently integrated into the bacterial regulatory systems, points towards the advantageous contributions and the competitive advantage afforded by these regulators to the bacteria.

**Acknowledgments**

We thank Brandon McKethan for assistance with the figures in this manuscript. We also thank the members of the laboratory for their thoughtful insights and input during the preparation of this review. The work in our laboratory is supported by the National Science Foundation grant MCB-9733532, and by the National Institutes for Health grant 1R01GM069925 to J.E.G.

# CHAPTER II

## *Sinorhizobium meliloti* AND ITS SYMBIOTIC ASSOCIATION

***S. meliloti* and its host.** Availability of nitrogen is often a limiting factor in plant growth. Members of the soil bacterial family Rhizobiaceae namely *Sinorhizobium*, *Bradyrhizobium* and *Azorhizobium* form nitrogen fixing associations with members of the plant family Leguminosae. In this symbiotic association the bacteria enter the plant roots where specialized organs called nodules are formed. Within the nodules, the bacteria fix atmospheric nitrogen for the plant, which in return provides carbohydrates to the bacteria (21, 22). *S. meliloti* is a gram negative bacterium, from the α-Proteobacteria family that associates with its legume host plant *Medicago sativa* (alfalfa). The sequenced genome of *S. meliloti* consists of a 3.65 Mb chromosome that codes for most of the house keeping genes. In addition, it contains two mega plasmids, pSymA and pSymB that are 1.35 and 1.68 Mb respectively. The pSymA plasmid encodes for genes required for nodulation and nitrogen fixation. The genes in the pSymB plasmid are responsible for nutrient uptake and successful invasion of the plant host (75).

**Symbiosis and nitrogen fixation.** The symbiosis between the soil bacteria and its legume host begins with the chemotatic attraction of the bacteria towards the plant root exudates (Fig. 1). The roots of leguminous plants secrete chemicals called flavonoids, which are three ring aromatic compounds derived from plant metabolism (118).

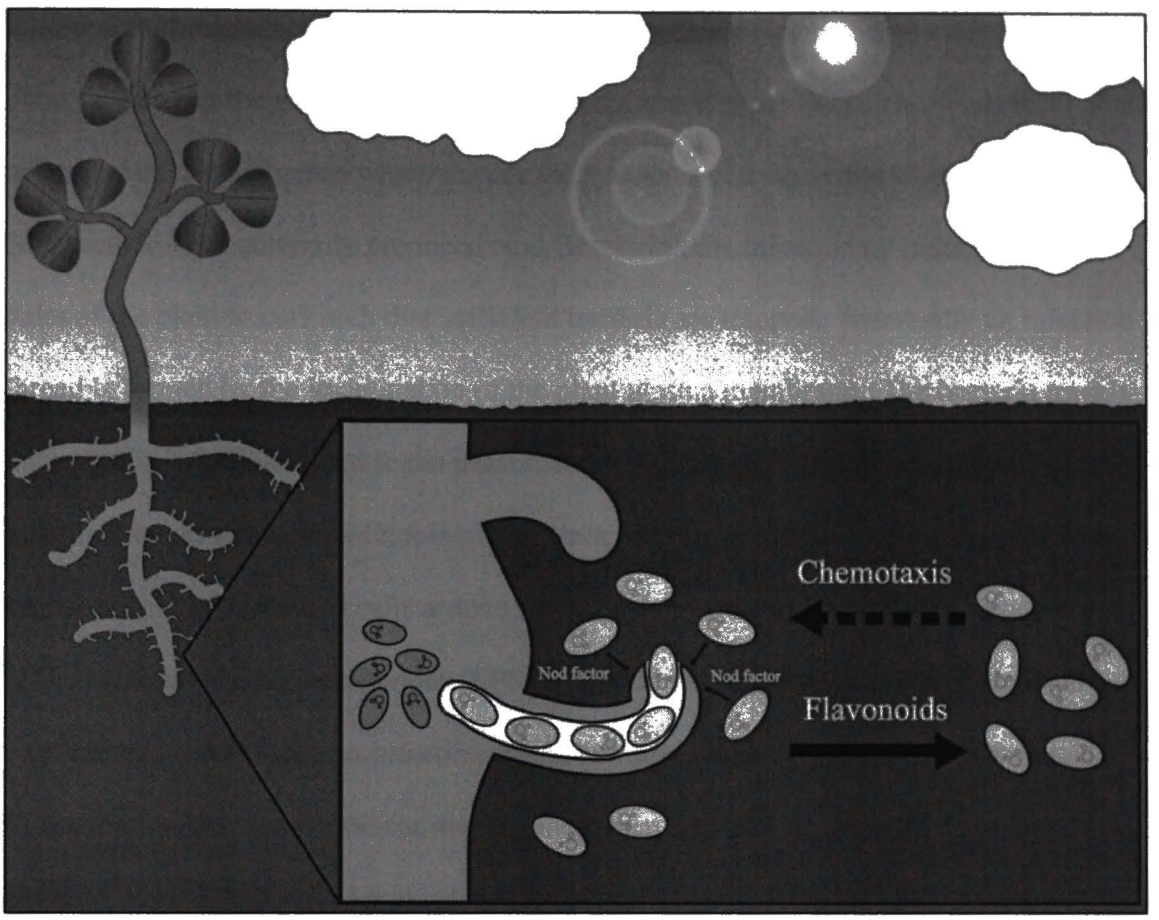



**Figure 1. Symbiosis between alfalfa and *S. meliloti.*** Flavonoids produced by the plant attract the bacteria towards the roots and activate the *nod* genes. The *nod* gene product, the Nod factor, induces the root hairs to curl. An infection thread which allows the bacteria to move into the root hair and to other plant root cells is subsequently formed. Multiplication of the bacteria and proliferation of the plant root cells around it ultimately lead to nodule formation. The bacteria inside the nodules actively fix nitrogen for the plant.

The flavonoids bind to NodD, a bacterial regulator that serves as a sensor of the plant signals and the transcriptional activator of the *nod* genes (22). This leads to activation of the *nod* genes which initiate the synthesis of a lipo-chito-oligosaccharide, or Nod factor. The bacterially produced Nod factors initiate infection by inducing the root hairs of the plant to curl such that sufficient bacteria are trapped. Eventually an infection thread is formed by the invagination of the plant plasma membrane. The bacteria travels through this infection thread to the plant root cortex. There, it proliferates within the infection thread network, continuing to produce Nod factor which, concurrently induces proliferation of the cortical cells around the bacteria to from nodules (190). Production of the bacterial exopolysaccharides (EPS) is critical in order for the symbiont to enter a root hair and travel along an infection thread towards a developing nodule. Two main EPS are important for symbiosis, are succinoglycan and EPS II. Inability to produce either of these exopolysaccharides leads to deficient nodule invasion (21).

The final stages of the nodule formation occurs when the bacteria is released into the plant cell by endocytosis of the plant plasma membrane. Here, the bacteria differentiate into morphologically distinct forms called bacteroids that actively fix nitrogen for the plant (118). Nitrogen fixation is directed by two sets of genes, the *nif* genes and the *fix* genes. The *nif* genes encode the structural proteins of the nitrogenase enzyme that converts atmospheric nitrogen into a source of reduced nitrogen for the plant. The *fix* genes encode regulatory proteins and the membrane-bound cytochrome oxidase that are required for respiration by the bacteria at low oxygen tension. In addition, plant cells in the nodule synthesize large amounts of leghemoglobin, whose role

is to buffer the oxygen concentration and control the diffusion of oxygen to the bacteroids (21). The plant also produces dicarboxylic acids which serve as the major source of carbon and energy for the bacteroids. Thus, the successful establishment of rhizobium-plant symbiosis depends on the release of a wide array of chemicals by the host and the bacteria and their appropriate responses to these chemicals.

***S. meliloti* and quorum sensing.** *S. meliloti* uses yet another form of chemical signaling called quorum sensing to regulate processes as varied as nodulation efficiency, exopolysaccaride production, motility and chemotaxis. Regulation of these phenotypes has been attributed to the Sin/ExpR quorum sensing system of *S. meliloti* (92, 122). However, *S. meliloti* contains four additional putative quorum sensing regulators whose regulatory network remains to be identified (75). In addition to the previously characterized long-chain AHLs, *S. meliloti* seems to synthesize short-chain AHLs as well (122). The identity of these AHLs is as yet unknown. Moreover, based on the sequenced genome analysis of *S. meliloti*, it appears that the bacteria possess homologs of other mediators of quorum sensing, that might be responsible for the synthesis of novel signal molecules (75). Therefore, the overall goal of this research is to further elucidate the regulatory role of quorum sensing in *S. meliloti*.

## CHAPTER III

## AN ORPHAN LUXR HOMOLOG OF *Sinorhizobium meliloti* AFFECTS STRESS ADAPTATION AND COMPETITION FOR NODULATION.

The Sin/ExpR quorum-sensing system of *S. meliloti* plays an important role in the symbiotic association with its host plant *Medicago sativa.* The LuxR-type response regulators of the Sin system include the synthase (SinI)-associated SinR and the orphan regulator, ExpR. Interestingly, the *S. meliloti* genome encodes for four additional putative orphan LuxR homologs whose regulatory roles remain to be identified. These response regulators contain the characteristic domains of the LuxR family of proteins which include an N-terminal autoinducer/response regulatory domain and a C-terminal helix-turn-helix domain. This study elucidates the regulatory role of one of the orphan LuxR-type response regulators, named NesR. Through expression and phenotypic analysis, *nesR* was determined to affect the active methyl cycle of *S. meliloti.* Moreover, *nesR* was shown to influence nutritional and stress response activities in *S. meliloti.* Finally, the *nesR* mutant was deficient in competing with the wild-type strain for plant nodulation. Taken together, these results suggest that NesR potentially contributes to the adaptability of *S. meliloti* when it encounters challenges such as high osmolarity, nutrient starvation and/or competition for nodulation, thus increasing its chances for survival in the stressful rhizosphere.

## Introduction

Bacteria are constantly subjected to environmental changes that require the coordination of their behavior in order to adapt and survive. This is particularly crucial for bacteria that interact with eukaryotic hosts during the establishment of a pathogenic or symbiotic association. One way to achieve this coordination is through the regulatory system known as quorum sensing. This form of regulation mediates gene expression through the interaction of signaling molecules and regulatory proteins in a population density-dependent manner. Quorum sensing is often found in host associated bacteria where, it plays a role in human pathogenesis (*Pseudomonas aeruginosa*), symbiosis (*Sinorhizobium meliloti*), plasmid conjugation (*Agrobacterium tumefaciens*), and antibiotic production (*Erwinia carotovora*) (55, 122, 195, 197). Quorum sensing was first discovered and described in the gram-negative marine bacterium *Vibrio fischeri*, where the regulation of bioluminescence by LuxR/I serves as a paradigm for most gram-negative quorum-sensing systems (59, 60). According to this model, production of the autoinducer signal, *N*-acyl homoserine lactone (AHL), is controlled by proteins from the LuxI family of synthases, which bind to and activate specific regulators belonging to the LuxR family which then go on to control specific target genes (60). Typically members of the LuxR family of proteins share two conserved domains, an amino-terminal, autoinducer binding domain, and the carboxy-terminal, DNA binding domain (69, 72, 197). Binding of the LuxR-type protein to its cognate autoinducer causes a conformational change which results in multimerization and stabilization of the protein (207). The LuxR-AHL complex that is formed recruits RNA polymerase and regulates

the transcription of target genes by recognizing and binding to specific DNA sequences at quorum sensing-regulated promoters (72, 197).

In the typical LuxR/I-type quorum-sensing circuit, the gene encoding the response regulator (*luxR*) is in close proximity to the gene that encodes the AHL synthase (*luxI*). In several instances, no gene encoding for LuxI homologs can be found associated with those encoding the regulators, and thus these are commonly referred to as orphan LuxR regulators (68). Numerous bacteria have been reported to carry orphan LuxR homologs where they regulate a variety of genes and/or functions (2, 39, 63, 140, 206). One example of an orphan LuxR regulator is QscR, which responds to the AHLs produced by the LasR/I quorum sensing system of *P. aeruginosa* and regulates the synthesis of virulence factors such as phenazine and hydrogen peroxide (39, 112). QscR thus integrates into the complex quorum-sensing regulatory network of *P. aeruginosa* (109, 112). Another example of an orphan LuxR-type regulator is the TrlR of *A. tumefaciens* (140). Transfer of the virulence genes encoded by the Ti plasmid in *A. tumefaciens* is controlled by the TraR/I quorum-sensing system (70). The Ti plasmid also encodes for *trlR*, and in the absence of conditions conducive for conjugation (e.g., rich carbon sources), TrlR binds to TraR and prevents this resource-intensive process (31, 140). Interestingly, in some bacteria, orphan LuxR regulators are responsive to exogenous signal molecules. For example, though *Escherichia coli* and *Salmonella enterica* do not normally produce AHLs, they possess LuxR-type regulators that respond to quorum-sensing signals produced by other microbial species (2). Similarly, the orphan LuxR

homologs, XccR of *Xanthomonas campestris* pv. *campestris* and OryR of *Xanthomonas oryzae* pv. *oryzae*, respond to host plant exudates in order to mediate virulence (63, 206).

*Sinorhizobium meliloti* is a gram-negative soil bacterium that can exist in a free-living state or can form a symbiotic association with the plant *Medicago sativa* (alfalfa). During this association, bacteria fix atmospheric nitrogen for the plant, often a limiting factor in plant growth, and in return receive nutrition from the host (75). Quorum sensing has been extensively characterized in *S. meliloti* where SinI and SinR are the homologs of LuxI and LuxR, respectively (75, 121, 122, 183). SinI is involved in the production of a range of AHLs and SinR serves to regulate AHL production in response to population density (121, 122, 183). In addition to SinR/I, *S. meliloti* has a gene encoding an orphan LuxR-type response regulator called *expR* (149). The ExpR regulator in conjunction with SinR/I controls a myriad of genes involved in motility, chemotaxis, and production of the symbiotically relevant exopolysaccharides EPS II and succinoglycan by *S. meliloti* (76, 80, 94). In addition to the SinR and ExpR response regulators, the *S. meliloti* genome contains four additional orphan LuxR homologs (encoded by *SMc04032*, *SMc00878*, *SMc00877*, and *SMc00658*) that are not associated with a known AHL synthase of *S. meliloti* (75).

Here, we explore the regulatory role of SMc04032, one of the orphan LuxR homologs of *S. meliloti,* via global expression analysis and phenotypic studies. Disruption of the *SMc04032* locus which we have named *nesR*, leads to the inability of the bacteria to cope with specific nutritional, environmental and stress conditions. We show that *nesR* is involved in the active methyl cycle within the methionine biosynthesis

pathway and impacts general stress and metabolic responses. In addition, we show that the *nesR* mutant has a competitive disadvantage in nodulation when compared to the wild-type strain.

## Materials and Methods

**Bacterial strains and media.** The bacterial strains and plasmids used in this study are listed in Table 1. The wild-type strain used in this study is Rm8530 (SU47, str-21, *expR*$^+$). The *nesR* (2011mTn*5*STM.1.11.H02) mutant was obtained from Anke Becker, who generated it by random Tn*5* transposon mutagenesis in Rm2011 (154). The *nesR* mutation was then transferred into different strain backgrounds by generalized transduction of 2011mTn*5*STM.1.11.H02 using phage ϕM12 as described previously (79, 154). *S. meliloti* strains were grown at 30°C in Luria-Bertani (LB) medium supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ for routine cultures. Starter cultures for RNA purification were grown in tryptone yeast-extract (TY) medium and sub-cultured in minimal mannitol glutamate low-phosphate medium (MLP) [50 mM MOPS, 19 mM sodium glutamate, 55 mM mannitol, 0.1 mM $K_2HPO_4/KH_2PO_4$, 1 mM $MgSO_4$, 0.25 mM $CaCl_2$, 0.004 mM biotin, pH 7] as described previously (92). For growth with glycine betaine as the sole source of carbon, *S. meliloti* was grown aerobically at 30°C in MLP supplemented with 1 mM glycine betaine (Sigma) instead of mannitol. Antibiotics were used in the following concentrations, streptomycin (Sm) 500 μg/ml, gentamycin (Gm) 5 μg/ml (for *E. coli*) or 50 μg/ml (for *S. meliloti*), and neomycin (Nm) 200 μg/ml in LB and 25 μg/ml in MLP.

**Table 1. Bacterial strains and plasmids used in this work.**

| Strains or plasmids | Relevant characteristics | Reference or source |
|---|---|---|
| *Sinorhizobium meliloti* | | |
| Rm8530 | Su47 *str-21*, *expR*$^+$, Sm$^r$ | (149) |
| 2011mTn*5*STM.1.11.H02 | Rm2011 *SMc04032*:: Tn*5* | (154) |
| Rm8530 *nesR* | *SMc04032*::Nm | This work |
| Rm8530 *nesR* + pJNesR | *SMc04032*::Nm + pJNesR | This work |
| Rm8530 *nesR* + pJN105 | *SMc04032*::Nm + pJN105 | This work |
| *Escherichia coli* | | |
| DH5α | See source | Life technologies |
| MT616 | MT607(pRK600) | (79) |
| Plasmids | | |
| pJN105 | *araC*-$P_{BAD}$ cassette cloned in pBBR1MCS-5 | (137) |
| pJNesR | pJN105 containing the *SMc04032* gene, Gm$^r$ | This work |

**Construction of pJNesR.** To complement the *nesR* mutation, *SMc04032* was cloned into the arabinose-inducible plasmid pJN105 (137). An 800 bp fragment was amplified from Rm8530 chromosomal DNA by using the following primers: 5'-CGCGAATTCCCTGTGACCGGACCGGAGAC-3', and 5'-GCGTCTAGAGGGGTTTCGAGCCGTGGCAG-3', where the underlined nucleotides are EcoRI and XbaI restriction enzyme sites, respectively. The plasmid was then transformed into *E. coli* and candidates were selected with gentamycin. The recombinant plasmid was introduced into Rm8530 *nesR* by triparental mating and transconjugants were selected with neomycin and gentamycin (79). For the vector-only control, the pJN105 plasmid was introduced into Rm8530 *nesR* by triparental mating and selected with gentamycin (79). All strain constructs were confirmed by PCR.

**Sensitivity to detergent stress.** The ability of *S. meliloti* to grow in the presence of the hydrophobic detergent sodium deoxycholate (DOC) was tested by plating dilutions of the bacterial strains on LB agar containing 0.1%, 0.2%, 0.4% and 0.6% of DOC. Viable counts were expressed as colony forming units (cfu) and were plotted against the concentration of DOC. The assays were conducted as three independent experiments.

**Growth analysis.** A single colony of each *S. meliloti* strain was inoculated into 2 ml of TY medium in the presence of the appropriate antibiotics and grown at 30° C. Saturated cultures were then washed and subcultured (1:100) into MLP, MLP+0.5 M NaCl or MLP (without mannitol) + 1 mM glycine betaine. Cell density was measured by monitoring the $OD_{600}$, and all growth curves were performed in triplicates.

**AHL extraction and TLC analysis of AHL production.** AHLs were extracted from 25 ml cultures of *S. meliloti* grown to an $OD_{600}$ of 2 with the appropriate antibiotics as described previously (122). Five ml aliquots of the cultures were acidified to pH< 4 and were extracted twice with equal amounts of dichloromethane (DCM) as described previously (170). The AHL containing DCM extracts were dried in a Speed-Vac (Labconco), resuspended in 20 µl of 70% methanol and spotted on a Whatman $LKC_{18}$ analytical TLC plate. The TLC plates were resolved in a 70% methanol chamber and after drying, the plate was overlaid with the *A. tumefaciens* $NTL_4$ (pZLR4) indicator organism as described previously (121).

**RNA purification for microarray and real-time PCR analysis.** Bacteria were grown to saturation in TY medium. A (1:100) dilution was used to inoculate 25 ml of MLP media with the appropriate antibiotics. Cultures were then grown at 30° C aerobically until they reached an $OD_{600}$ of 1.2. These culture conditions and optical density have been previously established as optimum conditions for differential expression of quorum sensing-regulated genes (80, 94). Cells were harvested by centrifugation (10,000 x g for 2 min. at 4° C), and the cell pellets were immediately frozen in liquid nitrogen. Total RNA was purified using the RNeasy Mini Kit (Qiagen). Cells were resuspended in 10 mM Tris HCl (pH 8) and in the RLT buffer (supplemented with β-mercaptoethanol) provided with the RNeasy kit. The cells were transferred to Fast Protein tubes (Qbiogene) and disrupted using the Ribolyser (Hybaid) (45 sec, level 6.5) prior to spin column purification according to the RNeasy Mini Kit RNA purification protocol. The RNA samples were then treated with the Qiagen on-column RNase-free

DNase. Samples were DNase treated a second time with the TURBO RNase-free DNase from Ambion according to the manufacturer's protocol, and an additional RNA clean-up step was performed (92). RNA integrity was determined by using an Agilent 2100 Bioanalyzer.

**Affymetrix GeneChip hybridization and expression analysis.** The cDNA synthesis and hybridization to the GeneChip Medicago Genome Array (Affymetrix, Santa Carla, CA) were performed as described previously, at the Core Microarray facility at UT Southwestern Medical Center (Dallas, TX) (80, 94). Ten μg of RNA were used for each experiment and the GeneChip Scanner 3000 was used to measure the signal intensity of each array. The .CEL files generated were processed by the Affymetrix GeneChip Operating Software, (GCOS v 1.4). Comparative analysis of the control and experimental expression were expressed in terms of an M value (signal log ratio) which indicated an increase, decrease or no change in expression of a gene in the mutant with respect to the wild-type strain. An M value $\geq 1$ (2 fold change) and *P* value of $\leq 0.05$ were considered significant.

**Quantitative real-time PCR analysis.** cDNA from each strain was prepared with the RETROscript kit from Ambion by using 1 μg of total RNA per reaction, and 1 μl of the cDNA reaction was used as a template for the real-time PCR reaction. The oligonucleotide sequences for real-time PCR are listed in Table 2. For real-time PCR analysis using the SYBR Green dye, each reaction mixture contained 0.3 μM of the sense oligonucleotide, 0.3 μM of the antisense oligonucleotide, 0.5X of SYBR Green 1 (Sigma), 0.5 of the Omni Mix HS PCR beads (each PCR bead contains 1.5 U of *Taq*

**Table 2. Sequences of the oligonucleotides used for quantitative real-time PCR.**

| Gene | Forward Primer (5' to 3') | Reverse Primer (5' to 3') |
|---|---|---|
| *SMc03112* (*metH*) | CCCAATCTATCCGAAAGG | GAGTATCTGAAGGTTGCC |
| *SMc04325* (*bmt*) | GCCCTTTCCGATCTCCTC | TCCACGAAGTCCTGATGC |
| *SMc01109* (*metK*) | GGGCATCATGTTCGGCTATG | CGGTCACCTGGCTCTTGG |
| *SMc02755*(*ahcY*) | CGGACCCTGCTGCTTGAC | TCGGCATCCTTCTCCAACC |
| *SMc01843* (*metF*) | GATTATTGATCGGCAGTATCC | CTTACGCAGTTCTTCTTCG |

DNA polymerase, 10 mM Tris-HCl [pH 9.0], 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphate, and stabilizers, including bovine serum albumin) in a 25 μl-reaction volume. The experiment was performed in a Cepheid Smart Cycler version 2.0c as previously described (92). The expression of *SMc00128* was used as an internal control for normalization as described previously (80). This gene was used as a reference since its expression, as measured by real-time PCR, was at similar levels under different environmental conditions and/or mutant backgrounds (106).

