Regulatory mechanisms of the Sin Quorum Sensing System and its impact on survival of the soil-dwelling bacterium *Sinorhizobium meliloti*

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Chapter 1: Summary

The Sin Quorum Sensing (QS) system of the soil bacterium *Sinorhizobium meliloti* controls genes involved in a variety of cellular processes such as exopolysaccharide (EPS) production, motility, nitrogen fixation, and transport of metals and small molecules. The system consists of SinI, an *N*-acyl-homoserine lactone (AHL) synthase, SinR, the LuxR-type transcriptional regulator of *sinI*, and ExpR, the LuxR-type master transcriptional regulator. The aims of this study are to understand the mechanisms and functions of the Sin QS, as well as its importance to survival of *S. meliloti*.

Some of the regulatory target genes of the Sin QS have been previously shown to contain a promoter sequence that binds specifically to AHL-activated ExpR. In the first part of this study, the mechanisms of the ExpR transcriptional regulatory network were explored. The results confirmed 7 previously detected ExpR-DNA binding sites and added 26 novel sites, some of which regulate genes previously unknown to be members of the ExpR regulon. ExpR regulates the expression of the target genes in an AHL dependent manner. The data indicate that the location of the ExpR-binding site with respect to the relevant transcription start determines whether ExpR/AHL activates or represses promoter activity. Furthermore, the strength of the response is dependent upon the concentration of AHLs. This suggests a type of temporal gene expression program whereby the activity of each promoter is subjected to a specific range of AHL concentration since AHL accumulation and concentration varies with the age of the culture.

In the second part of this study, the regulation of the Sin QS itself was further investigated. Until recently, all LuxR-type proteins were thought to bind to AHLs as the inducer. Unexpectedly, the results confirm that, in contrast to ExpR, the activity of SinR on *sinI* expression is independent of AHLs. The results also indicate that RNase E, an endoribonuclease that is essential for cell viability, regulates *sinI* expression by specifically targeting the 5'-UTR of *sinI* mRNA. Overexpression of *rne* resulted in a shorter half-life of *sinI* mRNA and a strong reduction of AHL accumulation. The results suggest that RNase E-dependent degradation of *sinI* mRNA from the 5' end is one of the steps mediating a high turnover of *sinI* mRNA, which allows the Sin QS system to respond rapidly to changes in transcriptional control of AHL production. This is the first report of a specific regulatory interaction between QS and an essential component of cell viability in *S. meliloti*.

The last part of this study involves the impact of the Sin QS on fitness of *S. meliloti*. Cultivation under standard laboratory conditions demonstrated aggressive invasions of QS-deficient *expR* mutants in the QS-efficient wild type population. Various mutants were tested in a series of competition assays. The results suggest that ExpR has a negative effect on bacterial fitness under standard laboratory conditions and that this effect is dependent upon EPS and flagellum production. However, when bacteria were exposed to severe stress, i.e. desiccation, survival was mostly dependent upon ExpR. In contrast, symbiotic potential was not enhanced by ExpR. Altogether, the results reveal that QS can have either positive or negative impact on fitness, depending on the context. It is, on one hand, a beneficial trait that helps bacteria to survive from severe stress but, on the other hand, tends to be eliminated under low stress and nutrient rich conditions.

Zusammenfassung

Das Sin Quorum Sensing (QS) System des Bodenbakteriums *Sinorhizobium meliloti* steuert eine Vielzahl von zellulären Prozessen, wie zum Beispiel Exopolysaccharid (EPS) Produktion, Motilität, Stickstofffixierung oder den Transport von Metallen und kleinen Molekülen. Das System besteht aus drei Komponenten: (i) SinI, eine *N*-Acyl-Homoserin-Lacton (AHL) Synthase; (ii) SinR, ein LuxR-ähnlicher Transkriptionsregulator von *sinI*; und (iii) ExpR, der AHL-abhängige LuxR-ähnliche Haupttranskriptionsregulator. Das Ziel dieser Arbeit ist die Mechanismen und Funktionen des Sin-QS Systems zu verstehen, sowie deren Bedeutung für das Überleben von *S. meliloti* zu beschreiben.

Es wurde bereits gezeigt, dass durch AHL aktiviertes ExpR spezifische DNA-Sequenzen innerhalb des Promotorbereichs einiger Zielgene bindet. Der erste Teil dieser Arbeit befasste sich mit den regulatorischen Mechanismen innerhalb des ExpR-Regulons. Die Ergebnisse bestätigten zunächst sieben zuvor identifizierte ExpR-DNA-Bindestellen und fügten darüber hinaus 26 bisher unbekannte Bindestellen hinzu. Einige dieser Bindestellen befinden sich in Promotorbereichen von Genen, die daraufhin dem ExpR-Regulon zugeordnet werden konnten. Die Positionen der ExpR-Bindestellen relativ zum jeweiligen Transkriptionsstart bestimmen, ob ExpR/AHL die Promotoraktivität aktiviert oder reprimiert. Außerdem konnte gezeigt werden, dass die Stärke der Regulation abhängig von der AHL-Konzentration ist. Die Ergebnisse und die Tatsache, dass AHL-Akkumulation und -Konzentration innerhalb alternder Kulturen variieren, suggerieren ein temporäres Genexpressionsprogramm, in dem jeder Promotor auf eine bestimmte AHL-Konzentration reagiert.

Im zweiten Teil der Arbeit wurde die Regulation des Sin QS Systems selbst untersucht. Die Ergebnisse zeigten, dass, im Gegensatz zu ExpR, die Aktivität von SinR auf die *sinI*-Expression AHL unabhängig ist. Neben der Autoregulation des Sin QS Systems konnte eine zusätzliche Regulationsebene nachgewiesen werden, in der RNase E, eine für das Wachstum essenzielle Endoribonuclease, eine Rolle spielt. RNase E ist an der Regulation der *sinI*-Expression beteiligt und zielt spezifisch auf die 5'-UTR der *sinI*-mRNA. Überexpression von *rne* führte zu einer kürzeren Halbwertszeit der *sinI*-mRNA und einer starken Reduktion der AHL-Akkumulation. Durch diese post-transkriptionelle Kontrolle der *sinI*-mRNA ist eine schnelle Antwort des Sin QS Systems auf Änderungen in der Transkriptionskontrolle der AHL-Produktion möglich.

Im letzten Teil dieser Arbeit wurde untersucht, wie das Sin-QS System die generelle Fitness von *S. meliloti* beeinflusst. Kultivierungsexperimente unter Standardlaborbedingungen und Kompetitivitätsanalysen verschiedener Mutantenlinien zeigten, dass ExpR einen negativen Effekt auf die bakterielle Fitness unter Standardlaborbedingungen hat, und dass dieser Effekt abhängig von EPS-Produktion und Flagellensynthese ist. Im Gegensatz dazu erhöht die Anwesenheit von ExpR unter Stressbedingungen (Austrocknung) die Überlebensrate der Zellen. Die symbiotische Fitness wurde hingegen nicht durch ExpR verbessert. Die Ergebnisse zeigen deutlich, dass QS je nach Wachstumsbedingungen entweder positive oder negative Auswirkung auf die Fitness haben kann. Es ist einerseits eine vorteilhafte Eigenschaft, die das Überleben der Bakterien bei Stress unterstützt. Andererseits wird es tendenziell unter nährstoffreichen Bedingungen eliminiert.

Chapter 2: Introduction

Bacteria are constantly subjected to environmental fluctuations, including changes in temperature, osmolarity, pH, and nutrient availability. In response, bacteria have developed various systems that allow adaptation to these stimuli. For example, two-component signal transduction phosphorelay schemes allow bacteria to sense and respond to multiple environmental factors by the activation or repression of specific target genes (Stock *et al.*, 2000). Similarly, the expression of assorted sigma factors in response to various signals enables transcriptional specificity in bacteria (Wösten, 1998). Alterations to DNA topology, protein-mediated or otherwise, can also result in changes to the transcriptional profile of a bacterium (Atlung & Ingmer, 1997; Pérez-Martín & de Lorenzo, 1997). A further layer of bacterial sensing and response mechanisms is in the form of population density-dependent regulatory system known as quorum sensing (QS). The process relies on the bacterial production of a small signal molecule, the extracellular concentration of which is related to the population density. The signal molecule can be sensed by cells and this allows the whole population to initiate a cooperative behavior once a critical concentration has been achieved.