**Plant symbiosis assay.** To test the nodulation and nitrogen fixation ability of the *nesR* mutant, plant assays with the symbiotic host *M. sativa* were carried out in duplicates with at least 15 plants per strain per experiment. *S. meliloti* cultures were grown in LB/MC with the appropriate antibiotics at 30° C until saturation. A 1:100 dilution was used to inoculate 3 day old plant seedlings on Jensen's agar as described previously (111). The plates were incubated at 25° C with 60% relative humidity and a 16 hr light cycle. Plants were examined weekly, and after a 4 week period the plant height and the number of pink (nitrogen fixing) and white nodules were enumerated.

**Competition assay for plant symbiosis.** Five ratios (100:1, 10:1, 1:1, 1:10, and 1:100) of the wild-type to mutant strains were tested for competitive nodulation of the host. Saturated cultures of the two strains were washed with sterile water three times, diluted (1:100) and mixed in the appropriate amounts to achieve the desired inoculum ratios. Portions of the inoculums were used to estimate the number of viable bacteria by dilution plating on LB/MC/streptomycin and on LB/MC/neomycin plates. One ml of each inoculum was applied to 3 day old plant seedlings on Jensen's agar as described

previously (111). The plates were incubated at 25° C with 60% relative humidity and a 16 hour light cycle. Each combination of the strains was applied to 10 plants and 6 nodules per plant were harvested at 4 weeks post inoculation. Nodules from the crown region were picked and sterilized in a 50% bleach solution for 5 minutes. In a microtiter plate, the nodules were subsequently washed three times with water, and then crushed in LB/MC plus 0.3 M glucose. Bacteria released from the crushed nodules were diluted and plated on LB/MC plates. The bacterial strains were further identified by growth in LB/MC/neomycin and/or in LB/MC/streptomycin. In addition, PCR analyses were carried out to confirm the genetic background of the strains. Competitiveness for nodulation was carried out twice and assessed by the proportional representation of each strain in the inoculum to the proportional representation of each strain after recovery from the nodules (14).

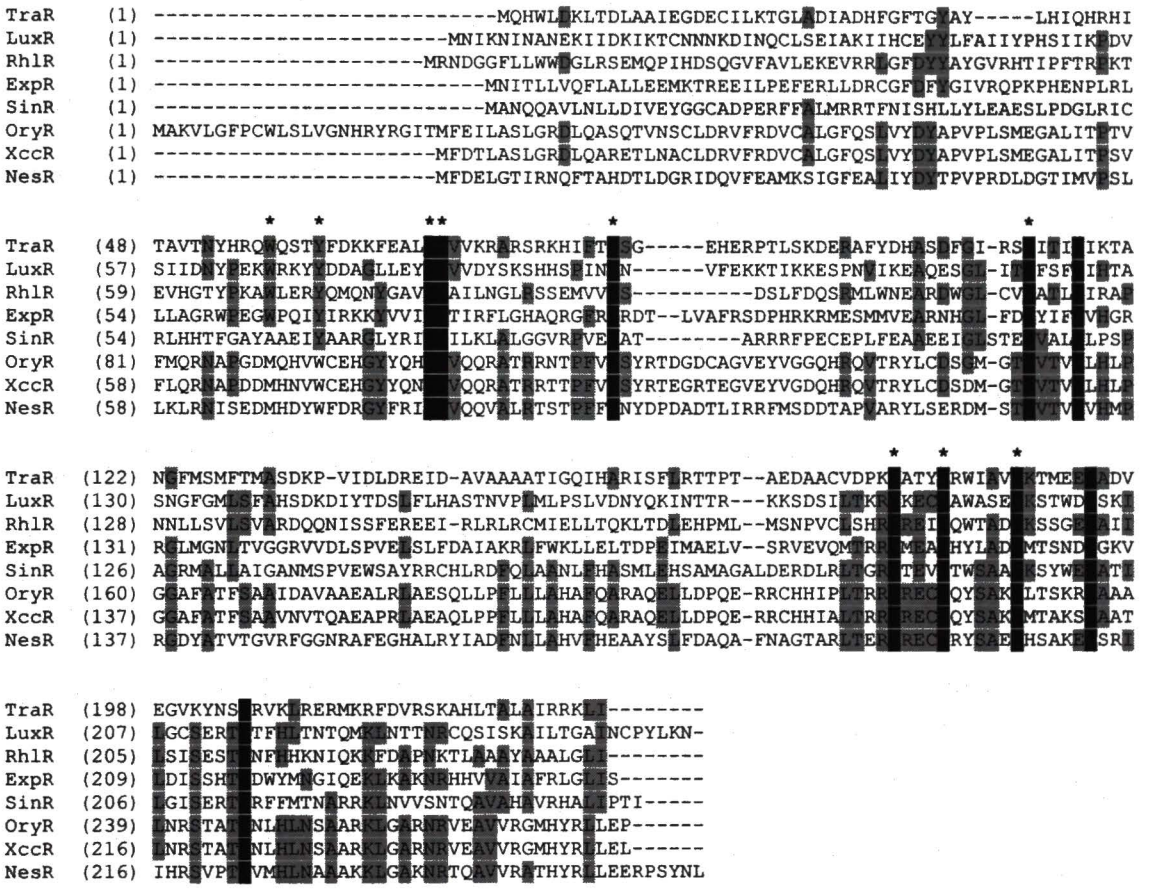
## Results

**Sequence analysis of the *SMc04032* locus.** In *S. meliloti*, the SinR and ExpR regulators have been extensively characterized as part of the quorum-sensing regulon (94, 120, 122). The *S. meliloti* genome revealed the presence of four additional *luxR*-type genes [*SMc04032* (*nesR*), *SMc00878*, *SMc00877*, and *SMc00658*] that share significant homologies to the typical quorum-sensing response regulators (75). These genes do not seem to be associated with a cognate *luxI*-type gene and could be considered to encode orphan LuxR regulators (68, 75). Genetic and biochemical evidence have identified that LuxR-type proteins share two conserved domains, the autoinducer binding N-terminal

domain and the DNA binding C-terminal domain (197). Analysis with SMART (Simple Modular Architecture Research Tool) revealed that the NesR protein contains an autoinducer binding domain (17-178 amino acids) and a helix-turn-helix DNA binding domain (189-246 amino acids), characteristic of the LuxR family of proteins (114). The NesR protein is predicted to encode a protein of 260-amino acids with a molecular mass of 29.7 KDa (114).

Crystal structure studies of the TraR quorum-sensing regulator of *A. tumefaciens* identified the amino acid residues that are involved in AHL binding, TraR dimerization, and DNA binding (207). Through protein sequence alignments of TraR with other LuxR-type proteins it was observed that nine residues are identical in at least 95% of LuxR-type proteins (197, 207). The amino acid sequence alignment of NesR with SinR and ExpR of *S. meliloti* as well as with other previously characterized LuxR-type regulators is shown in Fig. 1. The carboxy DNA binding domain of all LuxR-type proteins compared showed complete identity at E178, L182 and G188 (with respect to TraR). Among the six conserved residues in the amino domain, D70, P71, W85 and G113 also showed complete identity. At Y61, a substitution with a highly similar amino acid (Y61W) occurs in NesR. Interestingly, both XccR and OryR, which are LuxR-type regulators from *Xanthomonas* species, also have a similar Y61W substitution. The W57 residue was not conserved in NesR, SinR, XccR and OryR. Overall, it seems that the predicted DNA binding domain of NesR is generally conserved whereas its predicted signal binding domain shows some degree of variability. In TraR, the Y61 and W57 residues

**Figure 1. Alignment of the deduced protein sequence of NesR with selected LuxR-type proteins.** Sequence alignment of, TraR [14%] from *Agrobacterium tumefaciens*, LuxR [20%] from *Vibrio fischeri*, RhlR [19%] from *Pseudomonas aeruginosa*, ExpR [17%], and SinR [22%] from *Sinorhizobium meliloti*, OryR [42%] from *Xanthomonas oryzae* pv. *oryzae*, and XccR [44%] from *Xanthomonas campestris* pv. *campestris*, with SMc04032 (NesR) from *S. meliloti*. The alignment was performed using the Vector NTI Advance 10 (Invitrogen) software. Percentages of identities with NesR are shown in square brackets. Highly similar residues are shaded in grey and identical residues are shaded in black. * = LuxR-type proteins conserved residues (207).



were shown to be required for binding to AHLs (207). The observation that W57 and Y61 were not conserved in NesR, correlates with our findings that NesR does not depend on binding to AHLs for its regulatory role (see below).

**NesR affects the active methyl cycle of *S. meliloti*.** In order to determine the regulatory role of NesR in *S. meliloti*, we conducted a genome-wide expression analysis utilizing the GeneChip Medicago Genome Array. The gene expression profile of the *nesR* mutant was compared to that of the wild-type (Rm8530) strain. The data generated from the microarray analysis was mined to determine the regulation of genes with similar functions, or genes belonging to a single metabolic pathway. We found genes that were differentially expressed in the absence of *nesR* mainly belonged to the active methyl cycle of *S. meliloti* (Fig. 2). These genes include *metH*, *bmt*, *metK*, *metF*, and *achY* and their expression was downregulated in the absence of *nesR*. This observation was further validated by performing real-time PCR expression analyses (Fig. 3). We complemented the *nesR* mutant with a functional copy of the gene on a plasmid (pJNesR), or with the vector-alone control (pJN105) and compared the expression of the NesR-regulated genes in these constructs to the wild-type strain (Fig. 3).

The active methyl cycle involves the conversion of homocysteine to methionine, which in turn generates the potent methyl donor *S*-adenosyl-L-methionine (SAM) (Fig. 2). Donation of a methyl group from SAM regenerates homocysteine via the intermediate *S*-adenosyl-L-homocysteine (SAH) (Fig. 2) (124). In *S. meliloti*, the synthesis of methionine occurs via the cobalmin-dependent methionine synthase, *metH*

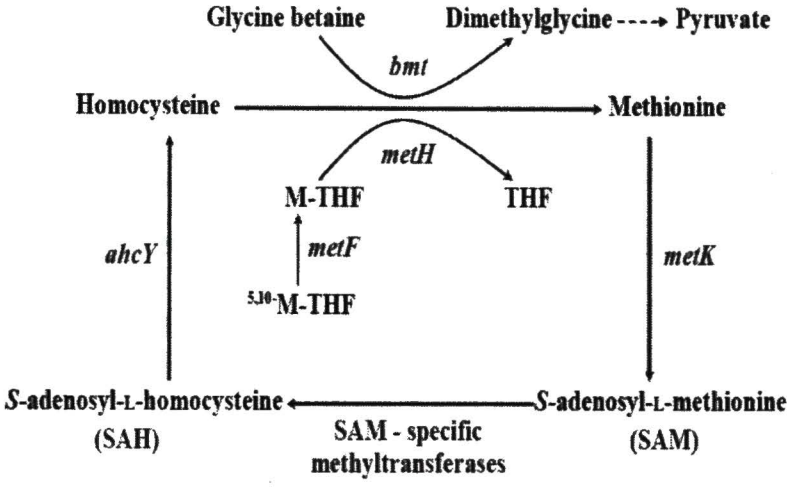



**Figure 2. Active methyl cycle and catabolism of glycine betaine in *S. meliloti*.** Cyclic synthesis of methionine to produce the potent methyl donor *S*-adenosyl-L-methionine is known as the active methyl cycle. In *S. meliloti*, methionine generated from either methyl tetrahydrofolate or glycine betaine is converted to *S*-adenosyl-L-methionine which, after sequential demethylations regenerates homocysteine. The production of methionine from glycine betaine also yields dimethylglycine and is the first step of the catabolic degradation pathway of glycine betaine to pyruvate. The pathway and its associated genes are adapted from information provided by the KEGG database, the *S. meliloti* genome sequence and from Barra *et al.* and Smith *et al.* (11, 75, 175). M-THF = methyl tetrahydrofolate, THF = tetrahydrofolate, $^{5,10}$-M-THF = methylene tetrahydrofolate.

(*SMc03112*), which transfers the methyl group from methyl tetrahydrofolate to homocysteine (75, 124). Recently, Barra et al. determined that in *S. meliloti* 102F43, the betaine methyl transferase enzyme encoded by *bmt* (*SMc04325*) can also serve as a methionine synthase by methylating homocysteine to methionine using glycine betaine as the methyl donor (11). This enzyme is unique amongst bacteria, and its closest homolog is the human *bmt* (11). Our analysis indicated that expression of both the methionine synthases of *S. meliloti, bmt* and *metH,* were decreased three- to five-fold in the *nesR* mutant respectively, and complementation of the *nesR* mutant increased the expression of *bmt* by four fold compared to the wild-type strain and restored expression of *metH* to wild-type levels (Fig. 3).

Further down the pathway, the amino acid methionine is converted to SAM by the activity of *S*-adenosyl-L-methionine synthase, m*etK* (*SMc01109*) (Fig. 2) (75). SAM is often referred to as the 'active methionine' since it serves as a methyl donor in key metabolic pathways, such as protein synthesis, nucleic acid methylation, and generation of metabolites like polyamines (115). On donation of a methyl group by various SAM-specific methyltransferases, SAM is demethylated into SAH. Homocysteine is reintroduced into the cycle by the cleavage of SAH into homocysteine and adenosine by S-adenosyl-L-homocysteine hydrolase, *ahcY* (*SMc02755*) (Fig. 2 ) (75, 124). Recycled homocysteine can then accept a methyl group from $N^5$, $N^{10}$ methylene tetrahydrofolate by the activity of methylene tetrahydrofolate reductase, *metF* (*SMc01843*), to generate methionine (75). We observed that the *metK* and *metF* exhibited a six- and five-fold, reduction of expression respectively, in a *nesR* mutant and complementation of the *nesR*

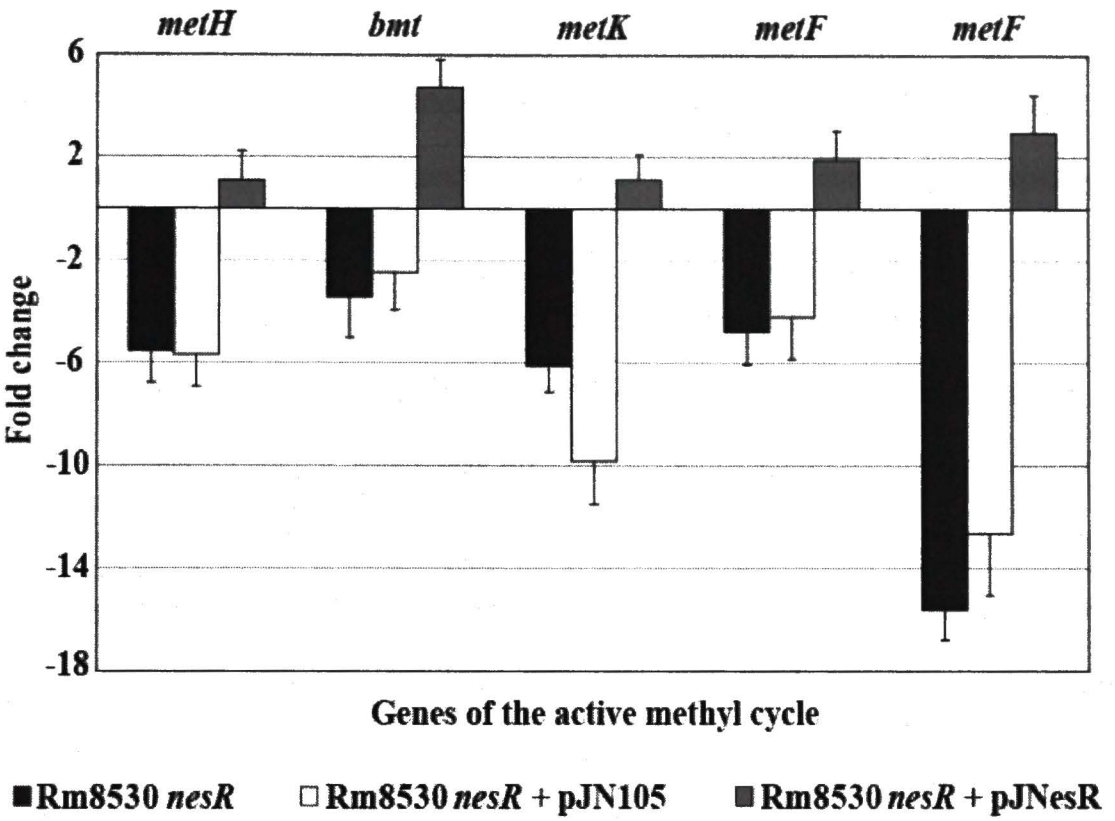



**Figure 3. *nesR* regulates expression of genes from the active methyl cycle of *S. meliloti*.** Quantitative real-time PCR assays were used to measure the expression of genes from the active methyl cycle. Fold changes were calculated as $2^{\Delta}$Ct from the Ct values obtained by real-time PCR analyses. Negative fold change values indicate downregulation in the *nesR* mutant (Rm8530 *nesR*) when compared to the expression of the wild-type (Rm8530) strain. The expression of the downregulated genes was restored when complemented with *nesR* on a plasmid (Rm8530 *nesR* + pJNesR). Rm8530 *nesR* + pJN105 served as vector-only control. Results are the average of a least three independent biological experiments, where within the replicates the coefficient of variance of the Ct values was <4%. The experiments include *SMc00128* as an internal control (106).

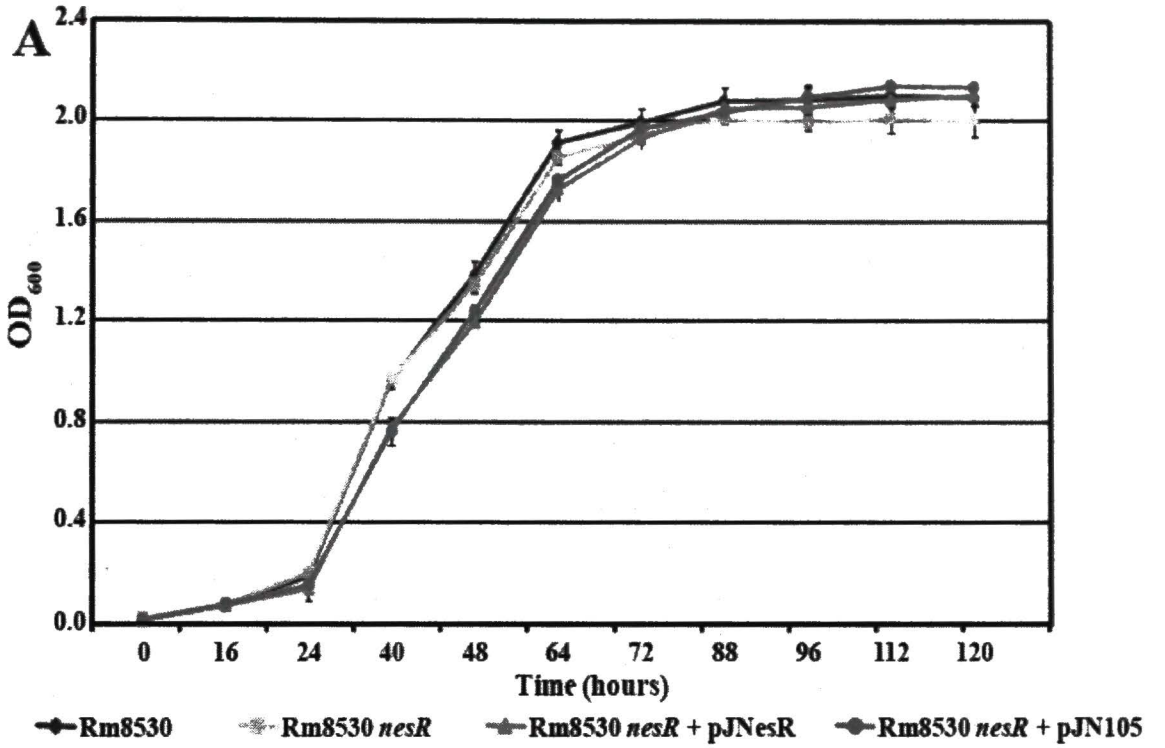
mutant restored expression levels close to that of the wild-type strain (Fig. 3). Expression of *ahcY* was downregulated by fifteen-fold in a *nesR* mutant, and complementation of the *nesR* mutant led to a three-fold upregulation of *ahcY* expression compared to the wild-type strain (Fig. 3).

**The *nesR* mutant does not exhibit a growth defect.** Since the *nesR* mutant affected the active methyl cycle, which is involved in the synthesis of methionine we wanted to determine if the mutant was auxotrophic for this amino acid. We measured the growth of Rm8530 (wild-type), Rm8530 *nesR*, Rm8530 *nesR* + pJNesR and Rm8530 *nesR* + pJN105 in minimal low-phosphate media (Fig. 4A). We observed that the growth of the mutant was similar to that of the wild-type strain indicating that the mutant was not auxotrophic and that any observed differences between the mutant and wild-type strains were not due to a growth defect (Fig. 4A).

**Sensitivity of the *nesR* mutant to salt and detergent stress.** Adaptation to environmental changes as diverse as high osmolarity, variations in pH, and nutrient starvation invoke a general stress response in bacteria (166). Alterations in bacterial membrane have also been observed as a general stress response to environmental insults such as pH changes and osmotic or heat stress (117, 204). The association of the active methyl cycle with general stress response has been observed in several instances (see discussion) (5, 100, 135, 164). Therefore, we sought to investigate if *nesR* played any role in surviving general salt and detergent stress.

Within the rhizobia, *S. meliloti* is relatively salt-tolerant even though within the species there is a high strain-dependent variation in the response to hyperosmolarity. In

**Figure 4. Analysis of growth and sensitivity to stress.** The *nesR* mutant did not exhibit a growth defect and had a reduced efficiency in adapting to an osmotic upshock and detergent stress. Growth curves of wild-type (Rm8530) strain and the *nesR* mutant along with the complemented strains are shown when grown in minimal low-phosphate medium alone (**A**) or when supplemented with 0.5 M NaCl (**B**). The results are the means of three independent experiments, and calculated standard errors are indicated. To test for detergent stress, the *nesR* mutant and the wild-type strain were subjected to increasing concentrations of deoxycholate (DOC) by plating on LB-DOC agar and the resulting colony-forming-units (cfu) were determined (**C**). The results are the means of three independent experiments; standard deviations from the means are as shown. The difference between the wild-type and the mutant cfu were significant at the level of $P<0.02$.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1.6
1.2
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0.4
0.0
OD600
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16
24
40
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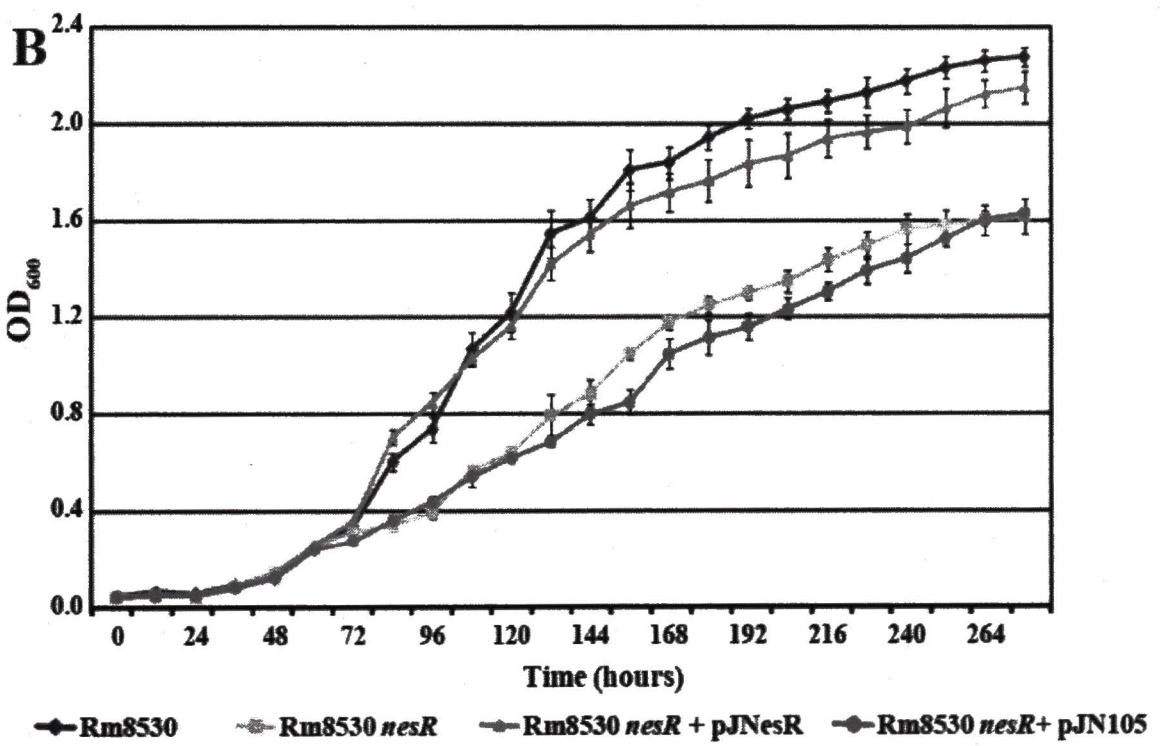
64
72
88
96
112
120
Time (hours)
Rm8530
Rm8530 nesR
Rm8530 nesR + pJNesR
Rm8530 nesR + pJN105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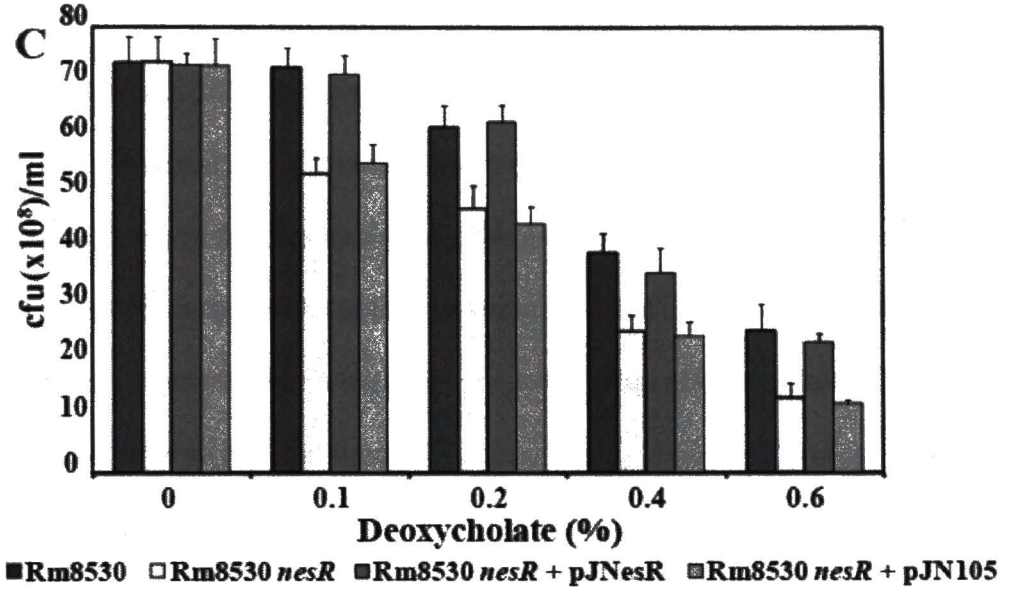
144
168
192
216
240
264
Time (hours)
Rm8530
Rm8530 nesR
Rm8530 nesR + pJNesR
Rm8530 nesR+ pJN105


C
80
70
60
50
40
30
20
10
0
cfu(x10⁸)/ml
0
0.1
0.2
0.4
0.6
Deoxycholate (%)
Rm8530
Rm8530 nesR
Rm8530 nesR + pJNesR
Rm8530 nesR + pJN105

general, different strains of *S. meliloti* can tolerate NaCl concentrations ranging from 0.3 to 0.7 M (130, 203). We compared the growth of the *nesR* mutant and the wild-type strain in minimal low-phosphate media that was supplemented with 0.5 M NaCl. We observed that in the presence of salt, the growth of the *nesR* mutant progressed at a much lower rate than that of the wild-type strain, and that, complementing the *nesR* mutant restored its growth rate to the wild-type levels (Fig. 4B), suggesting that the mutant is less adaptable to osmotic shock.

Gram-negative bacteria are fairly resistant to the membrane-dissolving properties of detergents (205). A change in the sensitivity to detergents like deoxycholate (DOC) typically occurs if the cell is exposed to stress that causes outer membrane erosion and indicates an alteration in the bacterial outer membrane (205). To determine the effect of DOC, the *nesR* mutant and wild-type strain were plated on LB agar plates containing a range of DOC concentrations. The mutant exhibited an increased sensitivity to DOC and after complementation, the effect of DOC was abolished (Fig. 4C). For example, at 0.1% DOC, a 26% reduction in the survival of the *nesR* mutant was observed compared to the wild-type strain. Similarly at 0.2%, 0.4% and 0.6% of DOC a 23%, 35% and 47% lower survival were observed for the *nesR* mutant respectively. Overall it was observed that, the sensitivity of the *nesR* mutant to DOC increased as the concentration of the detergent was increased.

**The *nesR* mutant is impaired in its ability to use glycine betaine as a sole source of carbon.** Usually bacteria exclusively utilize glycine betaine as an osmoprotectant (see discussion) but several studies in *S. meliloti* have shown its unique

ability to catabolize glycine betaine as a sole source of carbon (130, 175). In *S. meliloti*, glycine betaine can donate a methyl group to homocysteine to yield methionine and dimethylglycine as end products (Fig. 2) (11, 175). This reaction is also the first step in the catabolism of glycine betaine (175). *S. meliloti* can catabolize glycine betaine by successive demethylation reactions into pyruvate, which then feeds into the Krebs cycle to generate ATP (175). Therefore, the possible roles of glycine betaine in *S. meliloti* include osmoregulation and nutrition (11, 130, 175). Barra et al. reported that in Rm1021 (an *expR*$^-$ derivative of Rm8530) glycine betaine does not serve as an osmoprotectant and hence, its main role may rest on catabolic degradation (Fig. 2) (11). In order to assess the ability of the mutant and the wild-type strains to catabolize glycine betaine as a sole source of carbon we evaluated their growth in minimal low-phosphate media where mannitol was replaced with 1 mM glycine betaine. This concentration of glycine betaine has been previously shown to stimulate growth in *S. meliloti* (18). With respect to mannitol (Fig. 4A), glycine betaine proved to be a poor source of carbon for growth of the wild-type strain (Fig. 5). Compared to the wild-type strain, the ability of the *nesR* mutant to grow via the catabolism of glycine betaine was severely impaired (Fig. 5). Interestingly, when the *nesR* mutant was complemented, the ability to grow in the presence of glycine betaine as a sole source of carbon was enhanced. This also correlates with our real-time PCR expression analysis, which shows that the expression of *bmt* (involved in glycine betaine catabolism) increased above the wild-type expression levels in the presence of the *nesR*-complementing plasmid (Fig. 3).

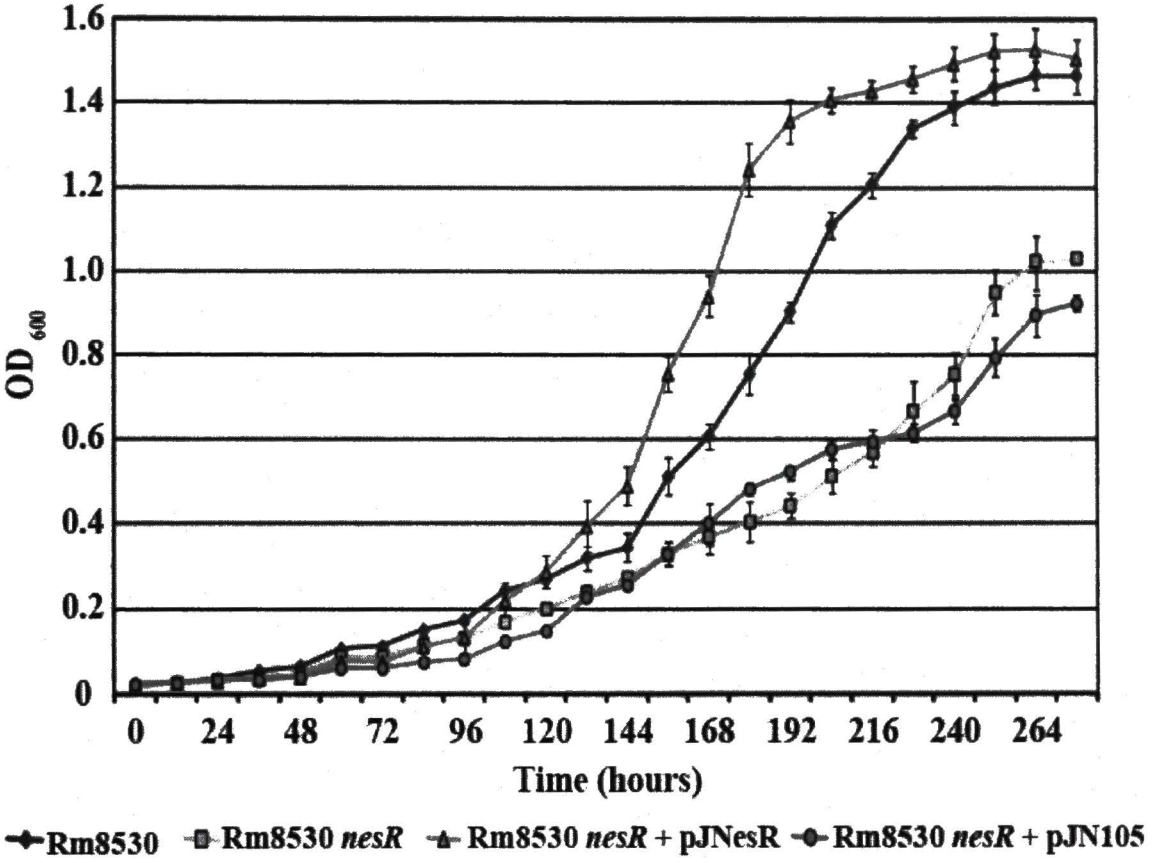
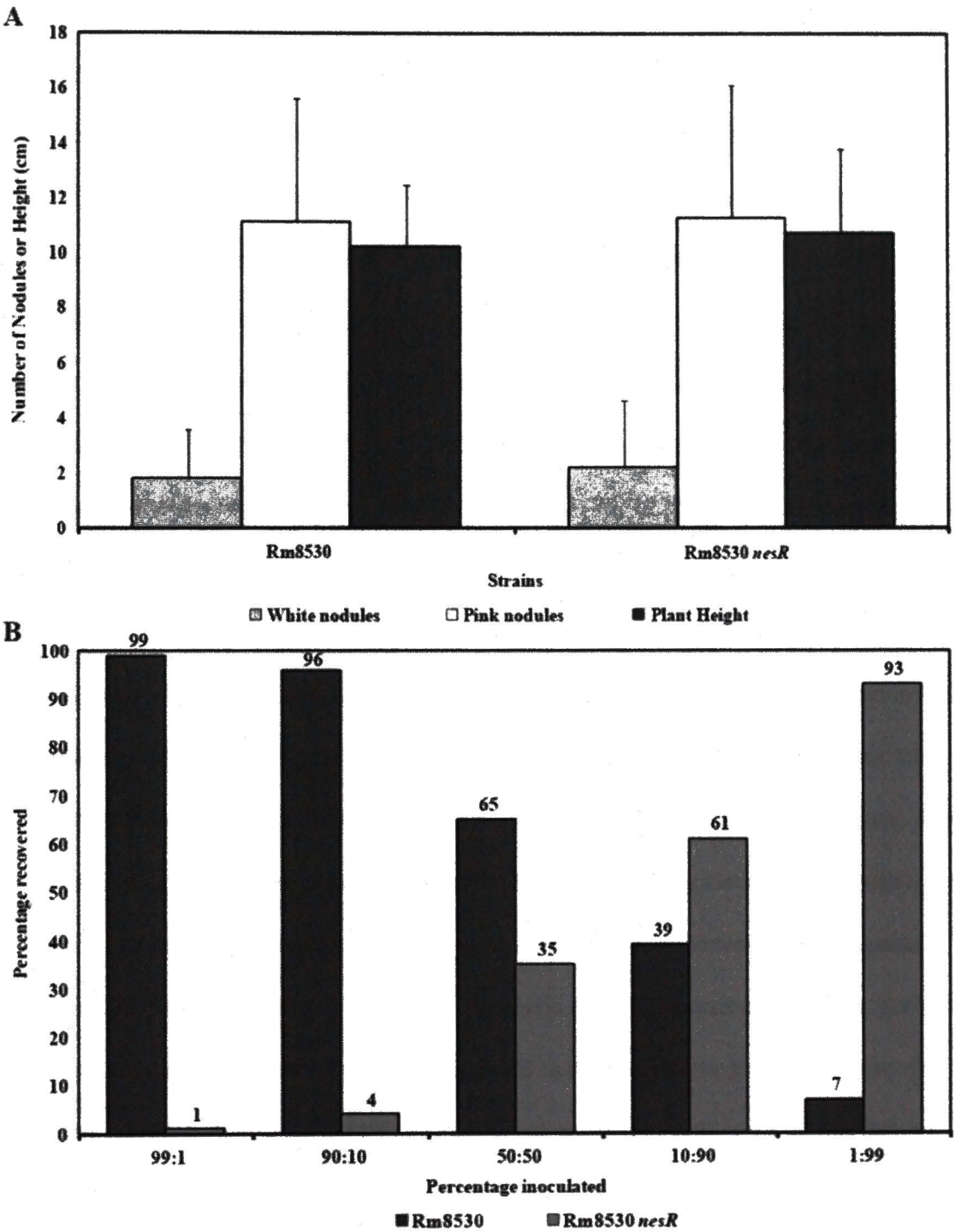



**Figure 5. Growth with glycine betaine as a sole carbon source.** *S. meliloti* has the unique ability to catabolize glycine betaine as a sole source of carbon and energy. Growth analyses of the wild-type strain, the *nesR* mutant and complemented strains when inoculated in minimal low-phosphate medium, where mannitol was substituted with 1 mM glycine betaine are shown. The results are the means of three independent experiments, and calculated standard errors are indicated.

**Individual and competitive establishment of symbiosis by the *nesR* mutant with the host plant.** Successful candidates for symbiosis in the wild must be effective in nitrogen fixation as well as be competitive against locally prevalent soil strains for nodulation (14). Mutants of *S. meliloti* susceptible to detergents or changes in osmolarity have been reported to display variable abilities to nodulate (11, 26, 107, 169). To evaluate the symbiotic and competitive characteristics of a mutation in the *nesR* gene, plant nodulation assays were carried out. Plants inoculated with the wild-type strain and the *nesR* mutant individually were proficient in establishing symbiosis as evidenced by healthy green plants with no significant differences in the number of pink or white nodules on the roots at the end of four weeks (Fig. 6A). However, in co-inoculation experiments, where the wild-type strain and the *nesR* mutant were combined and then applied to the plants in a range of ratios (100:1, 10:1, 1:1, 1:10, and 1:100), the wild-type strain was consistently more competitive than the mutant in nodule occupancy. When co-inoculated in a 1:1 ratio, the percentages of recovered bacteria from the nodules were 65% and 35% for the wild-type and mutant strains respectively (Fig. 6B). Mathematical models to estimate the competitive ability of strains have been described previously (4, 14). Competitive analysis using these models indicated that a significant linear relationship exists between the inoculum ratios and nodule occupancy ratios (data not shown). In addition, the competitive index $C_{wildtype::mutant} = 0.181 \pm 0.008$ had a positive value indicating that the wild-type strain was more competitive for nodulation than the *nesR* mutant (14).

**Figure 6-Symbiosis and competition for plant nodulation.** The *nesR* mutant was capable of establishing symbiosis but was less proficient at nodule occupancy when co-inoculated with the wild-type strain. **(A)** *M. sativa* roots were inoculated with the wild-type (Rm8530) strain and *nesR* mutants individually on Jensen's medium (111). The ability to establish symbiosis and fix nitrogen is indicated by healthy plants and the presence of pink nodules. The results are the means of two independent experiments and standard deviations are as shown. **(B)** Percentage of wild-type and mutant strains recovered from plant nodules at different inoculum-ratios. The X-axis represents the percentage of bacteria applied to the plants and the Y-axis represents the percentage recovered from crushed nodules.


A
Number of Nodules or Height (cm)
0
2
4
6
8
10
12
14
16
18
Rm8530
Rm8530 nesR
Strains
White nodules
Pink nodules
Plant Height
B
Percentage recovered
0
10
20
30
40
50
60
70
80
90
100
99
1
96
4
65
35
39
61
7
93
99:1
90:10
50:50
10:90
1:99
Percentage inoculated
Rm8530
Rm8530 nesR

**NesR and the Sin/ExpR quorum-sensing system of *S. meliloti*.** In gram-negative bacteria, LuxI homologs catalyze the synthesis of AHL molecules in the presence of SAM (*S*-adenosyl-L-methionine) and cellular pools of acylated acyl carrier proteins (ACP) from fatty acid metabolism (72). Moreover, LuxR homologs that are associated with LuxI homologs on their genome regulate the production of AHLs (72). In *S. meliloti* the Sin/ExpR quorum-sensing system is composed of three components: 1) SinI, the only LuxI homolog of *S. meliloti*, has previously been reported to produce a wide range of AHLs, (75, 122, 183); 2) SinR, the SinI-associated LuxR homolog that regulates production of AHLs via upregulation of *sinI* (75, 122); and 3) ExpR, an orphan LuxR homolog which in conjunction with the Sin AHLs, activates quorum-sensing-dependent gene expression (76, 92, 122). Since NesR, which is also an orphan LuxR homolog, was implicated in regulating SAM expression, we first examined its role in the production of AHLs from *S. meliloti*. AHL production profiles of the *nesR* mutant and the wild-type strain were examined in various quorum-sensing mutant backgrounds. We did not observe a difference in AHL production in strains lacking the *nesR* gene (data not shown). In addition, we examined the role of the Sin AHLs in the regulation of genes from the active methyl cycle of *S. meliloti*. Analysis of the expression of various genes from the active methyl cycle remained unchanged in the absence or presence of the *sinI* gene (data not shown); indicating that regulation of genes by NesR is not dependent on the Sin AHLs.

## Discussion

**NesR is an orphan LuxR-type protein.** Based on homology, NesR belongs to the LuxR family of proteins that share two regions of sequence conservation, an autoinducer/signal binding domain and a DNA binding domain. Generally, the DNA binding motif in this family of response regulators is highly conserved, whereas the autoinducer binding locus is similar but allows for related substitutions, perhaps reflecting the different types of signals to which the LuxR-type regulators can be responsive (54, 63, 206). For example, LuxR of *V. fischeri* is activated by AHLs, whereas FixJ, another LuxR-type protein of *S. meliloti* is activated in response to oxygen tension (56, 161). Additionally, these substitutions could reflect an alternative approach in achieving stability as binding of the signal to the regulator usually promotes multimerization and stabilization, both of which seem to be required for efficient DNA binding (54, 207). As NesR is predicted to be a LuxR homolog, we examined if it played a role in the production and regulation of the known autoinducers (Sin AHLs) of *S. meliloti*. We did not observe any difference in the AHL production pattern in strains lacking the functional *nesR* gene (data not shown). Usually, the response regulator flanking the AHL synthase on the genome is involved in the production of AHLs, as is the case of SinR in *S. meliloti* (122). Hence, it is not surprising that NesR is not associated with production of the Sin AHLs. Our genome-wide expression studies and real-time PCR expression analysis indicated that NesR played a role in regulating genes from the active methyl cycle of *S. meliloti* (Fig. 2, Fig. 3). We observed that the expression profile of the NesR-regulated genes did not change when measured in a *sinI*

mutant indicating that the AHLs provided by this synthase do not act as the activating signals for the regulatory role of NesR.

As quorum sensing is a population density-dependent regulatory system, we analyzed the expression pattern of the genes from the active methyl cycle in the wild-type strain (Rm8530) at optical densities ($OD_{600}$) of 0.2 and 1.2, which correspond to the early logarithmic phase and the early stationary phase of growth, respectively (N. Gurich, and J. González, manuscript in preparation). The expression of the active methyl cycle genes did not change as population density increased (data not shown), suggesting that quorum sensing per se does not play a role in their transcription. While based on its characteristic domains and homology NesR belongs to the LuxR family of proteins, it does not seem to rely on the hallmarks of quorum sensing (activation by AHLs and population density) for its regulatory activity.

**Activating signal of NesR.** Although AHLs are the most common signals for LuxR-type proteins, additional molecules such as cyclic dipeptides or plant-derived compounds have been shown to act as signals as well (63, 95, 206). In the *S. meliloti* genome, the *nesR* gene is flanked by two proline iminopeptidase genes (*pip2* and *pip3*) (75). PIP catalyzes the removal of the N-terminal proline from peptides, an activity found mainly in bacteria and some plants (128, 165). Association of PIP with LuxR-type proteins has been observed in several plant-related bacteria (63, 206). The role of one such association was characterized by Zhang et al. in the plant pathogen *X. campestris* pv. *campestris*, where plant-derived exudates activate XccR (an orphan LuxR homolog), which in turn upregulates transcription of the *pip* gene (206). Pip, as well as XccR, are

required for mediating bacterial virulence (206). Additionally, in the plant pathogen *X. oryzae* pv. *oryzae* the orphan LuxR homolog, OryR, is shown to be responsive to host plant exudates (63). Both XccR and OryR are highly homologous to NesR (Fig. 1), though overexpression of NesR in *S. meliloti* did not increase the transcription of the *pip* genes, as in *X. campestris* pv. *campestris* (data not shown). The role, if any, of plant-produced or other exogenous signals, in activating NesR remain to be explored.