QS was first described over 30 years ago in the luminous marine bacterium *Vibrio fischeri* (Nealson & Hastings, 1979). This bacterium lives in symbiotic association with the Hawaiian bobtail squid *Euprymna scolopes*. The squid supplies *V. fischeri*, inhabiting a specialized organ, with a nutrient-rich environment. In return, the bacterial symbiont produces light that helps the host to escape from predators by counterillumination (Nealson & Hastings, 1979; Ruby & McFall-Ngai, 1992; Ruby, 1996; Visick & McFall-Ngai, 2000). The enzymes responsible for light production are encoded by the luciferase structural operon *luxCDABE* (Engebrecht & Silverman, 1984; Miyamoto *et al.*, 1988), and light emission occurs only at high population density in response to the accumulation of secreted autoinducer signal molecules (Nasser *et al.*, 1998). The canonical QS circuit of *V. fischeri* is known as LuxRI QS.

2.1 LuxRI-type Quorum Sensing

The LuxRI-type QS systems contain, at a minimum, homologues of two *V. fischeri* regulatory proteins called LuxI and LuxR. LuxI/LuxR homologs have been identified in more than 100 Gram-negative bacteria (Case et al. 2008). The LuxI-like proteins are responsible for the biosynthesis of a specific *N*-acyl homoserine lactone molecule (AHL) known as autoinducer. The autoinducer concentration increases with increasing population density. The LuxR-type proteins bind cognate AHL autoinducers that have achieved a critical threshold concentration, and the LuxR-AHL complexes can then activate transcription of the target genes, e.g., the *luxCDABE* operon (Engebrecht *et al.*, 1983; Engebrecht & Silverman, 1984; Engebrecht & Silverman, 1987). The *V. fischeri* LuxRI QS system is shown in Fig. 1. Using this QS mechanism, bacteria can efficiently couple gene expression to appropriate population density.

LuxR-type regulators consist of an N-terminal AHL-binding domain and a C-terminal helix-turn-helix motif, which binds to DNA (Choi & Greenberg, 1991; Hanzelka & Greenberg, 1995). In *V.*

fischeri, a LuxR fragment containing only the C-terminal domain is constitutively active and unaffected by AHLs, indicating that the C-terminal domain contains all the sites essential for LuxR-DNA and LuxR-RNA polymerase interactions (Choi and Greenberg, 1991). Typically, AHLs stimulate dimerization of the N-terminal domain (Choi & Greenberg, 1992; Qin *et al.*, 2000; Ventre *et al.*, 2003). The LuxR-AHL complex, as a dimer, then binds to conserved palindromic sequences of the QS-controlled promoters and activates the expression of QS-dependent genes, including the genes encoding AHL synthase and LuxR-type regulator, to generate a positive feedback.

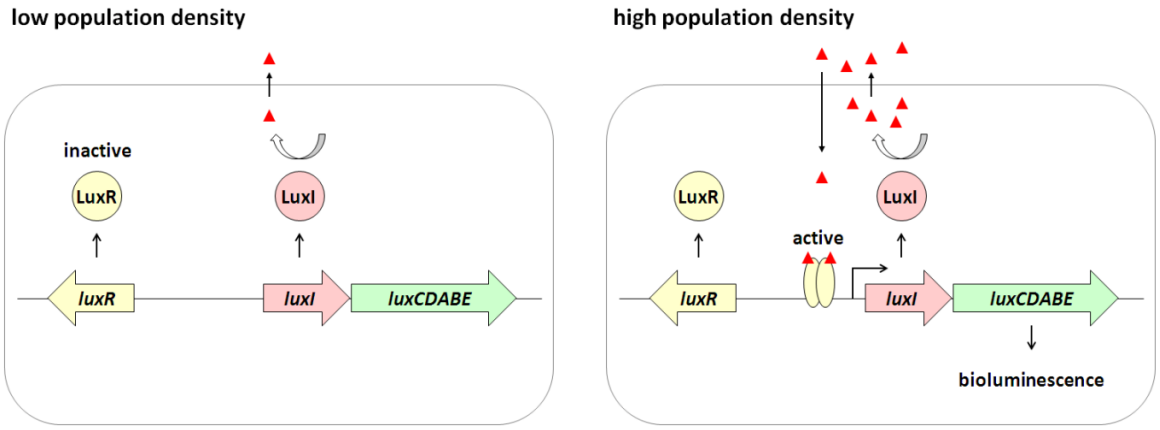

Fig. 1 Schematic representation of the LuxRI QS system in *V. fischeri*. At low population density, basal level production of AHL autoinducers (red triangles) results in the rapid dilution of the signals in the surrounding environment. At high population density, an increase in bacterial number results in accumulation of autoinducers beyond a threshold concentration, leading to the activation of the LuxR response regulator, which induces the expression of bioluminescence genes (*luxCDABE*).

All AHL autoinducer molecules share a common homoserine lactone moiety and differ only in their acyl side chain moieties (de Kievit & Iglewski, 2000; Fuqua *et al.*, 1996). LuxI-like proteins link the side chain group of specific acyl carrier proteins (ACPs) to the homocysteine moiety of S-adenosylmethionine (SAM) (Hanzelka & Greenberg, 1996; Moré *et al.*, 1996; Val & Cronan, 1998). Chain lengths vary from 4 to 18 carbon atoms, in saturation and in the substitution of a carbonyl or hydroxyl group at the third carbon (Kumari *et al.*, 2006)(see Fig. 2). *V. harveyi* and *V. fischeri* produce short chain AHLs, 3-hydroxy-C_4-HL and 3-oxo-C_6-HL, respectively (Bassler *et al.*, 1994; Eberhard *et al.*, 1981). The AHL with the longest acyl side chain (C_{18}-HL) was found in *S. meliloti* (Marketon *et al.*, 2002), suggesting that diffusion of these AHLs are restricted by the double membrane barrier. Consistent with this, the import of long chain AHLs in *S. meliloti* is facilitated by the outer membrane protein FadL, which also serves as a long-chain fatty acid transporter in various rhizobia and in *Escherichia coli* (Krol & Becker, 2014).

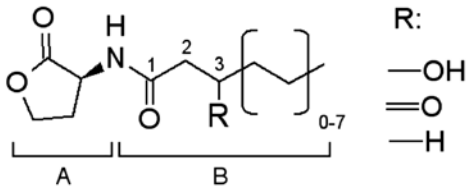

Fig. 2 Structure of an *N*-acyl-homoserine lactone. A: hydrophilic homoserine lactone ring; B: hydrophobic acyl side chain; R: possible modifications on the third carbon of the acyl side chain.

Beside the bioluminescence in *V. fischeri*, LuxRI-type QS is known to regulate many other physiological processes in different bacteria. Tra QS of the plant pathogen *Agrobacterium tumefaciens* controls horizontal transfer and vegetative replication of oncogenic Ti plasmids (Hwang *et al.*, 1994; Lang & Faure, 2014; Piper *et al.*, 1993). In *Pseudomonas aeruginosa*, a common opportunistic human pathogen, the production and secretion of multiple extracellular virulence factors is under the control of at least two sets of LuxRI homologues. The first of these, the Las system, was shown to regulate the expression of LasA elastase, LasB elastase, exotoxin A and alkaline protease (Gambello *et al.*, 1993; Jones *et al.*, 1993; Passador *et al.*, 1993; Toder *et al.*, 1991). The second QS system of *P. aeruginosa* is Rhl, initially shown to activate the production of rhamnolipid biosurfactants (Ochsner *et al.*, 1994). Subsequent studies have revealed that a functional Rhl system is also required to fully induce expression of other factors, including alkaline protease, pyocyanin, hydrogen cyanide, lectins and elastase (Brint & Ohman, 1995; Latifi *et al.*, 1995; Pearson *et al.*, 1997).

Rhizobia possess various QS systems affecting symbiotic interactions, surface polysaccharide, growth inhibition, stationary-phase adaptation, and plasmid transfer (González & Marketon, 2003; Wisniewski-Dyé & Downie, 2002). Several QS systems have been detected in *Rhizobium leguminosarum*, i.e., Tra, Cin, Rhl, and Rhi. The Tra system is responsible for the synthesis of 3-oxo-C_8-HL and controls conjugal transfer of symbiotic plasmid (Wilkinson *et al.*, 2002). 3-OH-$C_{14:1}$-HL (also known as *small* bacteriocin), produced by the Cin system, has an inhibitory effect on growth of several *R. leguminosarum* strains (Oresnik *et al.*, 1999; Schripsema *et al.*, 1996; Wisniewski-Dyé & Downie, 2002). Furthermore, addition of 3-OH-$C_{14:1}$-HL has been shown to promote starvation survival of *R. leguminosarum* cultures that enter stationary phase at low polulation density (Thorne & Williams, 1999). The Rhl system produces several short-chain AHLs and influences nodulation efficiency (Rodelas *et al.*, 1999). The Rhi system also produces several short-chain AHLs, however, little is known about the role of this QS system.