**Correlation between the active methyl cycle and stress response.** In higher eukaryotes and in prokaryotic microorganisms, similar biochemical and molecular mechanisms are induced in response to abiotic stresses such as high osmolarity, fluctuating temperatures, or water imbalance (20). Numerous studies in plants have revealed that a prominent physiological response to stress includes the accumulation of methylated metabolites especially those derived from the active methyl cycle (19). The active methyl cycle provides methyl groups to several key metabolic reactions, including DNA methylation and the production of glycine betaine, inositol derivatives, and polyamines (19, 115). Some of these products play a role in stress adaptation by acting as osmoprotectants (glycine betaine), and as radical scavengers (inositol derivatives) (19, 20). In several studies, changes in the transcription of genes from the active methyl cycle have been correlated with response to stress. For example, in tomato plants, production of *S*-adenosyl-L-methionine is increased in response to stress, which in turn leads to the increased production of protective lignifying tissue (164). Expression of *HvMS,* (*metH* equivalent), a methionine synthase gene from barley leaves, was shown to be upregulated in response to salt, drought, and cold stresses (135).

Association of the active methyl cycle to stress is not limited to plants, as it is observed in bacteria as well. In *Pseudomonas syringae*, methionine biosynthesis is required for epiphytic fitness on leaves and this requirement was increased under environmentally stressed conditions (5). Within rhizobia, a methionine synthase (*metH*) mutant of S*inorhizobium fredii* RT19 was highly sensitive to salt stress, while in *S. meliloti* 102F34, growth inhibition due to a *metH* mutation was relieved by the osmotic stress protector glycine betaine (11, 100). Overall, these studies indicate that the regulation of genes from the active methyl cycle play an important role in the cell's ability to cope with stress (5, 11, 100, 135, 164). In this study we provide yet another example of the connection between stress and the active methyl cycle. Through microarray and real-time PCR gene expression analysis we determined that mutation of the *nesR* gene leads to the decreased expression of the active methyl cycle genes (Fig. 3). The *nesR* mutant is also less able to cope with hyperosmotic and detergent stress (Fig. 4B, 4C); suggesting that the active methyl cycle may be critical for stress tolerance in *S. meliloti*. This study thus identifies the role of a previously unknown protein, NesR, as a modulator of metabolic fitness by its capacity to regulate the bacterial active methyl cycle.

***nesR* and the nutritional range of *S. meliloti*.** The complex soil-environment niche of *S. meliloti* selects for genes that provide the microorganism with a widespread ability to metabolize a variety of carbon sources (125, 130). We observed a metabolic defect in the *nesR* mutant in the form of a reduced capacity to catabolize glycine betaine as the sole source of carbon. Outside of nutrition, glycine betaine was recently shown to

be the methyl donor in the synthesis of methionine in *S. meliloti* (Fig. 2) (11). In addition, glycine betaine is a universal osmoprotectant that is used by both plants and a wide range of bacteria to counteract hyperosmotic shock (8, 103, 130). Typically, osmoprotectants are biologically inert; i.e., they accumulate at high osmotic conditions; but when normal conditions are resumed, the osmoprotectants are extruded out of the cells (103, 130). However, *S. meliloti* is atypical in its response to osmotic stress. Unlike most bacteria, the osmoprotectants are actively catabolized by *S. meliloti* even under hyperosmotic conditions, which indicates that these compounds are not inert (18, 130). Moreover, glycine betaine uptake in *S. meliloti* is constitutive in contrast to other bacteria where uptake is stimulated by high osmolarity (181). In rhizobia it has been suggested that the role of glycine betaine is more nutrition-focused rather than osmoprotective (192). The capacity to breakdown glycine betaine holds several potential benefits for *S. meliloti*. In its free-living form, it can expand its nutritional range, providing it with a competitive edge in the rhizosphere. In a study of several *Rhizobium* species, Wielbo et al. observed that strains which used a wider range of carbon sources were more competitive for nodulation (198). In its symbiotic association, glycine betaine produced by the plant could also be made available to the bacteroids, where its catabolism could be used for maintaining the symbiotic association (130). Interestingly, the ability to degrade betaines such as trigonelline, glycine betaine, and stachydrine has been shown to play a critical role in plant symbiosis where genes involved in the catabolism of trigonella and choline to glycine betaine are expressed at all stages of symbiosis, while stachydrine catabolism genes are required for nodulation (152, 181). Various potential sources of

glycine betaine exist in the soil for *S. meliloti*, including choline, the precursor of glycine betaine as well as preformed glycine betaine, which are released by surrounding plant and bacteria of the rhizosphere (103, 130). Given these facts, regulation of the active methyl cycle by NesR could serve to widen the nutritional range of *S. meliloti* in the free-living state and support its symbiotic association with the host.

**Competition for plant nodulation.** During plant nodulation assays, we observed that the *nesR* mutant was as proficient as the wild-type strain in establishing symbiosis or fixing nitrogen ($Fix^+$) (Fig. 6A). However, the *nesR* mutant was less efficient at nodulation when competing with the wild-type strain. The ability to compete for plant nodulation is typically measured by co-inoculating two strains in a 1:1 ratio under sterile conditions and comparing the relationship between representation in the nodule and representation in the inoculums (14). In this study we not only looked at competition between the mutant and wild-type when inoculated in a 1:1 ratio but we also tested four other ratios (Fig. 6B). In all the ratios tested, the *nesR* mutant was consistently the weaker competitor. Rhizobial competiveness is influenced by numerous factors such as metabolic fitness, survival under stresses in the rhizosphere, microbial interactions with other prevalent species, and the ability to utilize nutrients in the soil (7, 14, 198). Therefore, factors such as increased sensitivity to stress (osmotic or detergent) and/or reduced efficiency of the active methyl cycle could contribute towards the competitive defect of the *nesR* mutant.

Alterations in the bacterial membrane have been previously reported to occur as a response to stress conditions including ionic, osmotic or heat stresses (117, 204). Several

studies in *S. meliloti* have reported a correlation between sensitivity to membrane-solubilizing detergents such as deoxycholate (DOC) and a decreased ability to compete for nodulation (26, 107, 169). Increased sensitivity to DOC is either due to an altered LPS structure or due to loss of membrane integrity, probably due to a reorganization of the membrane components (138). For example, in previous nodulation studies of DOC-sensitive *S. meliloti* mutants, all of the strains were proficient at symbiosis and were $Fix^+$ when assayed individually. While some of the mutations were found to directly affect bacterial LPS structure, others had a non-LPS related effect on the membrane. Nonetheless, all mutants displayed a DOC sensitivity suggesting a compromised membrane integrity (26, 107, 169). Analysis of the LPS structure of the *nesR* mutant did not show any alterations when compared to the wild-type strain (data not shown) indicating that *nesR* has a non-LPS related effect on the membrane causing an increased sensitivity to detergent which could possibly be contributing to its competitive defect.

Studies in *S. meliloti* also report nodulation defects in strains affecting the synthesis of methionine. In *S. meliloti* 102F34, a *metH* mutant is $Fix^+$ but defective in competing with the wild-type strain for nodulation (11). Similarly, a *metA* mutant (involved in methionine biosynthesis) of Rm2011 is $Fix^+$, but exhibits delayed nodulation (11, 153). Auxotrophs of methionine have also been shown to affect nodulation not only in *S. meliloti* but in other rhizobia as well (1, 104, 105, 182). Therefore, through these studies, it is well established that methionine is critical for nodule invasion, and that the plant provides very low amounts of methionine to the bacteria (1, 11, 104, 105, 153, 182). The active methyl cycle of *S. meliloti* is the source of methionine, as well as stress

detoxifying metabolites (see above). Though the *nesR* mutant is not auxotrophic for methionine, the combined effect of reduced availability of methionine from the plant, the downregulation of methionine generating genes (*metH* and *bmt*) and the sensitivity to DOC stress could contribute towards its impaired nodulation while competing with the wild-type strain.

**Role of the functional *nesR* within the soil environment.** Chemoattractants secreted by the plants attract microorganisms from bulk soil towards the rhizosphere. The microbial activity around the plant roots is estimated to be several orders of magnitude higher than that in bulk soil (55, 130). In this environment, the metabolic and nutritional fitness of the bacteria equips them to compete for nutrition as well as for plant nodulation. Additionally during growth, plants cause an active uptake of water from the rhizosphere and an expulsion of toxic solutes (130). Due to the combined metabolic activities of the plant and the microorganisms, the rhizosphere tends to be high in osmolarity and presents a stressful environment for the bacteria (130). The nodulation process also contributes towards general stress for rhizobia in terms of high turgor pressure within the infection thread and elevated oxidative and osmotic stresses in the nodules (45, 130). In the rhizosphere, *nesR* of *S. meliloti* could play a role in the overall ability of the bacteria to tolerate stress and allow it to utilize variable sources of carbon. Moreover, within the plant environment *nesR* could provide the bacteria with the capacity to efficiently compete for symbiotic nodulation. Therefore, NesR could help *S. meliloti* to survive the rhizosphere environment and to establish an efficient interaction with the host plant.

In conclusion, we have identified the LuxR-type protein, NesR, as a regulator of the active methyl cycle of *S. meliloti*. The physiological processes modulated by NesR impacts the ability of *S. meliloti* to survive stresses, catabolize specific carbon sources, and ultimately compete for plant nodulation. As NesR is a transcriptional regulator, identification of the mechanisms by which it perceives signals and executes gene regulation could be the focus of future studies and may provide insight into alternative activating mechanisms of LuxR-type proteins.

**Acknowledgments**

We thank Brandon McKethan for assistance with the figures in this manuscript. We also thank the members of our laboratory for their thoughtful insights and input during the preparation of this manuscript. The work in our laboratory is supported by the National Science Foundation grant MCB-9733532, and by the National Institutes for Health grant 1R01GM069925 to J.E.

# CHAPTER IV

## DENITRIFICATION ACTIVITY OF *Sinorhizobium meliloti* AND ITS ORPHAN LUXR-TYPE REGULATORS

*Sinorhizobium meliloti* lives as a free living soil bacterium or forms a symbiotic association with the plant *Medicago sativa*. Coordination of gene regulation is vital to the bacteria in both these lifestyles. In *S. meliloti*, the population density dependent transcriptional regulatory system called quorum sensing controls several critical phenotypes. The LuxR-type response regulators, SinR and ExpR are the main modulators of control by quorum sensing in *S. meliloti*. Interestingly, *S. meliloti* contains additional LuxR-type homologs in its genome, which include the open reading frames of *SMc00878*, *SMc00877*, and *SMc00658*. In this study, the regulatory role of each of these additional LuxR-type proteins was elucidated via genome wide and gene specific expression analysis. These orphan LuxR-type regulators were shown to control the expression of the denitrification genes of *S. meliloti*. With phenotypic observations and enzymatic assays, the induction and activity of the denitrification pathway of *S. meliloti* Rm8530 (wild-type) and the orphan LuxR mutants was characterized.

### Introduction

Bacteria of the *Rhizobiaceae* family have the unique ability to establish nitrogen-fixing symbiosis with leguminous plants. *Sinorhizobium meliloti* belongs to this bacterial

family and is capable of living in a free-living state in the soil or in association with its legume host, alfafa (22, 118). During this association, bacteria exist in a morphologically distinct state called bacteroids within the plant root organs known as nodules. Rhizobia contribute to the global nitrogen cycle in various ways. On one hand, inside the nodules, bacteria synthesize nitrogenase, an enzyme which catalyzes the conversion of atmospheric nitrogen into ammonia (22, 118). On the other, as free-living organisms, or as bacteriods they are capable of reducing oxides of nitrogen via the denitrification pathway (84, 139). Bacterial denitrification is a major contributor in the return of nitrogen to the atmosphere by the metabolic reduction of nitrate ($NO_3^-$) or nitrite ($NO_2^-$) into nitric oxide (NO), nitrous oxide ($N_2O$) or into nitrogen ($N_2$).

Although the processes of nitrogen fixation and denitrification seem incongruous in rhizobia, they are both required for performing complementary functions that are relevant for survival in the soil and within the nodule environment (119). Within the rhizhobia, denitrification has been reported in several strains of *Bradyrhizobium*, *Mesorhizobium* and *Sinorhizobium* (84, 139, 141). In these organisms, the respiratory reduction of nitrate or nitrite is coupled with energy generation enhancing bacterial survival in the soil (139). Denitrification also augments the interaction with the plant host and symbiosis, since the presence of nitrate or nitrite in the soil inhibits the early stages of attachment to the host root hairs and nitrate within the nodule is toxic to nitrogenase activity (139). Denitrification typically occurs under low oxygen tension conditions when, oxygen cannot efficiently serve as the terminal electron acceptor (139, 141). Interestingly, denitrification has also been reported to occur in several bacteria in

the presence of oxygen (aerobic denitrification) (141, 211). The primary role of aerobic denitrification is believed to be in the removal of excess reducing power (27, 211).

The process of denitrification requires the activity of four enzymes; the nitrate- (Nar/Nap), nitrite-(Nir), nitric oxide- (Nor) and nitrous oxide- (Nos) reductases (211). The genome of *S. meliloti* comprises of the chromosome, the pSymA and pSymB megaplasmids (75). The denitrification pathway of *S. meliloti* has not been extensively characterized but several genes believed to be involved in this process have been mapped exclusively to the pSymA megaplasmid (34, 35, 48, 96). These include, the *napEDABC* genes ($NO_3^-$ to$NO_2^-$), the *nirKV* genes ($N_2O$ to NO), the *norECBQD* genes (NO to $N_2O$) and the *nosRZDYFLX* genes ($N_2O$ to $N_2$) (Fig. 1) (75).

In gram-negative bacteria, several communication systems have evolved to transform environmental cues into cellular responses. One such communication system, termed quorum sensing, governs the expression of genes in response to population density (180). Quorum sensing is a global regulatory mechanism that controls several pathogenic as well as symbiotically relevant characteristics (180). For example, in *P. aeruginosa* and *Vibrio cholerae* quorum sensing controls production of virulence factors which aid in establishment of a successful pathogenic infection (146, 210). In *S. meliloti* and *Vibrio fischeri* quorum sensing controls plant nodulation and bioluminescence respectively, both of which help interaction with their symbiotic hosts (59, 122). Quorum sensing is mediated via the activation of regulatory proteins by specific signaling molecules called autoinducers (69, 72). In gram-negative bacteria these autoinducers are usually *N*-acyl-L-homoserine lactones (AHLs), which are generated by a LuxI synthase

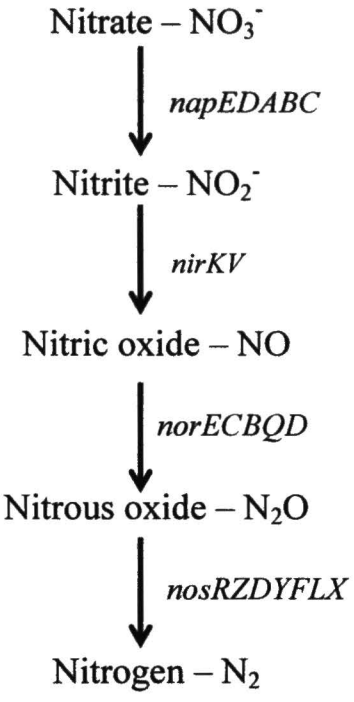



**Figure 1. The predicted denitrification pathway of *S. meliloti* and its associated genes** (48, 211).

(69, 72). The response regulators are characterized by two principal domains, an autoinducer or signal binding domain and a DNA binding, helix-turn-helix domain (69, 72, 197). The LuxR-type proteins can activate or repress target genes by interacting with specific quorum-sensing regulated promoters (72, 197). In most quorum-sensing systems, the *luxI*- and *luxR*-type genes are in close proximity to each other on their genome. Several recent studies have identified a number of genes which encode for additional *luxR*-type regulators, in excess of the *luxI*-type synthases (2, 39, 63, 140, 206). These additional regulators are termed orphan-*luxR*'s as they are not associated with a cognate synthase on their genome (68).

Global gene-regulation, controlled by quorum sensing has been extensively characterized in *S. meliloti* (30, 121, 170). SinR/I are the cognate LuxR/I pair while; ExpR is an orphan LuxR homolog of *S. meliloti* (75). Together the Sin/ExpR quorum-sensing system regulates over 250 genes including the production of symbiotically relevant exopolysaccharides and the control of motility and chemotaxis (75, 80, 94). The *sinI* gene is the sole *luxI*-type synthase encoded by *S. meliloti* (75). On the other hand, based on protein homology and domain characteristics, other than SinR and ExpR, the *S. meliloti* genome contains additional LuxR homologs (SMc04032, SMc00878, SMc00877, and SMc00658) (75, 149). Since the genes of these homologs are not associated with a known synthase on the genome, they are potentially orphan *luxR* regulators of *S. meliloti.*

In this study, we identify the regulatory role of *SMc00878*, *SMc00877,* and *SMc00658*, by creating mutants of these genes and then examining their role via genome-

wide gene expression analysis. We demonstrate that these three orphan LuxR-type homologs act upon the denitrification pathway of *S. meliloti* specifically under aerobic conditions. We also analyzed the nitrate- and nitrite reductase activities of the mutants and wild-type strains under aerobic conditions by enzymatic assays and the different physiological conditions that stimulate microaerobic denitrification in *S. meliloti* were characterized.

## Materials and Methods

**Bacterial strains and media.** The wild-type strain used in this study is Rm8530, and the relevant strains and plasmids used are listed in Table 1. *S. meliloti* strains were grown at 30°C in Luria-Bertani (LB) medium supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ for routine cultures. Starter cultures for RNA purification were grown in tryptone yeast-extract (TY) medium and sub-cultured in minimal mannitol glutamate low-phosphate medium (MLP) [50 mM MOPS, 19 mM sodium glutamate, 55 mM mannitol, 0.1 mM $K_2HPO_4/KH_2PO_4$, 1 mM $MgSO_4$, 0.25 mM $CaCl_2$, 0.004 mM biotin, pH 7], as described previously (92). Bacterial strains were grown on minimal glutamate media plates (MGM) (11 g $Na_2HPO_4$, 3 g $KH_2PO_4$, 0.5 g NaCl, 1 g glutamate, 10 g mannitol, 1.5% Agar, 1 mg/ml biotin, 27.8 mg $CaCl_2$ and 246 mg $MgSO_4$) for the screening of recombinant mutants. Antibiotics were used in the following concentrations, streptomycin (Sm) 500 μg/ml, kanamycin (Km) 25 μg/ml, ampicillin (Amp) 100 μg/ml, hygromycin (Hy) 50 μg/ml, spectinomycin (Sp) 100 μg/ml, neomycin

(Nm) 200 μg/ml in LB and 25 μg/ml in minimal media, and trimethoprim (Tp) 30 μg/ml (for *Escherichia coli*) or 1 mg/ml (for *S. meliloti*).

**Construction of the *SMc00878* and the *SMc00877* mutants.** An internal 400 bp fragment of the *SMc00878* open reading frame (ORF) was amplified from *S. meliloti* Rm8530 chromosomal DNA by using the following primers, 878-EcoRI-Fwd, 5'-CGCGAATTCGATATATCCTCATTGGTCCT-3'and 878-XbaI-Rev, 5-GCTCTAGAGGTGACCCGCGGCATCATGG-3', which contain EcoRI and XbaI restriction sites (underlined) at their respective 5' ends. The PCR fragment was purified, digested with EcoRI and XbaI and cloned into the suicide vector pVIK112, creating pVIK878. The recombinant plasmid was then transformed into S17-1λ*pir* and transferred into Rm8530 by biparental mating, where the wild-type copy of the gene was disrupted by single recombination integration. *S. meliloti* recombinant clones were selected by plating the mating mixture on MGM containing Nm.

A similar approach was used to disrupt the SMc00877 gene. Briefly, a 421 bp internal fragment of the *SMc00877* ORF was amplified from the *S. meliloti* Rm8530 chromosomal DNA using the following primers: 877-EcoRI-Fwd-5'-CGCGAATTCGATGATCACGATGGGGACGAC and 877-XbaI-Rev-5'-GCTCTAGAACCTTCGGCATCGTGGACCGG-3'. The resulting recombinant plasmid pVIK877, was mated into Rm8530 and mutant recombinant clones were selected on MGM containing Nm. All strain constructs were confirmed by PCR and the mutations were transduced using the phage ϕM12 into the wild-type strain (79).

**Table 1. Bacterial strains and plasmids used in this study.**

| Strain or plasmid | Relevant characteristics | Reference or source |
|---|---|---|
| *Sinorhizobium meliloti* | | |
| Rm8530 | Su47 *str-21*, *expR*$^+$ | (149) |
| Rm8530 *878* | SMc00878::Km | This work |
| Rm8530 *877* | SMc00877::Km | This work |
| Rm8530 *658* | SMc00658::Tp | This work |
| Rm8530 *878-877* | SMc00878::Km, SMc00877::Hy | This work |
| *Escherichia coli* | | |
| S17-1λ*pir* | See source | (171) |
| DH5 α | See source | Life technologies |
| MT616 | MT607(pRK600), helper strain | (79) |
| *Paracoccus denitrificans* | See source | (168) |
| Plasmids | | |
| pVIK112 | *lacZY* for transcriptional fusions, Km | (102) |
| pVIK878 | pVIK112 carrying the SMc00878 internal fragment | This work |
| pVIK877 | pVIK112 carrying the SMc00877 internal fragment | This work |
| pPCR-Script Amp SK (+) | See source | Stratagene |
| p658 | pPCR-Script Amp SK (+) carrying the SMc00658 ORF | This work |

| | | |
|---|---|---|
| pJQ200SmSp | Suicide vector, *sacB* | (121) |
| pMB419 | Carries Hy cassette | (10) |
| pK19-877-Km | pK19mobΩHMB carrying the SMc00877 internal fragment | Anke Becker |
| pK19-877-Hy | pK19-877-Km carrying the Hy cassette in the Km ORF | This work |

**Construction of the *SMc00878-SMc00877* double-mutant.** An internal fragment of the *SMc00877* ORF was cloned into the vector pK19mobΩHMB creating the recombinant vector pK19-877. This vector was kindly provided by Dr. Anke Becker from the University of Bielefeld, Germany. The kanamycin cassette of pK19-877-Km was disrupted by cloning a hygromycin cassette within the MscI restriction site. The hygromycin cassette was amplified from pMB419 by using the following primers with MscI (underlined) restriction ends, MscI-Hy-Fwd: 5'-GCTGGCCAGCTGCAGAAGGAATTACCAC-3'and MscI-Hy-Rev: 5'-GCTGGCCACTAGTAACATAGATGACACCGCGC-3'. The resulting plasmid, pK19-877-Hy was transformed into S17-1 λ*pir* and transferred into Rm8530 *878::*Km by biparental mating. Clones of the double mutant were selected by plating on media containing the appropriate antibiotics. The mutant construct was confirmed by PCR and was transduced using the phage ϕM12 into the wild-type strain (79).

**Construction of *SMc00658* mutant.** The *SMc00658* ORF was amplified from the Rm8530 chromosomal DNA using the following primers: 658-SpeI-Fwd 5'-GCACTAGTCGCAGACGCGGCGGGCGTCGTC-3' and 658-SpeI-Rev 5'-CGACTAGTGTGGCGATATTGCTGGATGCGCGTC-3'. The PCR product was cloned into the blunt EcoRV site of pPCR-Script Amp SK (+) (Stratagene) creating the vector p658. The *SMc00658* ORF was disrupted by transposon mutagenesis of p658 using the EZ::TN insertion kit (Epicenter). The disrupted ORF was then cloned into the SpeI site of the suicide vector pJQ200SmSp and the resulting recombinant plasmid was transformed into DH5α (121). This strain was then used to transfer the mutation to

Rm8530 by tripatental mating where MT616 was used as the helper strain (79). *S. meliloti* carrying the disrupted copy of *SMc00658* was selected by plating on media containing the appropriate antibiotics and 5% sucrose (158). The mutant construct was confirmed by PCR and was transduced using the phage ϕM12 into the wild-type strain (79).

**RNA purification for microarray and real-time PCR analysis.** Bacteria were grown to saturation in TY medium. A (1:100) dilution was used to inoculate 25 ml of MLP media with the appropriate antibiotics. Cultures were then grown at 30° C aerobically to an $OD_{600}$ of 1.2. These culture conditions have been previously established as optimum conditions for differential expression of quorum sensing regulated genes (80, 94). Cells were harvested by centrifugation (10,000 x g for 2 min. at 4° C), and the cell pellets were immediately frozen in liquid nitrogen. Total RNA was purified using the RNeasy Mini Kit (Qiagen). Cells were resuspended in 10 mM Tris HCl (pH 8) and disrupted in the RLT buffer (supplemented with β-mercaptoethanol) provided with the RNeasy kit. The cells were transferred to Fast Protein tubes (Qbiogene) and disrupted using the Ribolyser (Hybaid) (45 sec, level 6.5) prior to spin column purification according to the RNeasy Mini Kit RNA purification protocol. The RNA samples were treated with the Qiagen on-column RNase-free DNase. Samples were DNase treated a second time with the TURBO RNase-free DNase from Ambion according to the manufacturer's protocol, and an additional RNA clean-up step was performed (92). RNA integrity was determined by using an Agilent 2100 Bioanalyzer.

**Affymetrix GeneChip hybridization and expression analysis.** The cDNA synthesis and hybridization to the GeneChip Medicago Genome Array (Affymetrix, Santa Carla, CA) were performed as described previously, at the Core Microarray facility at UT Southwestern Medical Center (Dallas, TX) (80, 94). Ten μg of RNA was used for each experiment and the GeneChip Scanner 3000 was used to measure the signal intensity of each array. The .CEL files generated were processed by Affymetrix GeneChip Operating Software, (GCOS v 1.4). Comparative analysis of the control and experimental expression were represented in terms of M value (signal log ratio) which also indicated increased, decreased or no change in expression of a gene in the mutant with respect to the wild-type. An M value $\geq 1$ (2 fold change) and *P* value of $\leq 0.05$ were considered significant.

**Quantitative real-time PCR analysis.** cDNA from each strain was prepared with the RETROscript kit from Ambion by using 1 μg of total RNA per reaction, and 1 μl of the cDNA reaction was used as a template for the real-time PCR set up. The oligonucleotide sequences for real-time PCR are listed in Table 2. For real-time PCR analysis using the SYBR Green dye, each reaction mixture contains 0.3 μM of the sense oligonucleotide, 0.3 μM of the antisense oligonucleotide, 0.5X of SYBR Green 1 (Sigma), 0.5 of the Omni Mix HS PCR beads (each PCR bead contains 1.5 U of *Taq* DNA polymerase, 10 mM Tris-HCl [pH 9.0], 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphate, and stabilizers, including bovine serum albumin) in a 25 μl-reaction volume. The experiment was performed in a Cepheid Smart Cycler version 2.0c as previously described (92). The expression analyses were conducted as three

independent experiments. The expression of *SMc00128* was used as an internal control for normalization as described previously (80). This gene was used as a reference as its Ct values obtained by real-time PCR have been found to be at similar levels under different environmental conditions and/or mutant backgrounds (106).

**Methyl-viologen assay for measuring nitrate- and nitrite reductase activity.** Nitrate or nitrite reductase activities were spectrophotometrically measured by the methyl-viologen assay as described previously (17, 43). Under physiological conditions the reductases catalyze the reduction of nitrate or nitrite by accepting electrons and this property has been utilized for the enzyme assays, where methyl-viologen serves as a non-physiological electron donor. Methyl-viologen can be chemically reduced with sodium dithionite (17, 43). The oxidized form of methyl-viologen is colorless while the reduced form is blue (17, 43). Accumulation of the reduced form can be monitored spectrophotometrically at a wavelength of 600 nm while re-oxidation (change from blue to colorless) can be measured by following a decrease in absorbance at 600 nm.

Bacteria were grown aerobically at 30° C to an $OD_{600}$ of 1.2 in MLP media which contained no nitrate or nitrite. Cells were then harvested by centrifugation (10,000 x g for 2 min. at 4° C) and the cell pellets were immediately frozen in liquid nitrogen. The samples to be assayed were resuspended in 20 mM Hepes buffer (pH=7) and 1 mM methyl-viologen to a final volume of 1 ml in round-top plastic cuvettes (Brandtech) and the cuvettes were sealed with suba-seals (Sigma). As the reduction of methyl-viologen is susceptible to oxygen, the cuvettes were sparged with nitrogen gas for 15 mins. To initiate the reduction of methyl-viologen, freshly prepared sodium dithionite was added to

**Table 2. Oligonucleotides used in real-time PCR analysis.**

| Gene | Forward Primer (5'to 3') | Reverse Primer (5'to 3') |
|---|---|---|
| *napE* | GCTCTTCGTTAAGTTCGG | GTCACAACAATTTCCACAC |
| *napA* | ACGCCTTGTAGAGTTCCG | ATCTCCCGACGACGAATAC |
| *napB* | GGCAGGAAAGGCATCGGTTGG | ACCAGCCGCCAGTCATCCC |
| *nirK* | CCTTGTCATAGGTAATTGAATTGC | CCTCCAGGCATGGTTCCG |
| *nirV* | CAGCGGTAACCTGATATTTC | TCCTCGCACTTGAGACAG |
| *norE* | TGTTGGAAGCGGCTATTG | CCGATACTTTCATCCTCTGG |
| *norC* | CACCGCTTCCACACATTG | ACTATTACATGAAGACCGAATC |
| *norB* | ACATACCACCAGTACATCTTG | GCTACCTCCTCCACATCC |
| *norD* | CGAAGCGATTGAGGATGAAC | TGGCGATAGAGCGGAAGG |
| *nosR* | TTCAAGACGGCGATCATCC | AAGATAGCGGCAGTAGAACC |
| *nosZ* | GCATCCCTCGTTTACAGAC | AGTGTGCTGGTTCGGAAG |
| *nosY* | GGCAAGTTCCTCGGTCATC | ACACGAAGAACAGCCAGATAC |

the cuvette with a Hamilton syringe until a steady state absorbance at 600 nm was obtained. Appropriate substrates, $KNO_3$ for nitrate reductase or $NaNO_2$ for nitrite reductase, were injected through the seals to obtain a final concentration of 10 mM. The bacterial nitrate and nitrite reductase catalyze the reduction of the added substrate by accepting electrons from the reduced methyl-viologen. The resulting decrease in absorbance due to re-oxidation of methyl-viologen into a colorless form was measured in a continuous recording spectrophotometer (Cary 50 UV, Varian). The extinction coefficient of methyl-viologen is 13 $mM^{-1}$ $cm^{-1}$ which was used to calculate the specific activity of the reductase (101). The specific activity of the enzyme was calculated as (delta absorbance/min)/extinction coefficient of methyl-viologen. The enzyme activity was normalized to the protein concentration, measured by the Bradford method (Bio-Rad). The assays were conducted as two independent experiments.

**Growth assays to estimate denitrification activity.** *S. meliloti* strains were grown under microaerobic conditions in gas tight anaerobic culture tubes (Bellco Biotechnology). Cultures were inoculated in TY medium alone or supplemented with either 10 mM $KNO_3$ or 5 mM $NaNO_2$. The inoculated media was added to the 24 ml capacity anaerobic culture tubes in different volumes to achieve a range of approximately 4%, 2% and 0% oxygen concentration in the head space. Each anaerobic tube was sealed with a sterile butyl stopper and incubated under static condition at 30°C. Growth and gas production was ascertained periodically. The previously well characterized denitrifyer, *Paracoccus denitrificans* was used a the positive control for denitrification activity (168).

## Results

### Sequence analysis of the *SMc00878*, *SMc00877* and *SMc00658* loci.

Transcriptional regulators from the LuxR family of proteins contain a signature helix-turn-helix domain at their carboxy-terminus and a signal binding domain at their amino-terminus (69, 197) Analysis of the *S. meliloti* genome sequence reveals the presence of three *luxR*-type genes that correspond to the open reading frames of *SMc00878*, *SMc00877* and *SMc00658* (75). All three genes are located on the chromosome with *SMc00878* and *SMc00877* lying next to each other (75). Since these genes are not associated with a synthase on their genome, they are considered orphan LuxR-type regulators (68, 75). The general characteristics of these proteins are show in Table 3. SMART analysis of the three orphan LuxR regulators revealed the presence of the typical signal binding domain at the N-terminus and the presence of the helix-turn-helix domain at the C-terminus (Table 3). The amino acid sequence identities of SMc00878, SMc00877 and SMc00658 with some previously characterized response regulators are shown in Table 4. Previous studies with LuxR-type proteins indicate that they share an overall 18-25% sequence identity with other members of the family (69, 197). Our protein sequence comparisons indicated that the homology of most functional LuxR-type regulators with SMc00878, SMc00877 and SMc00658 are within that range (Table 4). Interestingly, amongst the functionally characterized regulators, AvhR of *Agrobacterium vitis* showed the highest homology to SMc00878, SMc00877 and SMc00658 (Table 4). AvhR is also an orphan LuxR-type protein and has been reported to affect necrosis and hypersensitivity response in the plant pathogen *A. vitis* (89).

**Mutants of *SMc00878*, *SMc00877* and *SMc00658* show decreased transcription of genes involved in the denitrification pathway.** In order to determine the regulatory role of *SMc00878*, *SMc00877* and *SMc00658* we disrupted their coding sequences to create strains with mutations in these genes. Since *SMc00878* and *SM00877* lie adjacent to each other on the genome and are highly homologous to each other we surmise they could work in conjunction with each other therefore; we constructed a double-mutant strain of their genes. We utilized the Affymetrix GeneChip Medicago Genome Array to generate genome wide expression profiles of the Rm8530 *878-877* (double mutant) strain and the Rm8530 *658* strain. We compared these profiles to that of the wild-type stain to determine which genes are differentially expressed due to mutations in the orphan *luxR* homologs. Preliminary analysis of the data generated by the Affymetrix GCOS analytical software indicated that several genes from the *S. meliloti* denitrification pathway were downregulated in the Rm8530 *878-877* strain and in the Rm8530 *658* strain. Figures 2A and 2B illustrate the gene map of denitrification genes on the pSymA plasmid of *S. meliloti* (75). The differential-expression of genes detected by the microarray analyses are indicated as M-values above each open reading frame (Fig. 2).

In order to verify the effect of the orphan LuxR homologs, as indicated by the genome wide microarray analysis, real-time PCR expression analysis were conducted with selected genes (*napE*, *napA*, *napB*, *nirK*, *nirV*, *norE*, *norC*, *norB*, *norD*, *nosR*, *nosZ*, and *nosY*) from the denitrification pathway of *S. meliloti*. These genes were selected

**Table 3. General properties of deduced protein sequence of SMc00878, SMc00877 and SMc00658 of *S. meliloti*.**

| Properties | Orphan LuxR-type Regulators of *S. meliloti* | | |
|---|---|---|---|
| | **SMc00878** | **SMc00877** | **SMc00658** |
| **Predicted protein size** | 246-amino acids | 277-amino acids | 247-amino acids |
| **Predicted molecular mass** | 27.1 KDa | 30.8 KDa | 27.4 KDa |
| **N-autoinducer binding domain** | 25 to 176 amino acids | 55 to 204 amino acids | 28 to182 amino acids |
| **C-DNA binding domain** | 182 to 239 amino acids | 213 to 270 amino acids | 184 to 241 amino acids |

**Table 4. Percentage identities of LuxR homologs.** Amino acid sequence comparisons of, LuxR from *Vibrio fischeri*, TraR from *Agrobacterium tumefaciens*, QscR, LasR and RhlR from *Pseudomonas aeruginosa*, SdiA from *Escherichia coli*, CinR and RhiR from *Rhizobium leguminosarum*, AviR and AvhR from *Agrobacterium vitis*, and ExpR, SinR, SMc00878, SMc00877, and SMc00658 from *Sinorhizobium meliloti*. Orphan LuxR-type proteins are underlined.

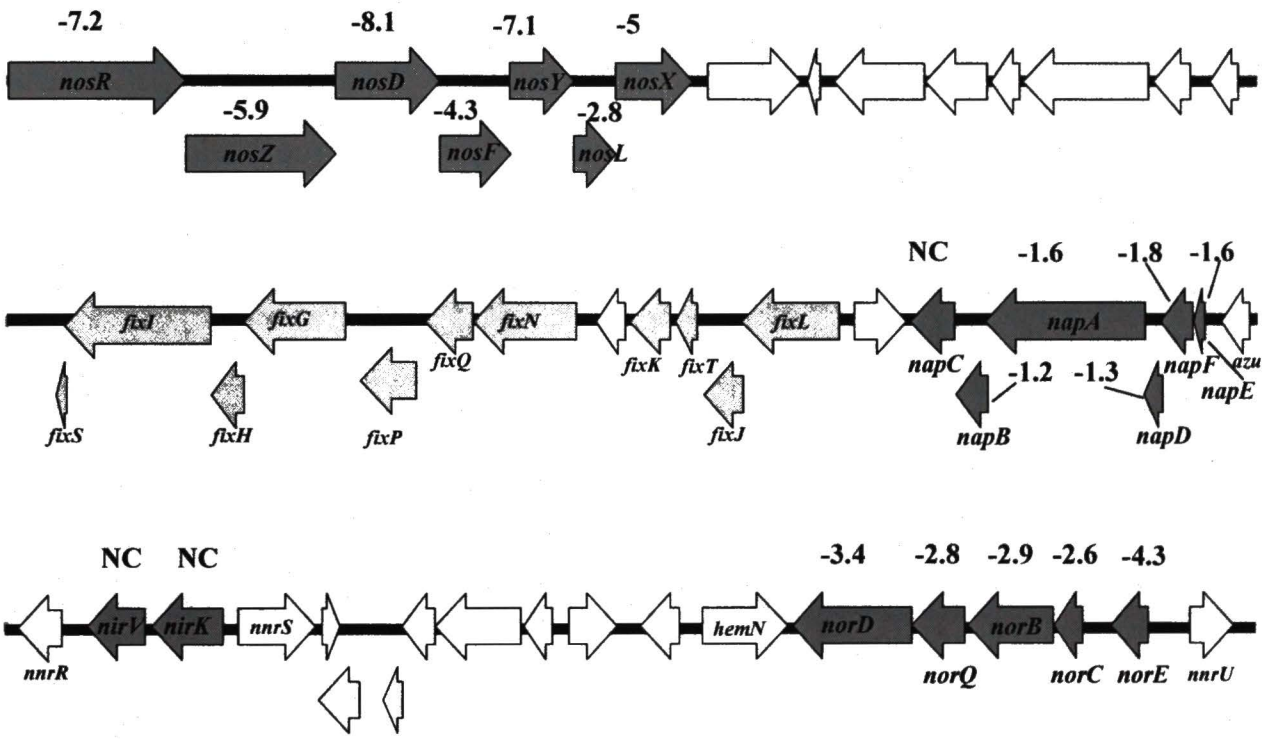
| | LuxR | TraR | OscR | LasR | RhlR | SdiA | CinR | RhiR | AviR | AvhR | ExpR | SinR | SMc00878 | SMc00877 | SMc00658 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **LuxR** | | 18 | 24 | 27 | 20 | 18 | 18 | 22 | 21 | 21 | 20 | 20 | 22 | 20 | 17 |
| **TraR** | | | 21 | 17 | 23 | 26 | 16 | 20 | 18 | 20 | 16 | 17 | 18 | 21 | 17 |
| **OscR** | | | | 27 | 32 | 30 | 22 | 23 | 21 | 21 | 22 | 21 | 22 | 19 | 24 |
| **LasR** | | | | | 29 | 24 | 19 | 23 | 20 | 22 | 24 | 23 | 21 | 21 | 23 |
| **RhlR** | | | | | | 40 | 20 | 25 | 24 | 23 | 25 | 22 | 19 | 23 | 20 |
| **SdiA** | | | | | | | 17 | 18 | 22 | 20 | 23 | 20 | 20 | 19 | 18 |
| **CinR** | | | | | | | | 20 | 18 | 21 | 20 | 21 | 18 | 20 | 22 |
| **RhiR** | | | | | | | | | 25 | 26 | 25 | 23 | 22 | 25 | 22 |
| **AviR** | | | | | | | | | | 24 | 56 | 19 | 21 | 24 | 20 |
| **AvhR** | | | | | | | | | | | 26 | 26 | 43 | 47 | 30 |
| **ExpR** | | | | | | | | | | | | 19 | 23 | 23 | 22 |
| **SinR** | | | | | | | | | | | | | 20 | 23 | 25 |
| **SMc00878** | | | | | | | | | | | | | | 34 | 28 |
| **SMc00877** | | | | | | | | | | | | | | | 30 |
| **SMc00658** | | | | | | | | | | | | | | | |

because they were either the first gene of an operon or were likely to be expressed as individual transcripts. We analyzed the expression of these genes in Rm8530 *658*, Rm8530 *878*, Rm8530 *877*, and Rm8530 *878-877* strain backgrounds. The results from the quantitative real-time PCR analysis are shown in Figure 3. The data generated from these analyses correlated with the microarray analysis. Interestingly, *SMc00878* and *SMc00877* individually affect the expression of the genes from the denitrification pathway of *S. meliloti*. In general, Rm8530 *658* and Rm8530 *878* had the greatest regulatory effect on the pathway (Fig. 3). We also observed that the magnitude of regulation increases for the latter reactions (*nor*, *nos*) of the pathway. Moreover, the first gene in the operon encoding for the reductase is most highly regulated as indicated by the maximal downregualtion of *napE*, *norC* and *nosZ*. In *S. meliloti,* the expression of the genes required for nitrous oxide reductase activity has been previously characterized (96). These genes were shown to be expressed as three individual transcripts in the form of *nosR*, *nosZ* and *nosDFYL*, where *nosR* is the regulator and the remaining genes encode for the structural units of the enzyme (96). In that context, we observed that each of these individual transcripts were highly downregulated in the various orphan LuxR mutants (Fig. 3).

**Mutants of *SMc00878*, *SMc00877* and *SMc00658* are proficient at denitrification under microaerobic conditions.** The known factors that influence the transcription the denitrification genes vary highly between species (84, 139) Therefore conditions for denitrification need to be established in a strain-specific manner. In most strains of bacteria, denitrification is mainly induced in response to external signals such

**Figure 2. Genes of the denitrification pathway of *S. meliloti* affected by the orphan LuxR homologs.** A gene map of the pSymA plasmid depicting the denitrification genes (dark grey) of *S. meliloti*. M-values of Rm8530 vs. Rm8530 *658* **(A)** or vs. Rm8530 *878-877* **(B)** obtained from microarray analysis (see Materials and Methods) are indicated above the genes. Negative M-values indicate a downregulation of expression in the mutant strain compared to the wild-type strain. NC = no change. Unlabelled open reading frames are genes with unknown functions. Overlapping open reading frames are shown below the axis.