S. meliloti harbors at least two QS systems: the Sin and the Tra system. The Tra system, which is carried on pRme41a and is present only in strain Rm41, produces short chain AHLs, including 3-oxo-C_8-HL, and controls the conjugal transfer of pRme41a. In the Sin system, several long chain AHLs, including C_{12}-HL, C_{14}-HL, oxo-C_{14}-HL, $C_{16:1}$-HL, oxo-$C_{16:1}$-HL and C_{18}-HL, are produced by SinI, whose transcription is controlled by SinR (Marketon *et al.*, 2002; Teplitski *et al.*, 2003). Unlike typical LuxRI-type QS, the Sin system has an additional LuxR-type transcription regulator called ExpR, which is required for EPS biosynthesis (Marketon *et al.*, 2003). In *S. meliloti* strain Rm1021, disruption of either *sinR* or *sinI* correlates with a delay in the appearance of nitrogen-fixing nodules, as well as with

an overall decrease in the number of pink nodules, suggesting a role of QS in establishing a successful symbiosis with the host plant alfalfa (Marketon *et al.*, 2002).

2.2 *S. meliloti* – Alfalfa Symbiosis

S. meliloti is one of the best studied rhizobia in the family *Rhizobiaceae*. This Gram-negative α-proteobacterium exists either in a free-living lifestyle or in symbiosis with leguminous plants from the genera *Medicago*, *Melilotus*, and *Trigonella*, including the model legumes alfalfa (*Medicago sativa* L.) and Barrel clover (*Medicago truncatula*). *S. meliloti* genome consists of three separate replicons, the chromosome (3.65 Mb) and the two megaplasmids pSymA (1.35 Mb) and pSymB (1.68 Mb). The genome sequence of the strain Rm1021 and Rm41 has been published in 2001 (Barnett *et al.*, 2001; Capela *et al.*, 2001; Finan *et al.*, 1986; Galibert *et al.*, 2001) and in 2013 (Weidner *et al.*, 2013), respectively. In addition to the three replicons, the strain Rm41 has two additional plasmids pRme41a and pRme41b. The latter carries genes involved in nodulation and nitrogen fixation (Bánfalvi *et al.*, 1981).

Fig. 3 Leaves and root nodules of *Medicago sativa* cv. Europe, 4 weeks after infection with *S. meliloti* Rm2011. The root nodules are approximately 2 mm long.

In general, successful rhizobium-legume symbiosis requires chemical communication between bacteria and host plant (Fisher & Long, 1992; Long, 1989). Flavonoids, phenolic signals produced by the plant roots, are recognized by the bacteria and induce the expression of bacterial nodulation genes, resulting in the production of Nod factors (D'Haeze & Holsters, 2002; Perret *et al.*, 2000; Peters *et al.*, 1986). Bacterial Nod factors then act on the plant roots to induce nodule formation and root hair curling. Bacteria trapped in the curled root hair can then access the host plant through infection thread (van Brussel *et al.*, 1992). In the commonly used *S. meliloti* laboratory strain Rm1021, this process requires the synthesis of symbiotically essential EPS (Becker *et al.*, 2002; Fraysse *et al.*, 2003; González *et al.*, 1996b; Skorupska *et al.*, 2006). Once inside, the bacteria differentiate into morphologically altered forms termed bacteroids and begin to synthesize nitrogenase and the other proteins required for nitrogen fixation. The symbiotic interaction results in the reduction of atmospheric dinitrogen to ammonia by the bacteroids, which is then utilized by the host plant. Fig. 3 shows leaves and root nodules of *M. sativa* infected with *S. meliloti*. Symbiotic nitrogen fixation is a finely tuned process, and

the inability to properly attach, produce EPS, travel through the infection thread, or fully develop into bacteroids may result in a failed host-bacterium interaction (Becker *et al.*, 2002; Ferguson *et al.*, 2002; Jones *et al.*, 2007).

As the bacteria move toward the host, they cluster around the roots and the population density rises. This increase in numbers leads to the coordinated regulation of bacterial genes by QS (Fuqua *et al.*, 1996; Whitehead *et al.*, 2001). In *S. meliloti*, the Sin QS controls a multitude of downstream genes, especially those responsible for the EPS production. *S. meliloti* is capable of producing two distinct EPS, succinoglycan and galactoglucan, either of which can function in symbiosis (Cheng & Walker, 1998; Glazebrook & Walker, 1989; González *et al.*, 1996b; Leigh & Walker, 1994). Succinoglycan is a polymer of an octasaccharide subunit, consisting of a backbone of one galactose and three glucose residues, a side chain of four glucose residues, and succinyl, acetyl and 1-carboxylethylidene (pyruvyl) modification in a ratio of approximately 1:1:1 (Reinhold *et al.*, 1994; Reuber & Walker, 1993) (Fig. 4A). Succinoglycan is produced in a high-molecular-weight form, as a polymer containing hundreds of the octasaccharide subunit, and a low-molecular-weight form that is composed of monomers, dimers, and trimers (Djordjevic *et al.*, 1987; González *et al.*, 1998; Wang *et al.*, 1999). Galactoglucan is composed of alternating glucose and galactose residues that are acetylated and pyruvated, respectively (Fig. 4B). The low-molecular-weight galactoglucan consists of 12-35 dimers (González *et al.*, 1996b). The low-molecular-weight fraction is an active biological form of EPS indispensable for successful infection.

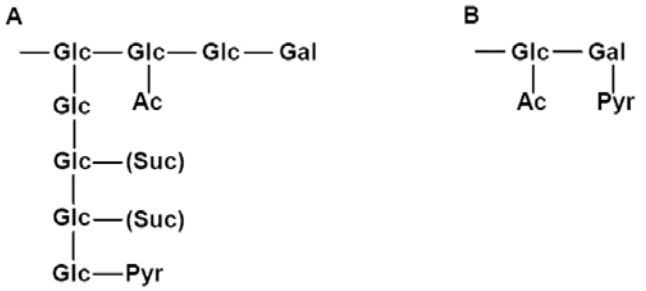

Fig. 4 General structure of the exopolysaccharide produced by *S. meliloti*. (A) Octasaccharide subunit of succinoglycan (B) Disaccharide subunit of galactoglucan

Succinoglycan biosynthesis has been extensively characterized (González *et al.*, 1998; Reuber & Walker, 1993). The genes involved in its production form a large *exo*/*exs* cluster (~35 kb), which is located on the pSymB megaplasmid (Finan *et al.*, 2001). In this region, 28 *exo*/*exs* genes organized in several operons have been identified, among them the genes encoding enzymes for the synthesis of nucleotide sugar precursors (*exoB* and *exoN*), enzymes involved in unit assembly (*exoY*, *exoF*, *exoA*, *exoL*, *exoM*, *exoO*, *exoU* and *exoW*) and modification (*exoZ*, *exoH* and *exoV*), and proteins responsible for polymerization of repeating units and transport of succinoglycan (*exoP*, *exoT*, *exoQ* and *exsA*) (Becker *et al.*, 1993a; Becker *et al.*, 1993b; Becker *et al.*, 1993c; Becker *et al.*, 1995; Glucksmann *et al.*, 1993; Jofré & Becker, 2009; Müller *et al.*, 1993). Moreover, other genes essential for sugar precursor synthesis (*pgm*, formerly *exoC*) and regulation of succinoglycan production (*exoD*, *exoR*, *exoS* and

mucR) are not linked with this region, but dispersed throughout the chromosome of *S. meliloti* (Doherty *et al.*, 1988; Keller *et al.*, 1995; Reed *et al.*, 1991; Uttaro *et al.*, 1990). The isolation of several *S. meliloti* mutants defective in the production of succinoglycan revealed a correlation between failure of bacteria to produce EPS and failure to invade nodules. With few exceptions, all of the mutants formed ineffective nodules, which are devoid of differentiated, nitrogen fixing bacteroids (Leigh *et al.*, 1985). The results indicate that succinoglycan is involved in nodule invasion, although not required for nodule formation.