A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-7.1
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-2.9
-2.6
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nnrS
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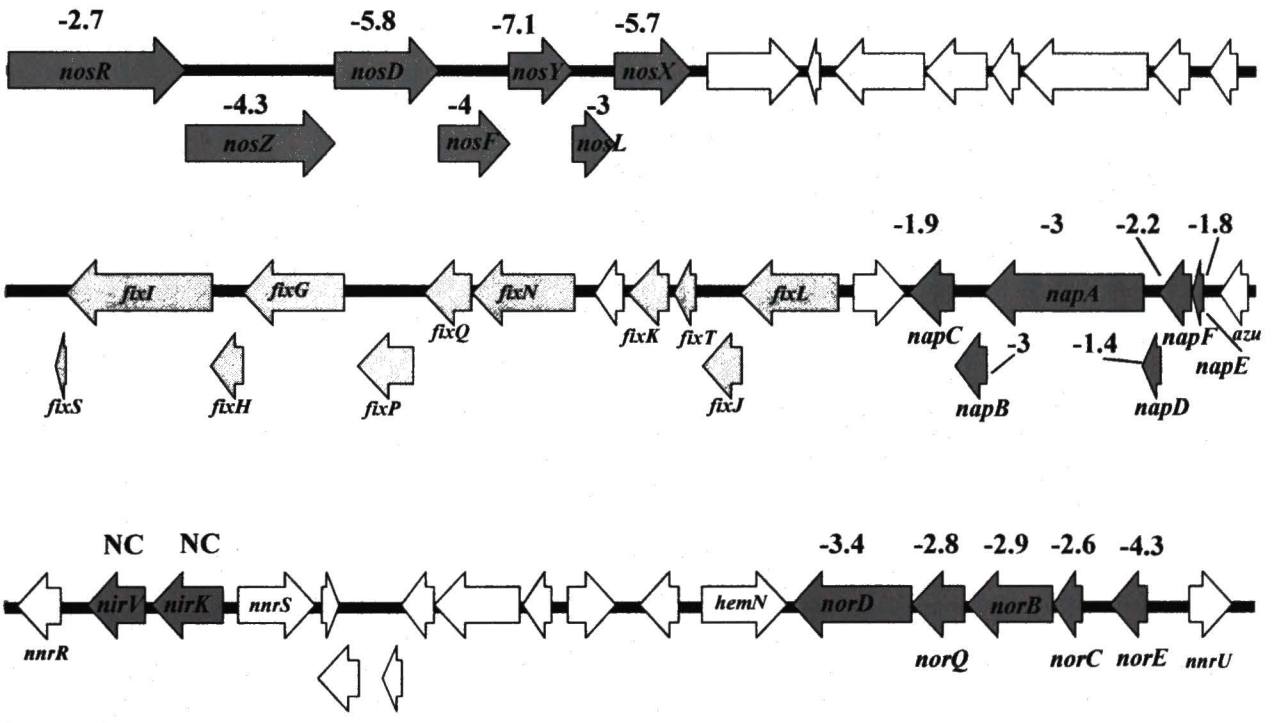
norD
norB
nnrR
norQ
norC
norE
nnrU

**B**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nosL
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-3
-2.2
-1.8
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fixG
fixN
napA
fixQ
fixK
fixT
fixL
napC
-3
-1.4
napF
azu
napE
fixS
fixH
fixP
fixJ
napB
napD
NC
NC
-3.4
-2.8
-2.9
-2.6
-4.3
nirV
nirK
nnrS
hemN
norD
norB
nnrR
norQ
norC
norE
nnrU

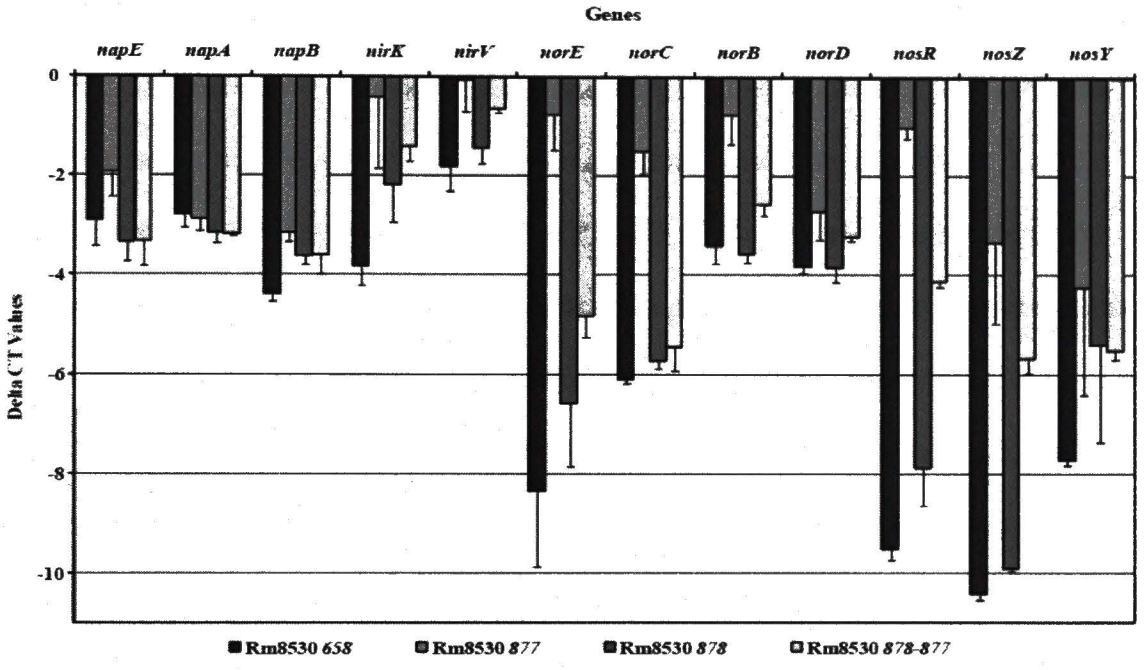



**Figure 3. Expression analysis of genes from the denitrification pathway.** Ct values (Fold change = $2^{\text{delta Ct}}$) were obtained by real-time PCR analyses where, genes of the denitrification pathway were downregulated in the mutants with respect to the wild-type.

as low oxygen tension, and/or the presence of appropriate substrates (nitrate or nitrite) (147, 211). In order to determine the effect of these factors on denitrification in *S. meliloti* Rm8530 we carried out growth assays under varying oxygen concentrations and in the presence of potassium nitrate ($KNO_3$) and sodium nitrite ($NaNO_2$) as substrates. Wild-type and orphan LuxR mutant strains were inoculated in TY media in anaerobic culture tubes, and were sealed with butyl stoppers (to prevent diffusion of air into the tubes). The media was filled in the anaerobic culture tubes to different levels in order to approximately achieve 4%, 2% and 0% oxygen conditions in the headspace. The TY media was supplemented with either 10 mM $KNO_3$ or 5 mM $NaNO_2$ as substrates for induction of denitrification. These concentrations have been previously shown to induce denitrification in some rhizobia (141, 150).

Denitrification activity was determined based on visual observation of gas production accompanied by growth (84). The results of the growth experiments (Table 5) showed that at 4% and 2% $O_2$ concentration, both, the wild-type and mutant strains induced denitrification within 64 hours in medium supplemented with 5 mM $NaNO_2$. In contrast, at these same levels of $O_2$ concentrations (i.e., 4% and 2%), 10 mM $KNO_3$ was unable to induce denitrification after 7 days of incubation (Table 5). Denitrification was not induced in *S. meliloti* strains even with increased amounts of $KNO_3$ [up to 50 mM KNO3] (data not shown). The control strain, *P. denitrificans* induced denitrification under all tested conditions (Table 5). Although the identity of the gas produced during growth was not established, it was deduced to be nitrogen gas from the reduction of nitrite by denitrification for the following reasons: a) *S. meliloti* does not produce gas

when grown aerobically b) the other gas intermediates of denitrification (i.e, NO and $N_2O$) are highly soluble and therefore do not accumulate in the headspace and finally, c) similar gas production was observed in microaerobic growth assays when the culture media was supplemented with $N_2O$ gas (data not shown).

Since denitrification is generally considered to be an anaerobic process, 0 % $O_2$ was used as one of the test conditions (211). All strains of *S. meliloti* tested did not denitrify under complete anaerobic conditions (0% $O_2$) (Table 5). Taken together these results indicate the following: 1) nitrate is unable to induce denitrification in *S. meliloti* Rm8530 2) Low oxygen tension in conjunction with nitrite can induce denitrification with the production of nitrogen gas in *S. meliloti* Rm8530, 3) complete anaerobic conditions do not support denitrification in *S. meliloti* Rm8530 and 4) the mutants of the orphan LuxR homologs are proficient at denitrification under microaerobic conditions, although transcription of the reductases was reduced under aerobic conditions. This indicates that the transcriptional control of these regulators is not dependent on reduced oxygen tension and substrate availability, and is potentially exerted mainly under fully aerobic conditions.

**The *nap* encoded nitrate reductase is non-functional in *S. meliloti* 8530.** The analyses of the wild-type and orphan LuxR mutants by growth patterns indicated that these strains were unable to utilize nitrate for denitrification activity. The rate of denitrification in rhizobia has been observed to differ in different strain backgrounds (77, 139). For instance, the denitrifying activity of *S. meliloti* is five to twelve times lower

**Table 5.** Assessment of denitrification in wild-type and orphan LuxR mutant strains.

| Bacterial strains | Concentration of $O_2$ in headspace | Denitrification* | |
|---|---|---|---|
| | | 10 mM $KNO_3$† | 5 mM $NaNO_2$§ |
| **Rm8530** | 4% | – | + |
| | 2% | – | + |
| | 0% | – | – |
| **Rm8530 *658*** | 4% | – | + |
| | 2% | – | + |
| | 0% | – | – |
| **Rm8530 *878*** | 4% | – | + |
| | 2% | – | + |
| | 0% | – | – |
| **Rm8530 *877*** | 4% | – | + |
| | 2% | – | + |
| | 0% | – | – |
| **Rm8530 *878-877*** | 4% | – | + |
| | 2% | – | + |
| | 0% | – | – |
| ***P. denitrificans*** (control) | 4% | + | + |
| | 2% | + | + |
| | 0% | + | + |

*Denitrifying activity indicted by growth accompanied with the production of gas.

†No growth observed for 7 days.

§Growth with gas production in 64 hours.

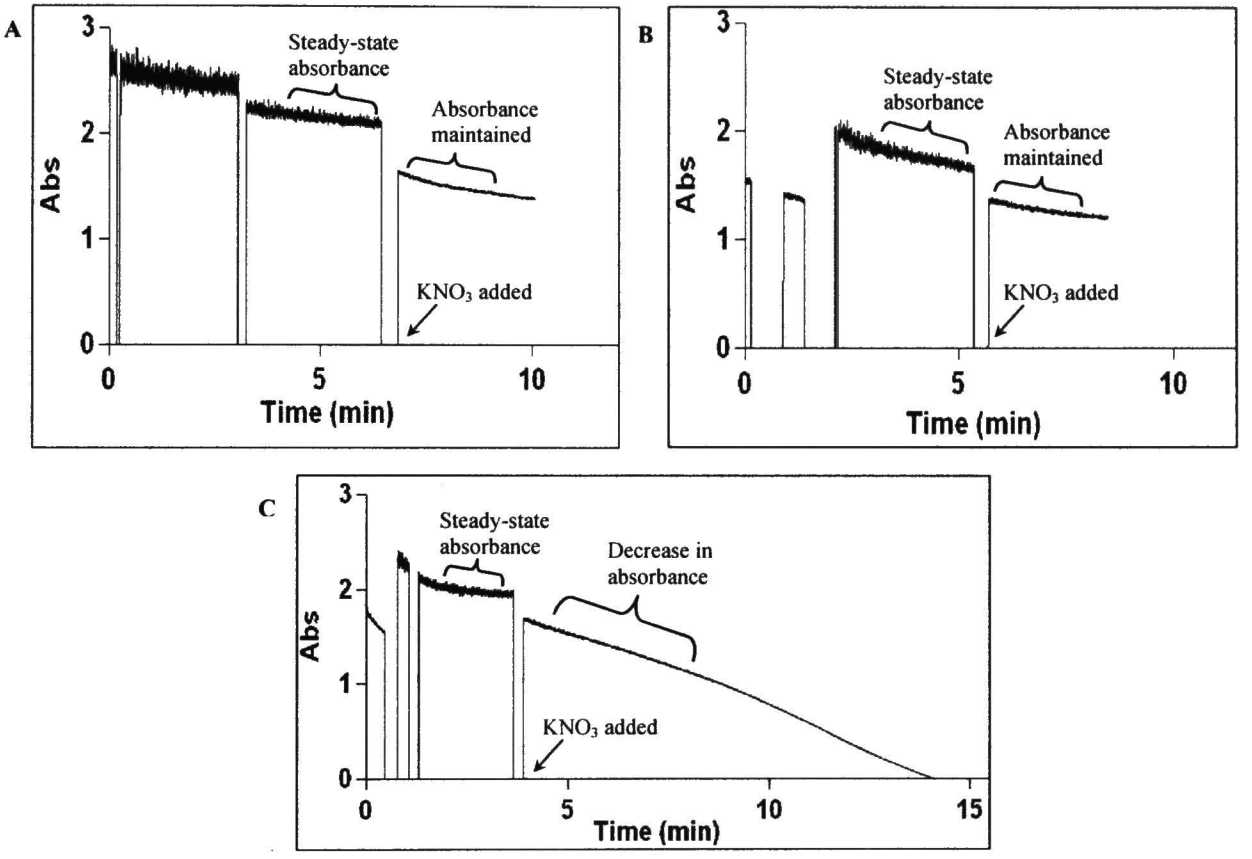
than that of *B. japonicum* which could explain the lack of, or slow growth of *S. meliloti* strains when using nitrate as the terminal electron acceptor (77). The reduction of nitrate is carried out by nitrate reductase and in *S. meliloti* the *nap* operon encodes for the structural components of periplasmic nitrate reductase (see discussion) (75, 211). Studies in several bacteria (including *S. meliloti*) have indicated that the expression of the *nap* operon is constitutive regardless of fully aerobic or microaerobic conditions of growth (77, 84, 211). The microarray and real time analysis conducted in this study corroborated this observation under aerobic conditions, where in the wild-type strain; the various genes of the *nap* operon were actively transcribed (Fig. 1, 2 and 3). Interestingly mutants of the orphan LuxR homologs exhibit a downregulation of expression of the *nap* operon under aerobic conditions (Fig. 3). Whether these transcripts were translated into functional nitrate reductase in *S. meliloti* was not known especially since phenotypically denitrification was not supported with nitrate as a substrate (Table 5). Therefore in order to measure nitrate reductase activity, we conducted enzymes assays utilizing methyl-viologen as the electron donor (see materials and methods). Figures 4A and 4B show nitrate reductase activity of the wild-type strain and the Rm8530 *658* strain as representative examples of the enzyme assay results. Upon introducing $KNO_3$ as the substrate for nitrate reductase, a decrease in absorbance was not observed with either the wild-type strain or with Rm8530 *658* indicating that these strains did not produce an active nitrate reductase enzyme.

Similar results were obtained with the other orphan LuxR mutants as well (data not shown). These results lead to the conclusion that in Rm8530 though the genes for

nitrate reductase are expressed they do not lead to production of a functional enzyme. This observation also reinforces the fact that Rm8530 is a partial denitrifier where the denitrification does not initiate from the reduction of nitrate but from the subsequent steps of the pathway.

**The orphan LuxR homologs have reduced nitrite reductase activity when grown under aerobic conditions.** Our genome wide and real-time PCR expression analysis revealed that the denitrification pathway was affected in the orphan LuxR homologs. The pathway can be initiated by the reduction of nitrate or nitrite. Form our growth and enzyme analysis we deduced that the wild-type and orphan LuxR mutant strains were unable to induce denitrification in the presence of nitrate but were proficient at inducing nitrite reductase activity under microaerobic conditions. In *S. meliloti*, the copper-containing *nirK* is predicted to encode for nitrite reductase and as this enzyme is located on the periplasm, its activity can be evaluated with methyl-viologen as an electron donor (see discussion) (17, 75). To test the enzyme activity, we analyzed the methyl-viologen-dependent nitrite reductase activity of whole cells in the wild-type or mutant strains grown aerobically in minimal medium. Five mM $NaNO_2$ was used as substrate in the assay and the resulting reduction of the substrate was followed spectrophotometrically as decrease in absorbance. On preliminary observation of the rate of decrease in absorbance it appeared that the wild-type strain had a higher nitrite reductase activity compared to that of the orphan LuxR mutants (Fig. 5). Representative rates of absorbance reduction for the wild-type strain and the Rm8530 *878-877* strain are shown in Figure 5. Similar results were obtained with the other orphan LuxR mutants as

**Figure 4. Nitrate reductase activity.** Enzyme activity was assayed for the wild-type strain Rm8530 (100 ul of culture) (A), and Rm8530 *658* (100 ul of culture) (B) with $KNO_3$ as the substrate and methyl-viologen as an electron donor. A reduction in the rate of absorbance was not observed with either strains indicating that they lacked functional nitrate reductase activity. Nitrate reductase activity of *P. denitrificans* (25 ul of culture) (C) was used as a control. Due to the high nitrate reductase activity of this strain, a one in four dilution of the culture was used for the enzyme assays.


A
Steady-state absorbance
Absorbance maintained
KNO3 added
Abs
Time (min)
B
Steady-state absorbance
Absorbance maintained
KNO3 added
Abs
Time (min)
C
Steady-state absorbance
Decrease in absorbance
KNO3 added
Abs
Time (min)

well. The rate of change in absorbance (delta absorbance/min) for each strain was used to calculate the specific activity of the enzyme. Nitrite reductase activity was expressed as μmol of methyl-viologen oxidized per minute and was normalized to one mg of protein. The nitrite reductase activities of various strains are shown in Table 6. It appears that nitrite reductase activity in the wild-type strain is around ten times higher than the various mutants. The nitrite reductase activity was not completely abolished in the mutants indicating that additional regulatory signals could control expression of nitrite reductase.

## Discussion

In this study, we report the identification and characterization of three novel transcription regulators of the denitrification pathway of *S. meliloti*. Based on genetic and biochemical analysis, it has been previously established that LuxR-type proteins of bacteria are composed of two functional domains: an amino-signal binding domain and a carboxy-DNA binding domain (72, 74). The deduced protein sequences of SMc00878, SMc00877 and SMc00658 have high similarity to the LuxR family of transcriptional regulators and are predicted to contain both the signature domain of this class of proteins (Table 4) (75, 114). In *S. meliloti*, two LuxR-type regulators (SinR and ExpR) have been previously characterized as modulators of quorum-sensing regulation (122, 149). Both of these regulators respond to the characteristic features of quorum sensing-regulation such as population density and the presence of AHL signal molecules. As, SMc00878, SMc00877 and SMc00658 are predicted to be LuxR-type proteins and were shown to

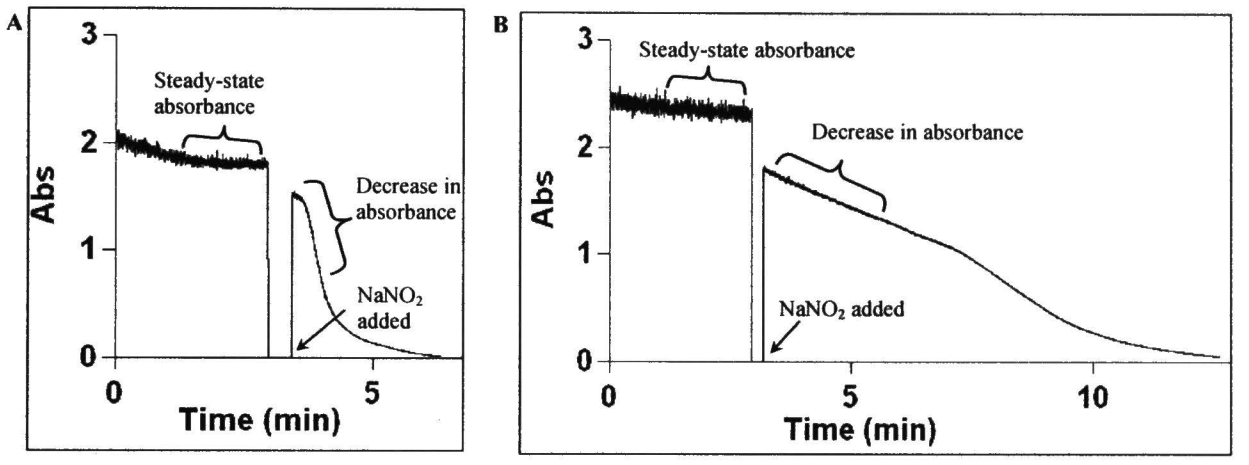



**Figure 5. Nitrite reductase activity.** Enzyme activity was assayed for the wild-type strain (A) and Rm8530 *878-877* (B) with $NaNO_2$ as the substrate and methyl-viologen as an electron donor. Both the wild-type and mutant strains showed a decrease in absorbance on addition of the substrate. A sharper reduction in the rate of absorbance was observed with the wild-type strain than the mutant either indicating that the wild-type had a higher enzymatic activity.

**Table 6. Specific activity of nitrite reductase enzyme of aerobically grown cells estimated by reduction of methyl-viologen.**

| Strain | Nitrite reductase activity |
|---|---|
| | μmol/min/mg of protein |
| **Rm8530** | 0.372 ± 0.0311 |
| **Rm8530** ***658*** | 0.0416 ± 0.0004 |
| **Rm8530** ***878*** | 0.03204 ± 0.0028 |
| **Rm8530** ***877*** | 0.04405 ± 0.0062 |
| **Rm8530** ***878 877*** | 0.07105 ± 0.0231 |

regulate the transcription of genes of the denitrification pathway we examined if population density or the presence of AHLs affected their regulatory role. We observed that the expression of genes from the denitrification pathway did not change at different stages of growth (early logarithmic and stationary) or in the absence of AHLs (*sinI* mutant) (data not shown) (N. Gurich, and J. González, manuscript in preparation). Taken together this indicates that, SMc00878, SMc00877 and SMc00658, while homologs of the LuxR family of proteins, do not rely on the distinctive properties of quorum sensing for controlling gene expression.

In order to gain further insight into the regulatory role of the orphan LuxRs, we conducted genome-wide microarray analyses and compared the expression profiles of the *SMc00658* mutant strain or the *SMc00878-SMc00877* double mutant strain to that of the wild-type strain. These transcriptome analyses indicated that genes from the denitrification pathway of *S. meliloti* were downregulated in the orphan LuxR mutant strains. The genome-wide analyses were supplemented with real-time PCR expression tests of a selected group of denitrification genes. Together, from these examinations it appears that the *SMc00658*, *SMc00878*, *SMc00877* or *SMc00878-SMc00877* loci control the expression of various genes from the denitrification pathway of *S. meliloti* (Fig 2 and Fig. 3).

Typically, in bacteria with high denitrification activities which serve as model systems for studying denitrification, (e.g., *Pseudomonas aeruginosa*, *Pseudomonas stutzeri* and *Paracoccus denitrificans*), the genes for the pathway are located on the chromosome (211). In contrast, the operons for various genes of the denitrification

pathway in *S. meliloti* are located on the pSymA plasmid (75). Rhizobia are considered to have evolved into nitrogen fixing symbionts by acquiring genes on the pSymA plasmid by horizontal gene transfer (155). Genes for several processes critical for establishment of symbiosis and nitrogen fixation (e.g. *nif, fix*,) are located on this episome (75). The location of the denitrification genes on the pSymA plasmid interspersed with the *fix* genes indicates that they too were potentially acquired by horizontal gene transfer (Fig. 2) (75). A high variation in the denitrification activities of *S. meliloti* strains, and their plasmid location points towards the fact that these strains probably acquired their denitrification genes from different sources (33, 84).

Several factors, including oxygen, nitrate, temperature, moisture and organic matter control rhizobial denitrification in the soil as well as under laboratory conditions though, a high strain-dependent variation to each of these responses is observed (78, 139). In several bacteria, denitrification is invoked in response to anaerobic conditions where the nitrogen oxides serve as alternate electron acceptors to generate energy (139). In this study, the predominant factors i.e., low oxygen tension and nitrate/nitrite substrate effect on denitrification in *S. meliloti* 8530 (wild-type) and mutants of the orphan LuxR homologs were analyzed. Denitrification was not induced in strains of *S. meliloti* under complete anaerobic conditions presumably due to the fact that *S. meliloti* Rm8530 is an aerobe that is unable to transition to respiration via denitrification under complete anaerobisis (75). This observation emphasizes the strain variation in denitrification activities of rhizobia. For example, in a study of denitrifying ability of thirteen *S. meliloti*

strains, only three strains failed to grow anaerobically using nitrate as the terminal electron acceptor (77).

In biological processes, nitrate is reduced by the activities of one of three different enzymes. The first is via the assimilatory nitrate reductase, where $NO_3^-$ is converted to $NH_4^+$ (84). The second is dissimilatory nitrate reductase which is further divided into respiratory nitrate reductase and periplasmic nitrate reductase (84, 211). Bacteria can encode for one or all of the above nitrate reductases (17, 155). The *S. meliloti* genome does not encode for the respiratory reductase while the periplasmic reductase is predicted to be located on the pSymA plasmid and is encoded by the putative *nap* operon (23, 75). The periplasmic nitrate reductase is typically expressed under aerobic conditions and its physiological role is detoxification by removal of excess reducing power and providing nitrite for aerobic denitrification (17, 211). The biochemical properties of reductases located in the periplasm can be assayed with the artificial electron donor methyl-viologen (17). In whole cells, reduced methyl-viologen does not cross the bacterial membrane but can donate electrons to enzymes located in the periplasm and therefore is specific for these reductases (17).