The biosynthesis of galactoglucan is directed by *exp* genes resided in a 27-kb cluster on pSymB, at a distance of 160 kb from the *exo/exs* cluster (Becker *et al.*, 1997; Moreira *et al.*, 2000). This cluster contains 22 genes organized into five operons: *wga* (*expA*), *wgcA* (*expC*), *wggR* (*expG*), *wgd* (*expD*) and *wge* (*expE*) (Bahlawane *et al.*, 2008a). Among them, four genes (*wgaG*, *wgaH*, *wgaI* and *wgaJ*, formerly *expA7*, *expA8*, *expA9*, and *expA10*, respectively) are involved in the synthesis of deoxythymidine diphospho-sugar precursors (dTDP-rhamnose and dTDP-glucose), and five genes encode potential glycosyltransferases: WgaB (ExpA23) and WgeB (ExpE2) β-glucosyltransferases and WgcA (ExpC), WgeD (ExpE4) and WgeG (ExpE7) galactosyltransferases. Other genes of this cluster are potentially engaged in the polymerization or secretion (*wgdA* and *wgdB*, formerly *expD1* and *expD2*, respectively) and regulation of galactoglucan synthesis (*wggR*) (Becker *et al.*, 1997; Moreira *et al.*, 2000). Strains producing only galactoglucan form nitrogen-fixing nodules, suggesting that galactoglucan can substitute for succinoglycan in nodule invasion (Glazebrook & Walker, 1989).

Regulation of EPS biosynthesis seems to be controlled, at least in part, by environmental signals, such as phosphate and nitrogen availability (Doherty *et al.*, 1988; Mendrygal & González, 2000; Summers *et al.*, 1998; Zhan *et al.*, 1991). It was shown that galactoglucan synthesis is stimulated by low-phosphate conditions but repressed in high phosphate (Summers *et al.*, 1998; Zhan *et al.*, 1991). In contrast, succinoglycan synthesis is increased under high phosphate conditions (Mendrygal & González, 2000). Interestingly, the galactoglucan made by the commonly used strain Rm1021 in low phosphate is of high molecular weight and therefore is not active in nodule invasion (González *et al.*, 1996b).

Previous studies have shown that the Sin QS system plays a key role in regulation of EPS production. The strain Rm1021 contains an IS*Rm1* element inserted in *expR*, resulting in disruption of this gene, while a related strain Rm8530 has an intact *expR* gene (Pellock *et al.*, 2002). The strain Rm8530 has been shown to produce both high- and low-molecular-weight galactoglucan (González *et al.*, 1996a; González *et al.*, 1996b). Thus, a functional copy of the *expR* gene is required for the biosynthesis of the symbiotically essential galactoglucan (Pellock *et al.*, 2002). The *sinR* and *sinI* genes are also required for almost all of the biosynthesis of galactoglucan by the strain Rm8530. However, the residual galactoglucan produced by the *sinI* mutant is inadequate to support nodulation since the disruption of *sinI* combined with the disruption of succinoglycan production (i.e., *sinI/exoY* double mutant) results in inability to form nitrogen-fixing nodules (Marketon *et al.*, 2003). Altogether, the results confirm that galactoglucan regulation by the Sin QS is important for a successful symbiosis.

2.3 The Sin QS System in *S. meliloti*

The Sin QS system depends on at least three genes, *sinR*, *sinI* and *expR*. The expression of *sinI*, the AHL synthase-coding gene, is regulated by two LuxR-type transcriptional regulators SinR and ExpR (Glazebrook & Walker, 1989; McIntosh *et al.*, 2008; Pellock *et al.*, 2002). *sinR* is located upstream of *sinI*, separated by an intergenic region of 156 bp, while *expR* is located approximately 1.5 Mb distant from the *sinRI* locus. An ExpR binding site has been reported in the promoter region of *sinR* (McIntosh *et al.*, 2009), as well as in the *sinR-sinI* intergenic region (Bartels *et al.*, 2007). Separated from the ExpR binding site by about 15 bp, a predicted SinR binding site has been located in this *sinR-sinI* intergenic region (Bartels *et al.*, 2007).

Expression of *sinI* and the consequent AHL production is strongly reduced upon disruption of *sinR*, suggesting a high level of dependence of *sinI* on *sinR* (Llamas *et al.*, 2004; Marketon *et al.*, 2002; McIntosh *et al.*, 2008). The result of a functional *sinR*, *sinI* and *expR* combination is a high expression of *sinI* and thus an increase in AHL production, which in turn stimulates a higher expression of *sinI* through AHL-activated ExpR. This process forms a positive feedback-loop that serves to increase AHL concentrations in growing populations (McIntosh *et al.*, 2009). Under phosphate limiting conditions, the effect of a combination of AHLs and ExpR is to reduce *sinR* expression. Addition of synthetic $C_{16:1}$-HL to the *sinI* mutant demonstrates that AHL-activated ExpR simultaneously mediates both the activation and inhibition of *sinI* (through *sinR*). If AHL levels are under the *sinR*-repression threshold, *sinI* expression is activated by positive feedback through a combination of SinR and AHL-activated ExpR. If AHL levels are above the *sinR*-repression threshold, negative feedback is mediated by AHL-activated ExpR that represses *sinR* expression. The activation threshold for *sinI* induction is 1-5 nM AHLs, while that of *sinI* repression is > 40 nM AHLs (McIntosh *et al.*, 2009). In addition, *sinR* is induced under phosphate limiting conditions, possibly through the response regulator PhoB of the PhoR/PhoB two-component system (Krol & Becker, 2004).

Previous studies have shown that ExpR and AHLs control the expression of multiple genes, including genes involved in EPS production, motility, chemotaxis, nitrogen fixation, carbon and nitrogen metabolism, metal transport, nutrient acquisition and many other cellular processes (Gao *et al.*, 2005; Gurich & González, 2009; Hoang *et al.*, 2004). Activated LuxR-type regulators usually bind to a consensus DNA sequence known as the lux box, typically located upstream of the promoters of its target genes (Stevens *et al.*, 1994). Prior to this study, ExpR-binding sites had been identified in the promoter region of seven target genes. These include, in addition to *sinR* and *sinI*, EPS biosynthesis genes (*exoI, exsH, wgeA* and *wgaA*) and the master regulator of flagellum production and motility genes (*visN*) (Bahlawane *et al.*, 2008b; Bartels *et al.*, 2007; McIntosh *et al.*, 2008; McIntosh *et al.*, 2009). Nonetheless, the knowledge about how ExpR and the Sin system regulate the target genes needed to be extended, since this system controls almost 9% of the transcriptome which is far in excess of the genes controlling motility and EPS production.

QS systems are controlled by many factors at the levels of transcription, translation, protein activity, and ligand stability. In *A. tumefaciens*, QS can be quenched by enzymes such as lactonases, which

degrade the AHL (Haudecoeur *et al.*, 2009). Homeostasis of the *Pseudomonas* LasRI system is regulated by transcriptional repressors, RsaL and RsaM (Venturi *et al.*, 2011). Small regulatory RNAs (sRNAs) have also been found to regulate QS (Bejerano-Sagie & Xavier, 2007; Lenz *et al.*, 2004). Typically, sRNAs interact with mRNAs with the help of the RNA chaperone Hfq and influence the translation rate and/or half-life of the mRNA targets. Usually both the sRNA and the mRNA are degraded in an RNase E-dependent manner (Massé *et al.*, 2003; Morita *et al.*, 2005; Storz *et al.*, 2011). Recently, it was found that *sinI* mRNA levels are higher in an *hfq* mutant of *S. meliloti* (Gao *et al.*, 2010), suggesting the involvement of an sRNA and possibly of RNase E in the Hfq-dependent regulation of this gene. However, the role of RNase E in the Sin QS of *S. meliloti* was not addressed prior to this study.

While studies in QS have been mostly focused on its mechanisms of gene expression control, less attention has been paid on its costs and benefits to the organism (Schuster *et al.*, 2013). The QS machinery can confer a selective advantage in the pathogenic interaction of *P. aeruginosa* with the host through the regulation of many extracellular virulence factors (Favre-Bonté *et al.*, 2002; Middleton *et al.*, 2002; Winzer & Williams, 2001). In the plant pathogen *Erwinia carotovora*, QS enhances oxidative stress tolerance and virulence and prompts bacterial fitness *in planta* (Jones *et al.*, 1993; Sjöblom *et al.*, 2008). Loss of AHL production resulted in altered colony morphology and reduced epiphytic viability of *Pseudomonas syringae* (Dumenyo *et al.*, 1998). Short chain AHLs produced by *R. leguminosarum* inhibit growth by inducing the cells to enter stationary phase at low population density (Gray *et al.*, 1996). *S. meliloti* uses the Sin QS to activate the production of copious levels of symbiotically important succinoglycan and galactoglucan (Gurich & González, 2009). This type of cooperation, therefore, requires enormous amount of energy and valuable nutrients. In this study, the advantages and/or disadvantages of the Sin QS under specific environmental conditions will also be demonstrated.

2.4 Aims of this study

The aims of this study were to answer two questions; (1) what are the mechanisms of regulation by the Sin QS system and (2) how does this system enhance survival. The first two parts of this study deal with question 1 and involve the identification of target genes, the characterization of the molecular mechanisms by which ExpR and AHLs regulate the target genes (Charoenpanich *et al.*, 2013, see Chapter 7) and, in addition, regulation within the Sin system itself (Baumgardt *et al.*, 2014, see Chapter 8). The third part deals with question 2 and was focused on the impact of the Sin QS on the fitness of *S. meliloti* in the free-living state under specific conditions, as well as on its symbiotic potential (Charoenpanich *et al.*, 2014, see Chapter 9).

Chapter 3: Results and Discussion

3.1 The Sin/ExpR regulon

3.1.1 The Sin QS system regulates target genes through ExpR and AHLs

At least 570 genes have been identified as being regulated by ExpR and AHLs (Gao *et al.*, 2005; Gurich & González, 2009; Hoang *et al.*, 2004). However, only seven genes have been shown to contain an ExpR binding site in the promoter region as mentioned above. In this study, a consensus sequence (CCCANNATTNTATTGGGG), generated based on the alignment of the previously identified binding sites, was used to identify additional sites in the genome of the *S. meliloti*. A total of 129 DNA fragments were tested with purified His_6-ExpR and AHLs in a DNA-protein binding gel shift assay, but only 26 binding sites were confirmed. These binding sites are located in the promoter region of genes involved in the production and transport of EPS (*exoH, exoF3, exoP2*) and transport of small molecules (SMb21135, SMc02378, SMc03864), genes encoding calcium-binding proteins (SMb21543, SMa2111), transcriptional regulators (SMc03150, *phrR*, *cspA3, nolR*) and some hypothetical proteins of unknown function. Generally, a closer resemblance to the ExpR binding consensus correlates positively with a stronger shift. Typically, sites with 3 or 4 Cs on the gene-distal side and 3 or 4 Gs on the gene-proximal side separated by 10 to 12 nucleotides rich in A and T are better suited for binding (Table 1, Chapter 7). The presence of Gs or Cs in the A/T-rich regions appears to weaken the shift. Similarly, the presence of As and Ts in the G or C-rich regions may weaken the shift. A new consensus sequence was derived from all 33 binding sites in this study. It can be represented as CCCCAAAAATTTTTTGGGG.

Interestingly, an ExpR binding site was identified in the promoter region of *expR* although the DNA sequence only weakly resembles the ExpR binding site consensus (Fig, 3B, Chapter 7). When a single nucleotide was exchanged (T to C) within the binding site sequence to improve resemblance to the ExpR binding site consensus, a stronger binding to ExpR was not apparent (Fig. 3D and 3E, Chapter 7). However, when another nucleotide within this sequence was modified (C to T) to decrease similarity to the ExpR binding site consensus, the result was an almost complete lack of binding to ExpR (Fig. 3D and 3E, Chapter 7).

ExpR in the presence of AHLs regulates the promoter activity of *sinI, sinR, expR, wgaA, wgeA, wggR* (Gurich & González, 2009; McIntosh *et al.*, 2008; McIntosh *et al.*, 2009; Mueller & González, 2011), *exoI, exsH, exoH* (Glenn *et al.*, 2007), and *visN* (Bahlawane *et al.*, 2008b; Gurich & González, 2009). To establish the role of ExpR and AHLs in the regulation of promoter activity, a promoter-*egfp* fluorescence assay was applied to three *S. meliloti* strains, wild type (with functional *expR*), *sinI*, and *expR* mutant. The *expR* promoter (Fig. 5, Chapter 7) responds to ExpR and AHLs, as was previously reported (McIntosh *et al.*, 2009). Binding sequence modification correlated well with changes in promoter activity. The effect of the T-to-C change was not obvious in a gel shift assay, but did result in a significant promoter activity increase in response to ExpR and AHLs (Fig. 5, Chapter 7). Furthermore, the C-to-T change almost completely removed not only the binding to ExpR but also the

activating effect from ExpR and AHLs. These experiments increase the confidence in the location and function of this ExpR binding site.

Other promoter regions containing ExpR binding sites showed a variety of responses to the presences of ExpR and AHLs. These fall into several categories. In the first are those promoters which are upregulated in the presence of ExpR and AHLs ≥2-fold. For these promoters, maximal activation requires the presence of both ExpR and AHLs. In this category are, for example, the promoters of *sinI, expR,* and those of EPS synthesis genes (*exoI, exoH, exsH, wgaA, wgeA*). In the second category are those promoters where ExpR and AHLs downregulate activity ≥2-fold. In this category are the promoters of *sinR* and *visN* plus the promoters identified in this study, e.g., those of *phrR*, SMc01524, and SMc02378. Repression of these promoters requires the presence of both ExpR and AHLs. In the third category are promoters which contain a binding site but whose activity is affected by ExpR and AHLs, either negatively or positively, <2-fold. In this category are the promoters of *nolR*, SMb21135, SMc04246, *cspA3*, and *exoF3*. In a fourth category are DNA regions located upstream of an annotated gene which contain a binding site but do not contain detectable promoter activity under our conditions (see Table 1, Chapter 7). For these regions, it is possible that their downstream genes have falsely annotated translation starts or that these promoters are dependent upon external signals not present in these growth conditions.

3.1.2 Differential AHL sensitivity of ExpR-regulated promoters

Promoters that respond to the presence of ExpR and AHLs (category 1 and 2) were tested for the sensitivity to supplemented AHLs in a strain incapable of producing AHLs (*sinI* mutant). The resulting change in promoter activity relative to the concentration of $C_{16:1}$-HL, as determined by promoter-*egfp* fusions, is summarized in Fig. 6, Chapter 7. For example, similar to the *sinI* promoter, the promoters of *phrR* and SMb20911 responded to very low levels of AHLs (5 to 10 nM). However, unlike the *sinI* promoter, their response to the presence of AHLs was negative. All other promoters required higher levels of AHLs for a response. Addition of 50 to 100 nM was sufficient to induce regulation of the promoters of *expR*, SMc04237, SMb21543, SMa2111, and genes controlling EPS production (*exoH*, *exsH*, *exoI*, *wgeA*, and *wgaA*). Interestingly, all of these promoters respond positively to the addition of AHLs. In contrast, most of the promoters that are repressed by AHLs required a higher level (100 to 1,000 nM) before a response was observable. These include promoters of *sinR* which responded at 100 to 200 nM, and *visN* (master regulator of motility genes) which responded at 500 nM. Thus, from these data, a fascinating pattern emerges: promoters repressed by AHLs tend to require higher levels of AHLs for their response, while promoters activated by AHLs tend to begin responding at lower levels. Three exceptions to this pattern are the promoters of *phrR*, SMc04059, and SMb20911, which respond negatively to AHLs.

Another interesting observation regards the opposing effects on promoter activity from lower versus higher levels of AHL. For example, the promoter of *sinI* is activated by low levels of AHL while higher levels of AHL reduced activity. In a similar fashion, the activity profiles of the promoters of *wgaA* and *wgeA* were almost inactive in the absence of AHLs (Fig. 4, Chapter 7) and increasingly active with

increasing levels of AHL to a maximal activity at 500 nM. Intriguingly, as the levels of AHL were further increased to 2,000 nM, these promoters responded by decreasing in activity, so that both exhibited a lower activity at 2,000 nM compared to that at 500 nM. Of all the promoters measured in this study, only those controlling the *wga* and *wge* operons and *sinI* showed such clear double-response effects that depend on AHL levels. In the case of *sinI* promoter, the second (negative) response is mediated not by an ExpR binding site located upstream of *sinI* itself but by another site upstream of *sinR*. Binding of ExpR to the site upstream of *sinR* results in a decrease of *sinR* expression and thus a decrease in SinR-dependent *sinI* expression (McIntosh *et al.*, 2009). Likewise, in the case of *wga* and *wge* promoters, the second response may be due to the ExpR/AHL-dependent regulation of other genes related to the activity of these promoters.

These results reveal a variety of AHL sensitivities and suggest that these promoters are organized in a program of QS regulation. As AHLs accumulate in a growing population, positively regulated promoters are programmed to respond prior to the negatively regulated promoters. The clearest example of this is the activation of the expression of genes controlling EPS production and the repression of genes controlling motility. Such inverse regulation appears to be a general feature of many bacteria (Jonas *et al.*, 2009), including *Pseudomonas* (Caiazza *et al.*, 2007), where regulation is achieved via the signal molecule cyclic di-GMP. The QS program in *S. meliloti* provides evidence that QS regulation exhibits dynamic behavior and precision timing.