Transcription analysis of the *nap* operon in *S. meliloti* Rm8530 revealed that its genes are expressed in the wild-type strain and down regulated in the various orphan LuxR homolog mutants. Both wild-type and mutant strains did not exhibit denitrifying activity when nitrate was provided in growth assays. A similar observations was made in the *napA* gene analysis in *Pseudomonas* isolates where some strains posses and express *nap* genes but are incapable of aerobic denitrification (65). We conclude that *S. meliloti*

Rm8530 is a partial denitrifier, where the denitrification pathway is truncated. The denitrification pathway can be truncated due to one of two reasons: 1) when organisms are capable of catalyzing each reaction of the pathway but do not have access to appropriate substrates and 2) when organisms are genetically/biochemically incapable of carrying various steps of the pathway (99). The latter case applies to denitrification in *S. meliloti* Rm8530. Variation in the capacity to denitrify has been previously observed in *S. meliloti* strains. For example, in a denitrification study of 13 *S. meliloti* strains the bacteria could be divided into 3 groups based on their ability to produce nitrite, nitrous oxide or nitrogen gas as the end product (77). In a study of 57 field isolates of *S. meliloti*, rates and patterns of denitrification were highly variable and only one isolate was able denitrify nitrite but not nitrate (33).

The observation that expression of the denitrification pathway was affected in the orphan LuxR homologs and that nitrate reductase in *S. meliloti* Rm8530 is not active, led us to analyze the next step of the pathway, the nitrite reductase activity. Bacteria can have either the cytochrome $cd_1$-type or the copper containing nitrite reductase enzymes for the conversion of nitrite to NO. In *S. meliloti, nirK* is predicted to encode for the copper-containing periplasmic enzyme while, its downstream gene, *nirV* is a potential accessory gene required for nitrite reductase activity (Fig. 2) (34, 75). NO generated by nitrite reductase is further reduced to $N_2O$ by the membrane bound NO reductase encoded by the *nor* genes. The *nor* genes in *S. meliloti* are predicted to be *norECB* which encode for the enzyme subunits and the putative *norQD* which are required for the activity of NO reductase (Fig. 2). Both, *nir* and *nor* groups of genes are proposed to be

regulated by FNR-type regulators. The copper containing *nir* genes in most bacteria are separated from the *nor* genes by a few open-reading frames. In *Rhodobacter sphaeroides* these genes have been characterized to be the *nnrR* genes which encode for Fnr-type regulators and co-factor synthesis genes (*azu, hem*) (34). In *S. meliloti* a similar organization is found where *nir* and *nor* genes are separated by putative *nnr* and cofactor synthesis genes (Fig. 2) (34, 75). The order and organization of the *nir-nor* genes clusters in bacteria have a profound effect on the expression of these genes. For instance, in *P. denitrificans* inactivation of either *norQ* or *norD* simultaneously affects nitrite, NO and $N_2O$ reduction activities (47). Interestingly nitrite reductase proteins were present in these mutants (47). Similarly, in *P. stutzeri* a 10% reduction of nitrite reductase activity was observed in *nor* mutants (212). In *P. denitrificans*, mutation of *nir* genes led to a drastic reduction of NO reductase activity (46). These and several other examples of interdependence of nitrite and NO reduction point towards the tight co-regulation of these functional units of the denitrification pathway (211). In addition, since the *nir* and *nor* loci in most of these strains are independently transcribed (as is also the case in *S. meliloti*) the observed effects are proposed to be of an indirect nature (211). The rationale behind the co-regulation is believed to lie in the potent toxic effects of NO in bacterial cells (211). If a cell in incapable of reducing NO into $N_2O$ by the activity of the *nor* genes then the reaction which generates NO is also aborted to prevent toxicity. In our analysis, inactivation of the orphan LuxR homologs resulted in a substantial downregulation of various *nor* genes. A simultaneous reduction in the functional nitrite reductase activity was also observed even though the transcription of the *nir* genes was

not that severely repressed. It is possible that the observed reduction in the nitrite reductase activity is due to the reduced transcription of the *nor* genes which activate a form of indirect feed-back effect. Activity of the NO reductase activity can be monitored with a modified Clark-type electrode (185). Future experiments with the orphan LuxR homologs where the NO reductase activity is measured could provide critical insights into the functional dependence of nitrite and NO reductase in *S. meliloti.*

Regulators of the denitrification pathway in most bacteria lie in close proximity to the genes for the reductase enzymes; these include the gene *nosR, fixK, nnr.* In *S. meliloti* the activity of *nosR* and an *nnrR*-like genes have been experimentally demonstrated (Fig. 2) (48, 96). Regulatory genes are also known to be located outside of the denitrification loci and these typically include global transcriptional activators (211). The orphan LuxR homologs of *S. meliloti* are located on the chromosome as opposed to the pSymA-denitrification locus and could perhaps belong to such a group of regulators. Bacterial metabolism is maintained by balancing the oxidation-reduction reactions (redox state) in an organism (83). Consequently, each reducing reaction of the denitrification pathway is influenced by redox-sensors (83). Global regulation of denitrification, especially aerobic denitrification is suggested to be under the control of redox-sensing factors (211). They are proposed to convert cellular redox signals into regulatory outputs, typically at the level of transcription, subsequently enabling bacteria to adapt to the altered redox environment and prevent cellular toxicity (83, 211).

Several two-component global regulatory systems have been shown to regulate denitrification in bacteria. In *B. japonicum* the FixLJ system senses low oxygen tension

and initiates a regulatory cascade which leads to the activation of the denitrification pathway (6). The NarXL system controls denitrification in *E. coli* and *Pseudomonas* species, where NarL acts as the transcriptional activator of nitrate reductase genes (90, 178). Interestingly both FixJ and NarL belong to the LuxR superfamily of proteins which contain an amino sensory domain and a helix-turn-helix DNA binding domain (74). The orphan LuxR proteins of *S. meliloti* also belong to this family of regulators and their regulatory domains could perhaps play a role in activation of denitrification by similar mechanisms.

Our experimental growth-conditions for bacterial cultures were minimal-medium lacking supplementation with denitrification substrates (nitrate or nitrite) and a fully aerobic environment. Under these conditions the wild-type strain of *S. meliloti* exhibited constitutive expression of the denitrification genes indicating that unlike typical denitrifying strains there is no requirement for the presence of nitrogen oxides and low-oxygen tension to stimulate activity (147, 211). Analogous observations have been made in denitrification studies with other rhizobial species (84). It has also been suggested that the overall energy efficiency of denitrification is low in most rhizobial species, as they grow poorly under anaerobic conditions compared to other denitrifying bacteria (27). A similar observation was made in our studies where complete anaerobic conditions did not support growth or denitrification. The reduction of nitrate to nitrite by using nitrate reductase is the most energy conserving reaction of the entire denitrification pathway and this step is missing in *S. meliloti* Rm8530, suggesting that denitrification could have other functions in this strain (99). The regulatory effects of the orphan LuxR homologs were

observed under aerobic conditions and not under microaerobic conditions. They demonstrated an effect on the amount of functional nitrite reductase under aerobic conditions either through direct modulation of the *nir* genes or through an indirect effect via the *nor* genes. Moreover, the onset of aerobic denitrification has been reported to be dependent on the direct or indirect regulation of the reductases at the genetic level to balance the redox state in bacteria (83, 211). Taken together, the possible functions of the orphan LuxR homologs could lie in detoxification by removal of excess reducing power under aerobic conditions of growth and/or alleviation of nitric oxide toxicity in *S. meliloti* Rm8530.

**Acknowledgements**

We thank Bree Szoztek, an undergraduate student in our laboratory, for her invaluable assistance during the course of this study. We thank Dr. Stephen Spiro, for expert advice and assistance with the enzyme assays. The work in our laboratory is supported by the National Science Foundation grant MCB-9733532, and by the National Institutes for Health grant 1R01GM069925 to J.E.G.

# CHAPTER V

## CHARACTERIZATION OF NOVEL COMPONENTS OF QUORUM SENSING IN *Sinorhizobium meliloti*

Quorum sensing is a gene regulatory process that enables bacteria within a population to communicate via autoinducing signal molecules. The most common form of this signal is an *N*-acylhomoserine lactone (AHL) which is synthesized by an autoinducer synthase. As additional quorum sensing systems are identified, novel signals used for communication, come to light. The *S. meliloti* AHL profile indicates the presence of several long-chain (carbon $\geq$ 12) and short-chain (carbon $\leq$ 10) AHLs, of these, the long-chain AHLs have been identified by mass spectrometry (122). Disruption of the known quorum-sensing AHL synthase, *sinI*, abolishes production of only the long-chain AHLs. Therefore, this study focuses on the identification of the short-chain AHLs by mass spectrometry. Additionally, the *S. meliloti* genome contains several homologs of various novel signal molecule synthases. Mutants of these genes are analyzed for their putative role in the synthesis of AHLs.

### Introduction

Quorum sensing is a global regulatory mechanism utilized by bacteria to synchronize the behavior of a population. This process is mediated through small diffusible signal molecules that bind to specific regulators leading to the control of gene

expression in response to bacterial population density. The most common signal molecule in gram-negative bacteria is an *N*-acylhomoserine lactone (AHL), which consists of a hydrophilic homoserine lactone ring attached to a fatty acid side chain of variable length (167). The biosynthesis of AHLs can occur in one of three ways. It can be catalyzed by proteins from the LuxI family, and this mechanism is by far the most common (described in Chapter I) (60). In *Vibrio* species, AHLs can be synthesized by the LuxM/AinS/VanM-type synthases (13, 86, 87). Finally, in *Pseudomonas fluorescens* F113, AHLs can be produced by the activity of the HdtS protein (108). The HdtS protein sequence is not homologous to either the LuxI- or LuxM-type of AHL synthases, but is most closely related to the lysophosphatidic acid acyltransferase, a PlsC protein from *Escherichia coli* (108).

Though AHLs are the predominant signal molecules for quorum sensing, other forms of signaling molecules also exist. For instance, in *Pseudomonas aeruginosa*, in addition to the two types of AHLs that contribute to quorum-sensing regulon, a third signal molecule, 3,4-dihydroxy-2-heptylquinole (PQS), is an integral part of the quorum-sensing cascade (151). Cyclic dipeptides produced by *Pseudomonas* and *Vibrio* species activate AHL bioreporters and are considered signals that modulate quorum sensing by potentially cross-talking with AHL-based quorum-sensing systems (95, 143). In the plant pathogen *Ralstonia solanacearum*, 3-OH palmitic acid methyl ester (3-OH-PAME) produced by the *phcB* gene serves as a signal molecule in the regulation of virulence gene expression and is involved in the production of AHLs (66). *Xanthomonas* species do not produce AHLs but utilize diffusible fatty acid derivatives to control production of

virulence factors (156, 193). Therefore, it is evident that bacteria utilize a broad spectrum of novel molecules to communicate within their population.

*Sinorhizobium meliloti* is a gram-negative soil bacterium, that associates with the plant host *Medicago sativa* in a symbiotic relationship (22). As a part of this association, bacteria invade the root system of the plant to induce formation of structures called nodules. The bacteria within the nodules fix atmospheric nitrogen for the plant and in return are provided with readily utilizable carbon sources (22). Quorum sensing in *S. meliloti* has been extensively characterized and has been attributed mainly to the functions of the *sinI*, *sinR* and *expR* genes (92, 120, 122). SinI belongs to the LuxI family of proteins and, according to mass spectrometry analysis, produces several long-chain AHLs such as $C_{12}$-HSL, $C_{14}$-HSL, oxo-$C_{14}$-HSL, $C_{16:1}$-HSL, oxo$C_{16:1}$-HSL and $C_{18}$-HSL (120). SinR is the cognate response regulator of the Sin AHLs and serves to upregulate the expression of *sinI*. A second response regulator, ExpR, with no associated synthase on the genome, is an orphan LuxR regulator of *S. meliloti* (75). Together, the Sin/ExpR quorum sensing system regulates motililty, chemotaxis, and production of the symbiotically relevant exopolysaccharides EPS II and succinoglycan of *S. meliloti* (92, 94, 120). Moreover, ExpR binds upstream of the *sinI* promoter to activate the expression of *sinI* and enhance production of AHLs (12). Mutations in *expR* do not halt production of AHLs, but the absence of ExpR results in an overall reduced amount of AHLs (120).

AHLs are easily detected and characterized by several bioreporters (e.g., *Agrobacterium tumefaciens* NTL4 [pZLR4] or *Chromobacterium violaceum* CV026) and are structurally identified with a combination of high pressure liquid chromatography

purification, nuclear magnetic resonance and mass spectrometry (127, 170). Shaw *et al.* described a rapid method for determination of AHLs produced by an organism using a technique that combines thin layer chromatography (TLC) separation of bacterial extracts along with bioreporters for assessing the AHLs produced by a given organism (170). Based on the migration pattern and on comparison with synthetic standards via TLC, AHLs can be preliminarily characterized (170). The *S. meliloti* AHL profile indicated the presence of several long-chain (carbon $\geq$ 12) and short-chain (carbon $\leq$ 10) AHLs. Disruption of *sinR/I* genes abolished production of only long-chain AHLs (120). Therefore, in this study I aim to identify the short-chain AHLs by mass spectrometry. Interestingly, apart from *sinI* (the *luxI*-type synthase), the *S. meliloti* genome encodes for additional homologs of quorum-sensing signal producers including *hdtS* and *phcB*. Therefore, mutants of these homologs in *S. meliloti* will be analyzed for their contribution to the production of short-chain AHLs.

## Materials and Methods

**Bacterial strains and media conditions.** The strains used in this study are summarized in Table 1. The mutant of the *phcB* homolog of *S. meliloti* (*SMc03143*) was obtained from Anke Becker at the University of Bielefeld, where it was created by plasmid integration mutagenesis of Rm1021. *S. meliloti* strains were grown at 30°C in Luria-Bertani (LB) medium supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ or in minimal (M9) medium [11 g $Na_2HPO_4$, 3 g $KH_2PO_4$, 0.5 g NaCl, 1 g $NH_4Cl$, 2 g glucose, 1 mg biotin, 27.8 mg $CaCl_2$ and 246 mg $MgSO_4$ per one liter). *A. tumefaciens*

**Table 1. Bacterial strains and plasmids.**

| Strains and Plasmids | Relevant Characteristics | Reference or Source |
|---|---|---|
| *S. meliloti* | | |
| Rm8530 | Su47 *str-21*, *expR*$^+$ | (149) |
| Rm1021 | Su47 *str-21*, *expR*$^-$ | (111) |
| Rm1021 *sinI* | *sinI*::Km | (122) |
| Rm1021 *hdtS* | Δ*SMc00714* | This work |
| Rm1021 *phcB* | *SMc03143*::pK19mob2ΩHMB | Anke Becker |
| *A. tumefaciens* NTL4 (pZLR4) | *traG::lacZ* reporter fusion, *traI*,$^-$ Gm | (170) |
| Plasmids | | |
| pUnk | *unk* entry vector | This work, |
| pChaC | *chaC* entry vector | This work |
| pD1Unk | *unk* deletion vector | This work |
| pD2ChaC | *chaC* deletion vector | This work |

NTL4 (pZLR4) was grown at 30° C in LB medium or in minimal glutamate media (MGM) (11 g $Na_2HPO_4$, 3 g $KH_2PO_4$, 0.5 g NaCl, 1 g glutamate, 10 g mannitol, 1 mg/ml biotin, 27.8 mg $CaCl_2$ and 246 mg $MgSO_4$). IPTG was used at a concentration of 20 μg/ml, and X-gal was used at a concentration of 80 μg/ml. Antibiotics were used in the following concentrations: streptomycin (Sm) 500 μg/ml, chloramphenicol (Cm) 25 μg/ml, gentamycin (Gm) 20 μg/ml, kanamycin (Km) 75 μg/ml, rifampin (Rif) 100 μg/ml, spectinomycin (Sp) 100 μg/ml, tetracycline (Tet) 10 μg/ml and neomycin (Nm) 200 μg/ml.

**Disruption of the *hdtS* homolog of *S. meliloti*.** An in-frame deletion in the *hdtS* homolog of *S. meliloti* (*SMc00714*) was created by the method described by House *et al.* (97). Briefly, in this method, a single set of primers are used to generate a PCR product that is cloned into an initial entry vector by using lambda integrase-mediated recombination (Gateway Technology). Amplified ORFs flanked by bacteriophage lambda *attB* (bacterial attachment) sites are generated by a nested PCR protocol and cloned into an entry plasmid that contains the corresponding *attP* (phage attachment) sequences (*attB-attP* reaction). The result is a plasmid with two *attL* sites flanking the ORF of interest. The entry ORF sequences are then shuttled by a novel pentaparental mating procedure to destination vectors by lambda integrase-mediated technology. The destination vectors pMK2016 (pD1) and pMK2017 (pD2) are suicide vectors that possess an origin of replication unable to function in *S. meliloti* and contain strategically placed Flp recombinase target (FRT) sequences that bracket ORFs. Triparental matings are used to integrate each suicide vector sequentially into the *S. meliloti* genome

**Table 2. Primers used to generate entry vectors.**

| Primer Name | Sequence (5' to 3') |
|---|---|
| Pri-Unk-F | AAAAAGCAGGCTCGCCCGGAAGGCCATGAATT |
| Pri-Unk-R | AGAAAGCTGGGTCCTACTCCCCGGCCGGCGCA |
| Pri-ChaC-F | AAAAAGCAGGCTCTAGGGCCCGGAGCGACTCA |
| Pri-ChaC-R | AGAAAGCTGGGTGCTCTTGCCGCCGGGAGCGG |
| Secondary - F | GGGGACAAGTTTGTACAAAAAAGCAGGCT |
| Secondary -R | GGGGACCACTTTGTACAAGAAAGCTGGGT |

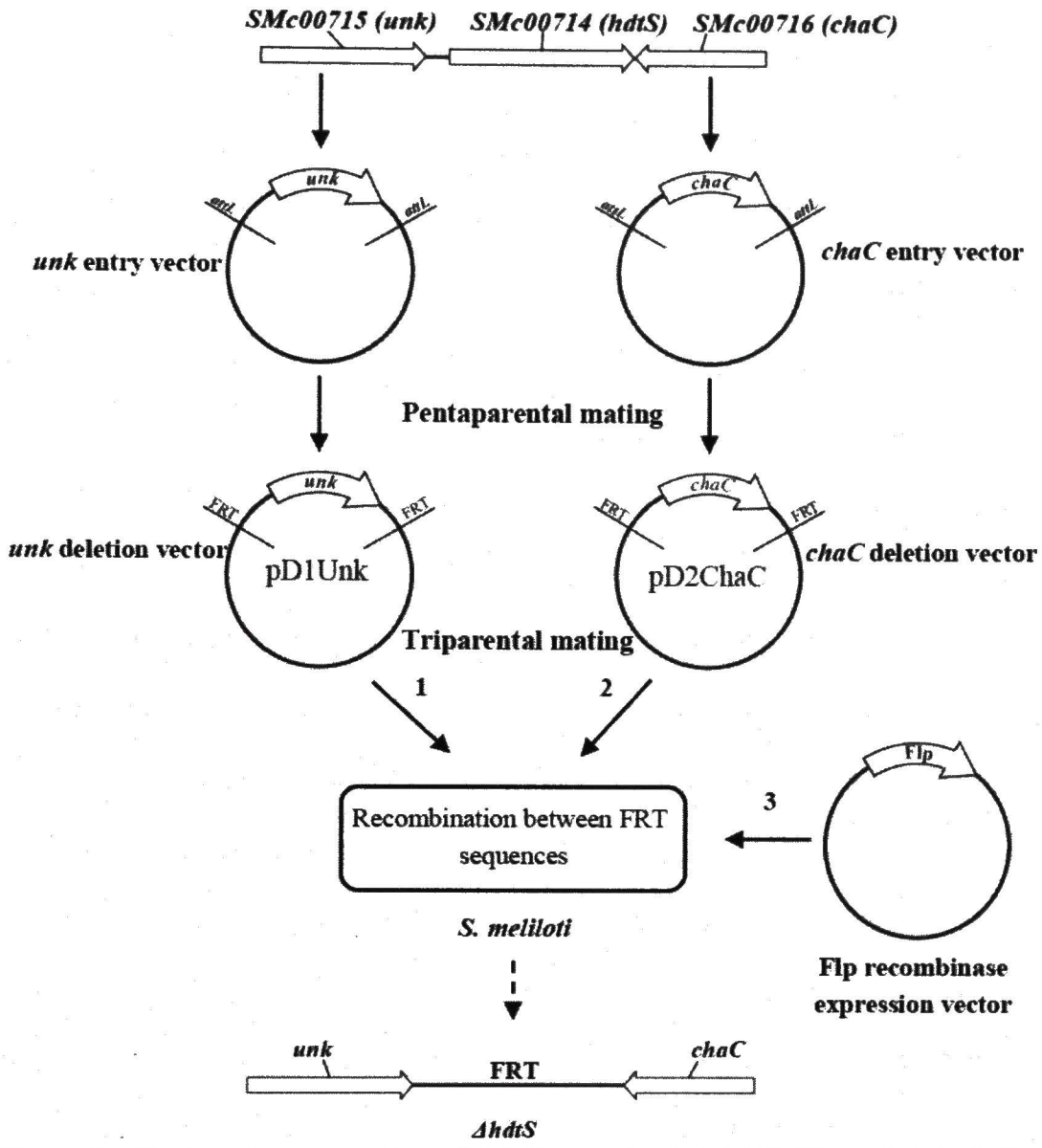



**Figure 1. Strategy for generation of deletion mutant of *hdtS*.** The *unk* and *chaC* open reading frames (ORF) were cloned into the entry vectors by lambda integrase-mediated recombination. The ORFs were shuttled into the deletion vectors via pentaparental matings. Consecutive mating of *S. meliloti*, with the deletion vectors and Flp recombinase encoding vector creates a strain in which recombination events between the flanking FRT sequences lead to the deletion of the intervening gene (i.e. *hdtS*) (97).

by gene-specific homologous recombination (97). Finally, a plasmid encoding the production of the Flp recombinase enzyme is introduced into the double integrants. Deletion mutants are then generated through site-specific recombination reactions between direct repeats of the integrated FRT sequences.

In the *S. meliloti* genome, the *hdtS* homolog (*SMc00714*) is flanked by *SMc00715*, a gene of unknown function (*unk*), and *SMc00716*, a cation transport gene (*chaC*) (Fig. 1). The primers listed in Table 2 were used to generate entry vectors; pUnk and pChaC, respectively (97). These vectors were then shuttled via pentaparental mating to generate destination vectors pD1Unk and pD2ChaC respectively (Fig. 1). Successive triparental mating with *S. meliloti* and introduction of the Flp recombinase enzyme-encoding plasmid yielded a strain with a deletion of the *SMc00714* ORF (Fig. 1).

**AHL extraction and TLC analysis of AHL production.** Saturated cultures of *S. meliloti* were used as starter inoculums (1:1000) in 25 ml of LB/MC medium with the appropriate antibiotics and were grown to an $OD_{600}$ of 2. Five ml of cultures were acidified to pH< 4 with 5 M HCl and were extracted twice with equal amounts of the organic solvent dichloromethane (DCM) as previously described (170). The AHL-containing DCM extracts were dried in a Speed-Vac (Labconco) and resuspended in 20 ul of 70% methanol and either half or whole extract was used to spot a Whatman $LKC_{18}$ analytical TLC plate and allowed to dry. The samples were then chromatographed in a 70% methanol-containing chamber and tested by overlaying with a reporter strain. The overlay was prepared by mixing a 4-6 hour culture of *A. tumefaciens* NTL4 (pZLR4)

(grown in MGM) with an equal volume of sterile MGM and 1% agar to which X-gal was added (121).