3.1.3 Mechanisms of promoter regulation by ExpR

The molecular mechanisms by which QS regulates its target genes are of considerable interest. A theoretical model of bacterial transcription found regulatory logic functions of plausible complexity by varying only two factors: strength of interaction between regulatory proteins and the relative positions of the relevant protein-binding DNA sequence in the *cis*-regulatory region (Buchler *et al.*, 2003). One example of this is the TyrR protein of *E. coli* (Pittard *et al.*, 2005), which can act as a repressor or activator of transcription for its eight known target promoters. Transcription activation and repression by TyrR are effected by binding to its TyrR box, and the direction of regulation is determined by the location of the TyrR box relative to the promoter. Tyrosine controls multimerization states of TyrR and affects binding to the TyrR box. The mechanism for repression can involve the exclusion of RNA polymerase from the promoter or interference with the ability of bound RNA polymerase to form open complexes or to exit the promoter. For transcription activation, TyrR can bind upstream of a promoter and interact with the α-subunit of the RNA polymerase. Finally, intracellular levels of TyrR protein are thought to be critical for determining regulatory outcomes.

Likewise, there are at least three factors that determined the strength of the regulatory effect of the *S. meliloti* ExpR/AHL combination on its regulon: (1) the abundance of ExpR, (2) the abundance of AHLs, and (3) the DNA sequence in and around each ExpR binding site. Evidence for factor 1 was reported in a previous study (McIntosh *et al.*, 2009), where levels of ExpR were controlled via expression from an IPTG promoter. In that study, various levels of ExpR intensified or weakened the promoter responses correspondingly. Evidence for the abundance of AHLs as a determinant of gene

expression was revealed in this study when AHL levels were varied in cultures carrying a promoter-*egfp* fusion. Promoter activity clearly depends upon the concentration of AHLs. However, in many cases, the effect of AHL addition on the ExpR-induced shift was only weakly apparent, if at all (Fig. 3, Chapter 7). Furthermore, a previous study using atomic force spectroscopy found that the strength of interaction between ExpR and its DNA binding site upstream of *sinI* was significantly increased upon the addition of AHLs (Bartels *et al.*, 2007). Evidence for the DNA sequence within and surrounding the ExpR binding site as one determinant of gene expression is suggested by the banding patterns in the gel shift assay (Fig. 3, Chapter 7).

ExpR-DNA binding is only one step in a multistep process of transcription activation and is therefore not necessarily a good indication of the strength of transcription. However, in both transcription activation and repression, the strength of the ExpR-DNA interaction is arguably one of the most critical steps in the regulation. This is supported by the study of the promoter of *expR*, in which alterations in the ExpR binding site affected not only the strength of the shift in a gel assay (Fig. 3B, Chapter 7), but also promoter activity in the presence of ExpR (Fig. 5, Chapter 7). Based on these data, a testable hypothesis can be proposed: at least one determinant of varying promoter sensitivity to AHLs is the DNA sequence to which ExpR binds, in which binding strength is stronger for sites that are more similar to the consensus.

Also relevant is the location of the binding site with respect to the promoter and transcription start, which may determine whether the regulation is positive or negative (Table 1, Chapter 7). The experimentally determined transcription starts were reported previously (Schlüter *et al.*, 2013). All of the ExpR binding promoter regions which were activated by ExpR contained a binding site either covering or upstream of the -35 regions. Two examples of this are the promoter of *sinI*, where the ExpR binding site is at -78, and the promoter of *expR*, where the binding site is at -41. In contrast to the ExpR-activated promoters, if the ExpR binding site is downstream of the -35 region, ExpR represses promoter activity. An example of this is the promoter of *sinR*. The ExpR binding site in the promoter of *sinR* is at -5. It is likely that the mechanism of repression is via ExpR covering the -10 region or the transcription start (+1). Two exceptions to these generalizations are the promoters of SMc04059 and SMc01524. In the case of SMc04059, the ExpR binding site is upstream of the -35 region. One possibility is that there is an alternative promoter with a transcription start that is closer to this binding site. In the case of SMc01524, the ExpR binding site covers the -35 region in a manner similar to the positively regulated promoters. It is possible that the binding site covers the transcription start of an alternative promoter.

3.2 The regulation of the Sin QS system

3.2.1 Autoregulation at transcriptional level

The expression of *sinI* is regulated by at least two proteins, SinR and ExpR. A previous study has shown that the loss of *sinR* results in a loss of detectable promoter activity of *sinI*, both in the presence and absence of *expR* or AHLs (McIntosh *et al.*, 2009). In agreement with that study, the result in this study showed that ExpR and AHLs activate the expression of *sinI* to a maximum at intermediate AHL

concentration (100 nM) but result in a gradually reduced expression when higher AHL concentrations are applied. Furthermore, the promoter activity of *sinR* is repressed by ExpR at higher AHL concentrations (≥200 nM) (see Fig. 6, Chapter 7). This reduced *sinR* expression, thus, results in a decrease in SinR-dependent *sinI* expression. In addition, ExpR shows a type of autoregulation at transcriptional level mediated by a binding of ExpR to its own promoter as presented above. Together with the other previously reported ExpR binding sites located upstream of *sinR* (McIntosh *et al.*, 2009) and *sinI* (Bartels *et al.*, 2007; McIntosh *et al.*, 2008), these three sites may explain the positive feedback loop (at low AHL levels) and the negative feedback loop (at high AHL levels) by which the Sin QS system appears to control AHL levels.

SinR and ExpR are both LuxR-type proteins, which typically contain a C-terminal DNA-binding domain and an N-terminal ligand-binding domain (Nasser & Reverchon, 2007). ExpR binds to the promoter of all the Sin system genes and regulates their expression in a fashion dependent on AHLs (as a ligand). A predicted SinR binding site has been located in the *sinI* promoter close to the -35 region (Bartels *et al.*, 2007). However, the question of whether SinR requires AHLs for its activation had not previously been addressed. Since SinR is encoded immediately upstream of *sinI*, it has been assumed to be dependent upon AHLs, but this has not been demonstrated. The results in this study show that in an *expR* mutant, the *sinI* promoter remained active in the absence of *sinI* and AHLs (Fig. 2, Chapter 7). Furthermore, addition of AHLs to the double mutant (*expR*/*sinI*) did not alter *sinI* promoter activity (Fig. 2, Chapter 7). This indicates that while ExpR is dependent upon AHLs for its activation of the *sinI* promoter, SinR-dependent activation is unaffected by the presence of AHLs. Also, ExpR/AHLs cannot activate the *sinI* promoter in a *sinR* mutant.

S. meliloti Rm1021 contains genes which code for at least 8 LuxR-type proteins but only one AHL synthase, SinI. Out of these LuxR-type proteins, only ExpR has been shown to be dependent upon AHLs. In addition to SinR, VisN and VisR are also LuxR-type regulators whose activities are independent of AHLs (Bahlawane *et al.*, 2008b). It is noteworthy that the expression of *sinR* and *visNR* is under QS regulation. Thus, it is plausible that these LuxR-type regulators have a non-AHL ligand. However, whether SinR binds to a ligand at all remains unknown. This is partly due to its extreme insolubility upon overexpression (Julia Mohr, 2006, Diploma Thesis).

3.2.2 Post-transcriptional regulation mediated by RNase E

To address the question of whether RNase E regulates the Sin QS in *S. meliloti*, AHLs harvested from the *rne* mutant and the mutant containing a plasmid with constitutive expression of *rne* (pRKrne) were compared to AHLs from the Rm2011 parent strain using a GFP reporter system in *E. coli*. The Rm2011 *rne* mutant contains a mini-Tn5 transposon insertion in the C-terminal region. Attempts to disrupt the N-terminal region of *rne* failed, suggesting that this region is essential for cell viability. Similar fluorescence levels were observed for the AHL extracts from the *rne* mutant and the Rm2011 parent strain, while extracts from a culture with the constitutive ectopic expression of *rne* resulted in a dramatic reduction of fluorescence (Fig. 2B, Chapter 8). As a control, qRT-PCR analysis showed that the level of *rne* mRNA increased in the mutant with the constitutive ectopic expression of *rne*

compared to the parent strain Rm2011 (Fig. 2C, Chapter 8). The result confirmed that ectopic expression of *rne* results in an elevated accumulation of *rne* mRNA and a corresponding strong reduction in AHL accumulation. Furthermore, the Rm2011 parent strain and the *rne* mutant, both carrying an IPTG-inducible pWBrne, were tested for AHL accumulation using an *A. tumefaciens* reporter system. Both strains exhibited a reduction of AHLs to non-detectable levels in the presence of IPTG (Fig. 2D, Chapter 8, upper panels). Altogether, these experiments demonstrate that overexpression of RNase E is responsible for the disruption of AHL accumulation.

To learn about the mechanism by which RNase E affects AHL accumulation, qRT-PCR analysis was performed on the *sinI* and *sinR* genes. In comparison to Rm2011, the *sinR* mRNA levels did not change significantly in the *rne* mutant or in the presence of pRKrne (Fig. 3A, Chapter 8). In contrast, a strong decrease in the amount of *sinI* mRNA was detected in the overexpressing strain (Fig. 3A, Chapter 8). Also, the IPTG-induced overexpression of *rne* in both the mutant and the parent strain resulted in decreased levels of *sinI* mRNA but not of *sinR* mRNA (Fig. 3B and C, Chapter 8). These results suggested that RNase E specifically degrades *sinI* mRNA but not *sinR* mRNA.

sinI mRNA stability was also measured in strain Rm2011 (pWBrne) grown without IPTG and compared to *sinI* mRNA stability in the same strain following the addition of IPTG. qRT-PCR was performed to determined the relative amount of *sinI* mRNA. The half-life of *sinI* mRNA was determined with two different primer pairs with very similar results (3.2 ± 0.4 min and 3.8 ± 0.2 min) As expected, the stability of *sinI* mRNA was significantly reduced upon overexpression of *rne* (1.9 ± 0.1 and 1.9 ± 0.2 min with each of the primer pairs, respectively) (Fig. 4, Chapter 8). In contrast, the stability of *sinR* and *rpoB* (internal references) mRNAs was not affected (Fig. 4C and D, Chapter 8). Using one of the primer pairs and cultures without IPTG, *sinI* mRNA stability was determined in two independent experiments at ODs of 0.5, 1.0, and 1.3. The *sinI* mRNA stability was comparable at all three ODs (Fig. 4A, Chapter 8, 0 mM IPTG). The results suggest that overexpressed *rne* specifically decreases the stability of *sinI* mRNA, leading to lower steady-state amounts.

The results from a plasmid-based reporter assay indicate that the activity of *rne* on *sinI* expression is independent of *expR* and AHLs since the *sinI* promoter activity reduced upon *rne* overexpression regardless of *expR* and *sinI* status (Fig. 6, Chapter 8). Moreover, when the 5' UTR of a control promoter (unaffected by *rne*) fused to *egfp* was replaced with the 5' UTR of *sinI*, the fluorescence from the control promoter decreased in a similar pattern as observed from the *sinI* promoter (Fig. 7, Chapter 8). But when the 5' UTR of *sinI* was substituted with that of the control promoter, the fluorescence remained unaffected. These experiments confirm that RNase E specifically targets the 5' UTR of *sinI* mRNA.

In many cases, an RNase E cleavage in the 5' UTR of bacterial mRNA is mediated by *trans*-encoded sRNAs, and the sRNA-mRNA interaction is usually Hfq dependent (Morita *et al.*, 2005). Hfq-dependent RNase E cleavage in the 5' UTR of *nifA* mRNA was also found in *Rhizobium leguminosarum*, which is important for the translation activation of NifA, the major transcriptional regulator of nitrogen fixation (Zhang & Hong, 2009). In this study, an Rm2011Δ*hfq* mutant was used

to check the involvement of Hfq. The fluorescence measured from the *hfq* mutant carrying *sinI* promoter-*egfp* fusion plasmid was reduced by 2.4 fold upon *rne* overexpression, comparable to the reduction of fluorescence in the parental strain Rm2011 containing the same plasmid (Fig. 5B, Chapter 8, data for *sinI* promoter-*egfp* fluorescence assay in Rm2011). Thus, overexpression of *rne* negatively influences *sinI* expression in an Hfq-independent manner. However, the Hfq-independent status of RNase E cleavage in the 5' UTR of *sinI* mRNA does not exclude the involvement of an sRNA in the regulation of *sinI*. A *trans*-encoded, Hfq-independent sRNA was also shown to regulate the expression of photosynthesis genes in *Rhodobacter sphaeroides* (Mank *et al.*, 2012).

Although overexpression of *rne* specifically destabilizes *sinI* mRNA, no differences in the stability of *sinI* mRNA at different points of the growth curve were detected when *rne* was not overexpressed. This shows that RNase E cleavage in the 5' UTR is an important factor in the high turnover of *sinI* mRNA but is not strongly modulated under the tested conditions. A mathematical model of the Sin QS system has been described which correlates predicted and observed behavior of the Sin QS system using the activity of the *sinI* promoter as the output and the relative abundance of ExpR, SinR, and AHLs as various inputs (McIntosh *et al.*, 2013). In that study, one basic assumption necessary for a workable model of the Sin System is that the gene products of both *sinR* and *sinI* should be rapidly degraded, allowing a finely tuned transcriptional control of AHL production that is sensitive to AHL levels. Consistent with this, the half-lives of both *sinR* and *sinI* mRNAs are in the range of typical mean chemical half-lives of RNA measured in bacteria (between 2.4 min in *Prochlorococcus* and 6.8 min in *E. coli*) (Evguenieva-Hackenberg & Klug, 2011).

3.3 Impact of the Sin QS on fitness of *S. meliloti*

3.3.1 Growth advantage of QS-deficient mutant under standard laboratory conditions

The Sin QS-activated EPS production contributes to a distinct mucoid phenotype on agar (Charoenpanich *et al.*, 2013; Glenn *et al.*, 2007; Pellock *et al.*, 2002). Under standard laboratory conditions, serial cultivations of mucoid isolates of *S. meliloti* grown as a bacterial lawn on agar appeared to gradually decrease in mucoid levels and eventually gave rise to a dry-colony phenotype. It is presumable that mutations were occurring that blocked EPS production. One possibility was that EPS production incurred a heavy cost, providing the mutant with a distinct growth advantage. In this study, a total of 30 mutants with dry phenotype were collected from different cultivations of three mucoid *S. meliloti* strains, Rm8530, Rm41, and Sm2B3001. Previously characterized dry phenotypes of *S. meliloti* strains were associated with mutations at the *expR* locus (Pellock *et al.*, 2002). Consistently, DNA sequence analyses showed that all the 30 mutants contained either a single nucleotide polymorphism or an insertion/deletion in the *expR* gene, leading to a frameshift, mismatch, or truncation. The nature of the mutations is consistent with random mutation and the locations of mutations are relatively evenly dispersed over the sequence of *expR*. The mucoid phenotype was restored via a plasmid-based copy of *expR*. Notably, *sinI* and *sinR* mutants did not appear in these experiments, perhaps because loss of *sinI* and *sinR* function can be compensated by AHLs from AHL-producing QS-efficient neighbors.

Although there are many genes essential to EPS production, only *expR* mutants arise during the serial cultivations, indicating a growth advantage of these *expR* mutants over the wild types under standard laboratory conditions. Furthermore, overexpression of *expR* seems to restrain growth. A *sinI* mutant overexpressing *expR* (through an IPTG-inducible plasmid, pBSαexpR) reaches a final OD_{600} of only 1.6, whereas the same strain overexpressing *egfp* (through pBSαegfp) reached the final OD_{600} of 2.5 after 5 days growth in TY broth supplemented with 1 μM $C_{16:1}$-HL and 0.1 mM IPTG. Normal morphology of *S. meliloti* is a 1-2 μm long rod-shape cell. In contrast, cells overexpressing *expR* showed a defective morphological phenotype (branched and/or crippled cells), while overexpression of *egfp* under comparable conditions did not affect cell morphology (Fig.5). These observations provide a hint to another possible role of ExpR in regulation of growth and cell division.

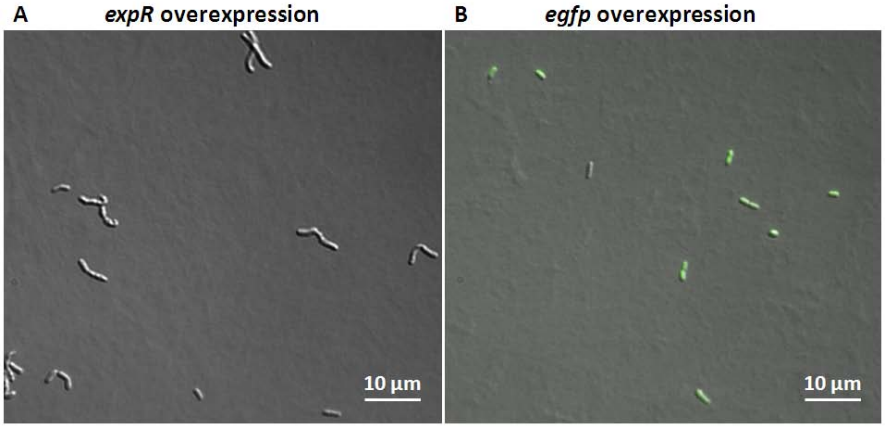

Fig. 5 Overexpression of *expR* resulted in morphological defects. Elongated, branched and/or crippled cells caused by *expR* overexpression (A) compared to normal rod-shaped cells upon *egfp* overexpression (B).