**Sample preparation for mass spectrometry analysis.** Rm1021 *sinI* was grown in 1 liter of LB, and Rm8530 was grown in 1.8 liters of M9. On reaching $OD_{600} = 2$, the culture medium was reduced to pH<4 with 5 M HCl. Whole cell cultures were then extracted twice with equal volumes of dichloromethane as previously described by Marketon *et al.* (121). To the pooled dichloromethane extract, $Na_2SO_4$ (1/20 the volume of the extract) was added to remove any aqueous residue. After an hour of incubation with $Na_2SO_4$, the extract was decanted into a fresh flask. The extract was then dried by rotary evaporation and resuspended in 2 ml of dichloromethane and centrifuged for 1 min at 12,000 rpm to remove any precipitate. Next, an SPE-$NH_2$ column (500 mg/3 ml) was conditioned with 2 fillings each of hexanes followed by ethyl acetate. The 2 ml dichloromethane extract was loaded onto the column, and the eluate was immediately collected. The column was further washed twice with 10 ml of ethyl acetate:ethanol (2:1). The consolidated eluate was dried completely by rotary evaporation. The extract from each strain was resuspended in dichloromethane and a small portion was tested on a TLC plate, as above, for activity with an *A. tumefaciens* NTL4 (pZLR4) reporter strain. The rest of the concentrated extract was analyzed by mass spectrometry by our collaborator, Dr. Anatol Eberhard, at Cornell University.

## Results and Discussion

**Contributions of the novel signal molecule producers to quorum sensing in *S. meliloti*.** In the absence of Sin AHLs, the TLC profile of Rm1021*sinI* showed spots that activated the *A. tumefaciens* NTL4 (pZLR4) bioreporter. The active compounds comigrated with $C_8$-HSL and $C_6$-HSL standards on the TLC plate (Fig. 2B and 2C). These additional spots could have been contributed by an additional synthase in *S. meliloti*. A bacterium can produce AHLs by novel non-LuxI type proteins such as the LuxM/AinS/VanM-type synthases or the HdtS-type synthase but the Rm1021 genome sequence indicates that *S. meliloti* codes for only one LuxI-type synthase (*sinI*) (13, 60, 75, 86, 87, 108). Although, *S. meliloti* does not encode for any LuxM/AinS/VanM-type synthase, it does possess a single open reading frame with 29% identity to an *hdtS* gene from *P. fluorescence* F113. This gene (i.e., *SMc00714*) could potentially be the synthase of the short-chain AHLs.

In *R. solanacearum,* the *phcB* gene catalyzes the production of a volatile signal molecule called 3-hydroxypalmitic acid methyl ester (3OH-PAME) (66). This molecule acts as a quorum-sensing signal since it accumulates in a population-density manner and controls expression of genes through a cascade of response regulators. PhcB was shown to control virulence-inducing exopolysaccarides and production of AHLs in *R. solanacearum* (66, 67). A Blastp analysis of the *phcB* from *R. solanacearum* against the *S. meliloti* genome picks out one open reading frame encoding for a hypothetical protein (*SMc03143*) with 25% amino acid identity, and suggests that it could also be involved in the production of AHLs (75).

Consequently, mutants of *SMc00714* and *SMc03143* were analyzed for the production of short-chain AHLs by TLC (Fig. 3.). The mutants were still proficient in the production of AHLs indicating that the short-chain AHLs were perhaps produced by an as-yet unidentified novel signal producer.

**Identification of the putative short-chain AHLs produced by *S. meliloti*.** The AHL profile (by TLC) of *S. meliloti* shows the presence of several long-chain and short-chain AHLs (Fig. 2). Long-chain AHLs produced by Rm1021 (when grown in LB medium) have been previously identified by mass spectrometry (122). They include $C_{12}$-HSL, $C_{14}$-HSL, 3O-$C_{14}$-HSL, $C_{16:1}$-HSL, 3O-$C_{16:1}$-HSL and $C_{18}$-HSL and one short - chain AHL, $C_8$-HSL (122). Interestingly, the TLC profile of Rm1021 shows several short-chain (shorter than $C_8$-HSL) AHLs, which activate reporter strains (Fig. 3). However, the structures of the putative AHLs cannot be estimated by chromatography alone and mass spectrometry of the AHLs is required. These short-chain AHLs had not yet been identified by mass spectrometry in *S. meliloti*. In order to identify the short-chain AHLs, initially I extracted a 1 liter culture of Rm1021 *sinI* grown in LB media. A strain lacking the *sinI* gene was used to eliminate the possibility of the already characterized long-chain AHLs. On examination of the extract by mass spectrometry, I did not detect any short-chain AHLs. Instead, I encountered several cyclic-dipeptide compounds called diketopiperazines (DKPs) (Table 4). There could be several reasons why the structures of the short-chain AHLs were not identified. First, the bacterial culture used for the structural analysis was grown in rich LB medium. Heat sterilization of rich

**Table 3. Homologs of quorum-sensing signal producers in *S. meliloti*.** Blastp results from the *S. meliloti* genome when HdtS of *P. fluorescens* F113 (A) or PhcB of *R. solanacearum* (B) were used as query sequences (66, 75, 108).

**A.**

| Strain | Gene | Annotation | % identity |
|---|---|---|---|
| *P. fluorescens* F113 | *hdtS* | *N*-acylhomoserine lactone synthase | 29% |
| *S. meliloti* | *SMc00714* | 1-acyl-glycerol-3-phosphate acyltransferase (PlsC) | |

**B.**

| Strain | Gene | Annotation | % identity |
|---|---|---|---|
| *R. solanacearum* | *phcB* | Regulatory protein | 25% |
| *S. meliloti* | *SMc03143* | Hypothetical protein | |

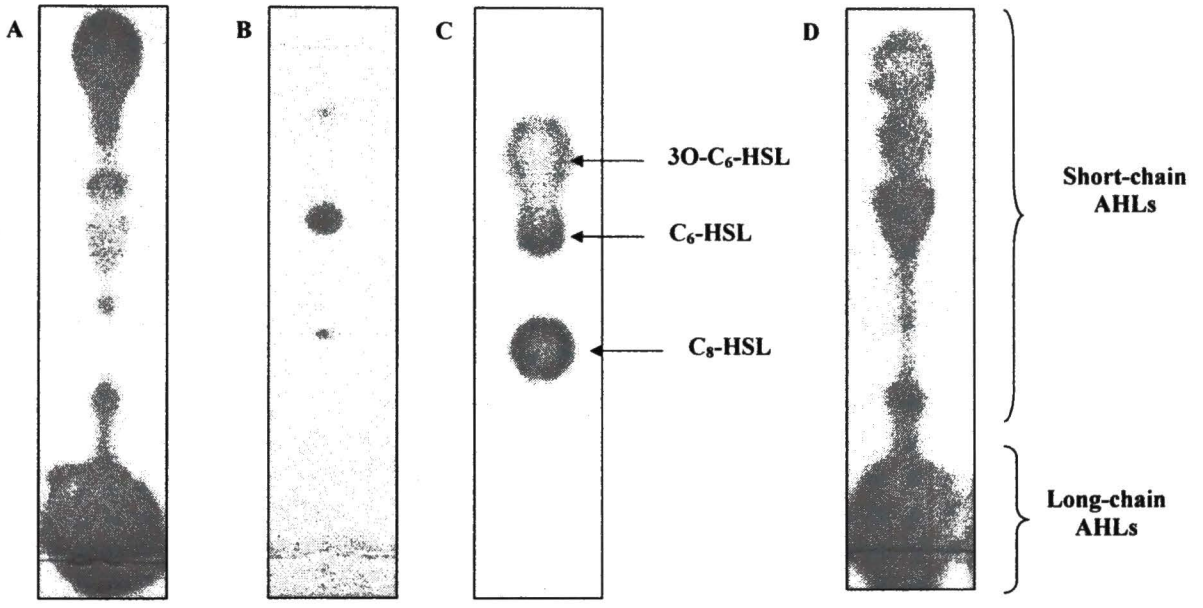



**Figure 2. TLC profile of *S. meliloti*** AHLs were extracted from whole cell lysates, spotted on $C_{18}$-reverse phase TLC plate, and overlayed with *Agrobacterium tumefaciens* NTL4 (pZLR4) reporter strain. **A**- Rm1021 (5 ml culture extract), **B**- Rm1021*sinI* (5 ml culture extract), **C**- Synthetic standards ($C_8$-HSL=31.6 pmol, $C_6$-HSL= 804 pmol and 3O-$C_6$-HSL=4.7 pmol), **D**= Rm8530 (2.5 ml culture extract)

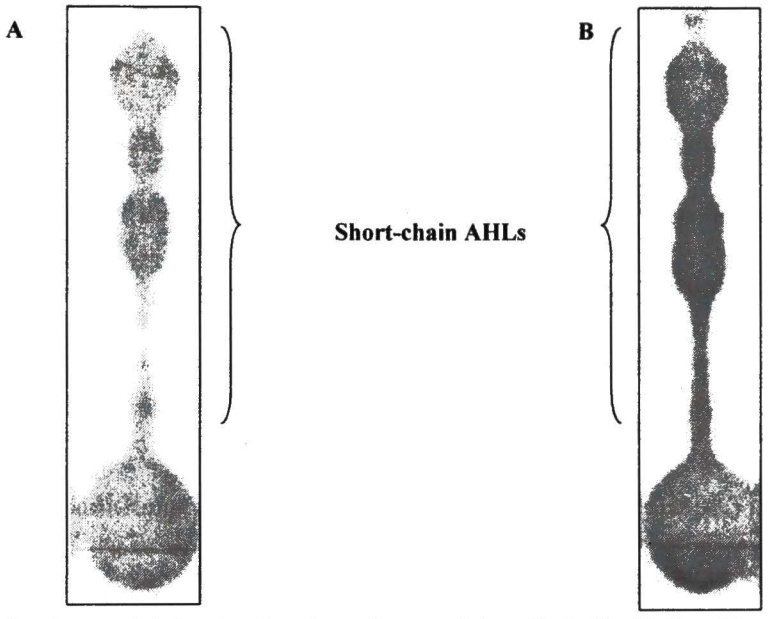



**Figure 3. TLC profile of potential signal molecule producers of *S. meliloti*.** The TLC profiles of mutants of *SMc00714* **(A)** and *SMc03143* **(B)** revealed they are capable of producing short chain AHLs. This indicates that synthesis of short chain AHLs is not dependent on these genes.

media are known to produce DKPs that have similar mobilities as short-chain AHLs and therefore mask or interfere with the peaks of short-chain AHLs in a GC/MS [(95) and personal communication A. Eberhard]. Second, the amount of AHLs could be less than the minimum required for efficient detection. The AHL production pattern of Rm1021 on TLC shows that the amount of long-chain AHLs produced is greater than that of the short-chain products (Fig. 3). To overcome the above mentioned problems associated with *S. meliloti* short-chain AHLs' structural identification; I first analyzed the production of AHLs in a minimal M9 medium to eliminate the interference, if any, by DKPs since minimal media does not contribute towards DKP production. I also observed that Rm8530, another strain that is frequently used to study quorum sensing has a TLC profile similar to Rm1021 but the overall amounts of AHLs (on the TLC) observed are relatively higher (Fig. 3). This increase in the amount of AHLs is perhaps due the presence of an additional quorum-sensing response regulator (ExpR) in Rm8530. ExpR has been previously shown to bind upstream of *sinI* and upregulates its expression, thereby increasing the production of AHLs (12). Subsequently, mass spectrometry analysis of extracts of Rm8530 grown in M9 media identified several long-chain AHLs (3O-$C_{14}$-HSL, $C_{16}$-HSL, $C_{16:1}$-HSL and 3O-$C_{16:1}$-HSL) as expected, but oddly no short-chain AHLs were detected (Table 4). Instead, several DKPs were identified, some of which have been previously reported as quorum-sensing signal molecules (Table 5). Holden *et al.* have reported that, DKPs purified from bacteria grown in minimal M9 media containing a single carbon source and no preformed amino acids are either

**Table 4. Results of mass spectrometry analysis.**

| Strain | Rm1021*sinI* | Rm8530 |
|---|---|---|
| Media | LB | M9 |
| Long-chain AHLs | – | 3O-$C_{14}$-HSL, $C_{16}$-HSL,$C_{16:1}$-HSL, 3O-$C_{16:1}$-HSL |
| Short-chain AHLs | – | – |
| Cyclic-dipeptides | cPF, cPL, cPI* | cPF, cPV, cPL, cPI, cPA, cPY* |

* cPF- cyclo (Pro-Phe), cPL- cyclo (Pro-Leu), cPI- cyclo (Pro-Ile), cPV- cyclo (Pro-Val), cPA- cyclo (Pro-Ala), cPY- cyclo (Pro-Tyr).

**Table 5. Characterization of DKPs in other bacteria.**

| DKP | Producing organism | Properties | Ref. |
|---|---|---|---|
| cyclo(Ala-Val), cyclo(Pro-Tyr) | *P. aeruginosa* | Activate bioreporter, compete with AHLs for binding regulators | (95) |
| cyclo(Pro-Phe) | *P. fluorescens*, *P. alcaligenes* | Activate bioreporter, compete with AHLs for binding regulators | (95) |
| cyclo(Pro-Leu), cyclo(Pro-Phe), cyclo(Pro-Tyr), cyclo(Val-Leu) | *P. putida* WCS358 | Activate bioreporter | (49) |
| cyclo(Pro-Phe) | *Vibrio* species | Pathogenicity | (143) |

synthesized de novo from the production of primary metabolism or generated via the metabolism of bacterial peptides (95).

DKPs are endogenous to different members of the animal and plant kingdom (157). They appear to be by-products of larger proteins and can have antifungal, antbacterial and antitumour activity (157). There are a few reports about the influence of DKPs on quorum-sensing phenotypes ranging from swarming motility of *Serratia liquefaciens* to modulation of pathogenicity of *Vibrio* species (49, 95, 143). Here, the DKPs accumulate in a population density dependent manner and activate quorum-sensing reporter strains (Table 5). Interestingly, DKPs also co-migrate with short-chain AHLs on a TLC plate and have also been shown to compete for AHL-binding sites on response regulator (LuxR-type) proteins (Table 5) (95). In *S. meliloti*, I did not detect short-chain AHLs by mass spectometery of purified AHL extracts even though these same extracts when separated by TLC, indicated the presence of *A. tumefaciens* NTL4 (pZLR4)-activating compounds. Moreover, I detected several DKPs even in minimal media; therefore, I conclude that the compounds co-migrating with synthetic short-chain AHLs on the TLC are indeed DKPs. Whether DKPs act as modulators of quorum sensing or whether their activity results from the cross-talk between bacterial signaling systems remains unclear.

## Conclusions

Quorum sensing in *S. meliloti* better adapts the bacteria to survival in the soil and to form a symbiotic association with its plant host (81, 120). The quorum-sensing circuit

of *S. meliloti* is quite unique and complex due to several factors. Essentially three genes, *sinR, sinI* and *expR* as opposed to the typical two genes (as in other quorum sensing systems), control the entire gamut of population density dependent regulation in *S. meliloti* (92, 120, 197). Additionally, the AHLs produced by the Sin system are some of the longest known in any organism (122). These distinctive properties of quorum sensing in *S. meliloti* indicate that it has the potential to recruit novel genes to expand its regulatory network. In addition, recruiting signal molecules like DKPs which are by products of existing metabolic processes could help in easing the cost associated with making newer signal molecules. Together these novel genes and/or signal molecules could serve to modulate the existing AHL-dependent quorum sensing of *S. meliloti* and perhaps provide an edge to occupy its ecological niche.

**Acknowledgments**

We thank Dr. Anatol Eberhard, at Cornell University for the mass spectrometry analysis. The work in our laboratory is supported by the National Science Foundation grant MCB-9733532, and by the National Institutes for Health grant 1R01GM069925 to J.E.G.

# CHAPTER VI

## SUMMARY

Bacteria that inhabit complex niches such as the soil or water are subjected to constant changes in the environment. In order to survive, bacteria respond to these external cues by appropriately regulating their gene expression patterns. Quorum sensing is one of the many processes that bacteria employ to coordinate this regulatory response. The mechanism of action of the typical quorum-sensing system includes the production and accumulation of signal molecules in response to population density. Specific response regulators, when bound to these signals, modulate transcription. Most of the quorum sensing response regulators in gram-negative bacteria are members of the LuxR family of proteins that are characterized by two functional domains, an N-terminal signal binding domain and the C-terminal DNA binding domain (74). Two varieties of these response regulators exist in quorum sensing systems; those that are associated with a cognate signal producer on their genomes and those that are not (68). The latter are referred to as orphan LuxR regulators and Chapter I of this thesis provides a comprehensive review of the various orphan LuxR homologs in gram-negative bacteria and their contributions to the regulatory network in those bacteria (68). I have discussed specific examples of how the orphan LuxR regulators not only expand the existing regulatory circuits in the bacteria that carry them but also enhance the ability of those bacteria to occupy their respective environmental niches.

Chapter II discusses the intricate signaling employed by *S. meliloti* to interact with its symbiotic plant host, alfalfa (*Medicago sativa*). *S. meliloti* is an ideal candidate to study host-bacterial interactions as it leads a dual lifestyle, one as a free-living soil bacterium, and another as a symbiont of an agriculturally important plant. *S. meliloti* is also a model organism for studying bacterial quorum sensing. It is well established that quorum sensing plays a critical role in establishing symbiosis and coordination of gene regulation in *S. meliloti* (92, 122). Chapter II also discuses the known molecular mechanisms of quorum sensing in *S. meliloti*. SinR/I are the cognate response regulator and signal producer respectively while, ExpR is an orphan LuxR homolog of *S. meliloti* (122, 149). Interestingly, the *S. meliloti* genome contains additional LuxR-type genes, which encode for orphan LuxR homologs and these include the open reading frames of *SMc04032*, *SMc00658*, *SMc00878,* and *SMc00877* (75). In order to characterize these response regulators, mutations in their respective open reading frames were created and genome-wide microarray analysis were employed to determine their regulon. For the microarray analysis, I employed the high-throughput Affymetrix GeneChip hybridization method. I compared the expression profile of the wild-type strain to that of the mutant when grown under conditions that cause the maximal quorum-sensing induced gene expression. The microarray data was analyzed to determine the regulation of genes with similar functions or genes belonging to a single metabolic pathway. The differential expression of these genes was further corroborated by real-time PCR expression analysis.

In Chapter III, I describe the regulatory role of the orphan LuxR homolog *SMc04032* which, we named *nesR* as it plays a role in the adaptation to nutritional,

environmental and stress conditions. I showed that a mutation in the *nesR* locus of *S. meliloti*, leads to a decrease in expression of the genes from the active methyl cycle, which in turn affects the mutant's nutrition and stress response abilities and its capacity to effectively compete with the wild-type strain for plant nodulation. These observations have profound implications on both the free-living and the symbiotic form of *S. meliloti*. In the soil and the nodule environment, *S. meliloti* is subjected to a range of challenges including, high osmolarity, nutrient starvation and/or competition for nodulation. NesR potentially contributes towards the overall ability of the bacteria to survive when faced with these and other stresses.

In Chapter IV, I describe the identification of the regulatory role of the other three orphan LuxR homologs of *S. meliloti*. Utilizing genome-wide microarray and real-time PCR expression analysis, the *SMc00658*, *SMc00878,* and *SMc00877* loci were shown to control the denitrification pathway of *S. meliloti*. Denitrification is the reduction of nitrate or nitrite substrate to generate nitrogen gas. The pathway is used by bacteria either as an alternative form of respiration during microaerobic growth or as a detoxification mechanism for the removal of excess reducing power during aerobic growth. I show by real-time PCR analysis that the expression of genes from the denitrificaton pathway of *S. meliloti* are highly downregulated when the orphan LuxR homologs are mutated. Additionally, the role of the orphan LuxR homologs in the denitrification pathway was confirmed phenotypically by enzyme assays using methyl-viologen as an artificial donor for nitrite and nitrate reductase activity. I showed that under aerobic conditions, the amount of functional nitrite reductase enzyme produced by

the mutants was decreased compared to that of the wild-type. I also showed that, although *S. meliloti* Rm8530 is predicted to encode for the periplasmic nitrate reductase, physiologically this enzyme is non-functional in this strain as is evident by the lack of nitrate reductase activity and the absence of growth when grown microaerobically with nitrate.

In Chapter V, I explored the potential of additional genes or factors in the quorum sensing network of *S. meliloti*. AHL profiles are routinely characterized by resolving culture-extracts by Thin Layer Chromatography (TLC). The TLC profile of *S. meliloti* culture-extracts contains compounds that comigrate with synthetic long-chain and short-chain AHLs. Production of long-chain AHLs in *S. meliloti*, is attributed to the *luxI*-type synthase, *sinI*, but, the production of short-chain AHLs is not affected in a *sinI* mutant (122). Since the genome did not reveal the presence of additional *luxI*-type genes novel AHL producing genes from other related bacteria were sought to be studied (75). The *hdtS* gene of *Pseudomonas fluorescens* F113 and *phcB* gene of *Ralstonia solanacearum* are involved in the production of the AHLs in their respective quorum-sensing systems (66, 108). I analyzed mutants of the *hdtS* (*SMc00714*) and *phcB* (*SMc03143*) homologs in *S. meliloti* for production of AHLs and found that the mutants were still proficient in the production of AHLs indicating that the short-chain AHLs were perhaps produced by an as-yet unidentified novel signal producer. In order to identify the chemical composition of the short-chain AHLs I utilized mass spectrometry analysis of the *S. meliloti* culture-extracts. I determined that the compounds which comigrated with short-chain AHLs were in fact cyclic dipeptides (DKPs). I identified the de novo production of

a range of DKPs in *S. meliloti*. DKPs have been shown to be a new class of signal molecules in quorum-sensing systems in several gram-negative bacteria, and this work reports the first example of their synthesis in *S. meliloti* (95).

The focus of this dissertation was to analyze the contributions of multiple regulators and signaling factors to the complex regulatory systems of *S. meliloti*. The task of deciphering regulons was highly aided by the sequencing of the *S. meliloti* genome and the availability of a DNA microarray chip. Most of the genes regulated by the orphan LuxR homologs of *S. meliloti* were pertinent to survival within its environmental niche. These orphan LuxR homologs represent a novel class of regulators that have the potential to respond to plant produced or other exogenous signal molecules. The nature of the activating signal of these regulators still remains unknown and future work could focus on identifying them by fusing reporter genes to the orphan LuxR regulators and evaluating which genes or signals induce activation. The downstream mode of regulation of the orphan LuxR regulators could be analyzed by conducting promoter-binding bandshift assays or DNA footprinting assays. Together, the above experiments could efficiently tease out the specific molecular mechanisms of the orphan LuxR regulators in *S. meliloti*. Another question that could to be addressed is whether the synthesis of cyclic dipeptides is population density dependent and what specific activity they modulate in *S. meliloti*.

The insights provided by this work are fundamental in understanding bacterial responses to environmental fluctuations, as well as interactions between bacteria and their hosts, and are crucial for a deeper understanding of prokaryote-eukaryote associations.

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