To study the invasion of mucoid cultures by the *expR* mutants, a set of *S. meliloti* strains labeled with mCherry were used. The strains included the wild type Sm2B3001 and its derivatives with disruptions in *exoB, wgeB, exoY,* and *visN*. Production of galactoglucan and succinoglycan requires *exoB*, encoding the UDP-glucose 4'- epimerase, an enzyme responsible for the production of UDP-galactose from UDP-glucose (Buendia *et al.*, 1991). *wgeB* and *exoY* are essential structural genes in the production of galactoglucan and succinoglycan, respectively (Becker *et al.*, 2002; Reuber & Walker, 1993). *visN* (downregulated by ExpR and AHLs) is a master regulator which is necessary for expression of genes related to flagella production and chemotaxis, so that a loss of *visN* results in a loss of flagella dependent motility (Nogales *et al.*, 2012; Sourjik *et al.*, 2000). After 24 days of incubation, colonies were harvested from the agar and the colony forming units (CFU) of the spontaneous *expR* mutants was estimated as a percentage of the total CFU (Fig. 1A, Chapter 9, below photo). In the wild type and *exoY* mutant colonies, *expR* mutant invasions were relatively high, at 22 ± 8 and 17 ± 4 CFU per 100 CFU. Invasions by *expR* mutants were severely restricted in the absence of *exoB, wgeB,* or *visN* (<1%, <1%, and <2%, respectively), suggesting that galactoglucan and flagella were both essential for the invasions under these conditions.

To learn more about how the *expR* mutant invades WT cultures, a two-strain competition assay was developed, in which the wild type Sm2B3001 and the competitor (labeled with mCherry) were mixed in a 9:1 ratio (wild type:competitor) before inoculation on agar plates. The competitors (referred to as strain R) included the wild type Sm2B3001, *expR, expR/exoB,* and *expR/visN* mutants. After 9 days, the mixed colonies were photographed (Fig. 1B, Chapter 9). When the wild type Sm2B3001 was included as competitor, red lines radiating from the center of the colony indicate the lines of expansion during competitive growth. Conversely, when the *expR* mutant was included as competitor, it revealed an aggressive invasion. Domination by the *expR* mutant was so strong that the mobility of the wild type was impeded. This is likely because the *expR* mutant produces flagella and thereby migrates rapidly to the expanding frontier of the colony (appearing as a red ring), presumably in search of nutrients. The *expR/exoB* double mutant exhibited a red ring similar to that observed from the *expR* mutant, while the *expR/visN* remained in the center of the mixed colony (site of inoculation) (Fig. 1B, Chapter 9). The mixed colonies were then recovered from the agar surface and the CFU of each strain was determined. The *expR* and *expR*/*exoB* mutants (as competitors) achieved >50% of the CFU, despite a 9:1 ratio at inoculation. In contrast, the wild type and the *expR*/*visN* double mutant remained at <1% of the CFU (Fig. 1B, Chapter 9, below photo), indicating that the invasion of *expR* mutant is dependent upon motility. This also confirms the previous observations that the loss of *expR* confers a strong selection advantage, since the *expR* mutant is relieved of the cost of galactoglucan production and simultaneously gains flagella to enhance motility towards nutrient.

However, the superior mobility alone does not seem to be enough to explain the invasion of the *expR*. To show this, a competitive liquid culture was applied, in which the bacterial cultures were maintained in a homogeneous mixture by constant shaking. The competing strains of interest, strain G and strain R, were mixed to a 9:1 (G:R) ratio at inoculation. After 2 days the first cultures in stationary phase were diluted in fresh medium to obtain the second cultures. The CFU of each strain in the final population of each culture was determined by serial dilution and plating to observe single colonies. The *expR* mutant displayed a clear numerical dominance over all *expR*$^+$ strains already in the final population of the first cultures (66-97%, Fig. 2A), regardless of their galactoglucan-producing status. Although *S. meliloti* has previously been shown to produce galactoglucan at high levels in liquid cultures (Hozbor *et al.*, 2004; Sorroche *et al.*, 2010), the genetic disruption of galactoglucan production (*exoB* or *wgeB*) did not prevent *expR* mutant invasions in liquid cultures. Furthermore, when both strains carried a functional *expR*, they maintained a G:R ratio of ≈9:1, regardless of the presence of *wgeB* or *exoB*. In the second cultures, the superior growth by the *expR* mutant compared to the WT was even more obvious, forming 97-99% of the CFU (Fig. 2A, Chapter 9). In contrast, strains carrying a functional *expR* performed poorly against the WT, remaining at 14-22%. Moreover, in the absence of AHLs, the *expR/sinI* double mutant was almost out-competed by the *sinI* mutant strain (Fig. 2B, Chapter 9). Invasion of *expR/sinI* double mutant could be rescued by addition of AHLs (≥50 nM) to the growth media. These results suggested that the invasions by the *expR* mutant were supported by an ExpR/AHL dependent restraint of growth.

Galactoglucan improves survival through bacterial autoaggregation and biofilm formation (Rinaudi & González, 2009; Sorroche *et al.*, 2012), colony expansion (Dilanji *et al.*, 2014; Gao *et al.*, 2012; Nogales

et al., 2012), and protection against predation (Pérez *et al.*, 2014). Yet QS-activated galactoglucan production implies heavy carbon consumption and thus likely incurs a significant metabolic cost. The loss of *expR* removes this cost, although some of it might be offset by the cost of flagella production, since this is downregulated by ExpR. During invasions on agar surface, the mutant requires its own flagella to move rapidly through the galactoglucan matrix to the expanding frontier of the colony and thereby benefits from fresh nutrients. Assisting the invasions is the ExpR-dependent restraint of growth. Thus, the regulation of multiple targets by ExpR helps explain the rapid invasions by the *expR* mutant, where the disruption of *expR* is a single event with multiple benefits. Alternative mutations to achieve the same outcome are significantly less probable since they would require multiple genetic events.

3.3.2 Survival advantage of QS-efficient wild type during desiccation

In the laboratory, bacteria are routinely cultivated under conditions that have been optimized for their growth, i.e., rich media, constant temperature and aeration. The results of this study, up to this point, showed that the Sin QS of *S. meliloti* is extremely unstable under these conditions. To be more exact, the loss-of-function mutation of *expR* is under strong negative selection pressure since *expR* restrains growth and represses bacterial mobility in the galactoglucan matrix towards the nutrient diffusion gradient. However, the presence of *expR* is evidence for its significance in nature. Therefore, an experiment was designed to simulate desiccation, a condition which frequently challenges rhizobia in natural habitats (Rinaudi & Giordano, 2010). Survival of desiccation was measured by comparing the CFU before and after a 7 day desiccation period, generating a survival rate in CFU per million CFU. The wild type Sm2B3001 showed the highest survival rate ranging from 2670 to 12630 CFU/million (average of 8300±5100) in three independent replicates (Fig. 6A). In contrast, the *expR* mutant exhibited a surprisingly low survival rate, approximately 280-fold lower than that of the wild type. In addition, the *exoB* mutant had an intermediate survival rate, approximately 6-fold lower than that of the wild type, but 45-fold higher than that of the *expR* mutant. Thus, *expR* strongly enhances fitness under desiccation, even in the absence of *expR* and EPS production.

The result also indicates that part of the fitness was lost through the disruption of *exoB*, suggesting an important role of galactose-containing macromolecules (e.g., galactoglucan, succinoglycan, glycoproteins and glycolipids) during survival of desiccation. Some of these macromolecules are secreted and thus become public goods in a cooperative population. To learn whether the fitness mediated by *expR*$^{+}$ strains could be shared with *expR* mutant individuals in the same colony, a competitive desiccation experiment was performed. For this, an *expR* mutant containing an mCherry marker and a kanamycin resistance (expRm) was used as an indicator. After desiccation and recovery on TY agar containing kanamycin, the survival rate from single strain colonies of expRm (13±5 CFU/million) is comparable to that of the *expR* mutant (31±12 CFU/million) (Fig. 6A). When grown in a competitive mixture (1:1) and subjected to desiccation, survival of expRm was moderately enhanced by the wild type and the *exoB* mutant (18-fold and 17-fold, respectively), but not by the *expR* mutant (Fig. 6B). This suggests that the *expR* mutant receives some protection from the wild type during desiccation and that this is mostly independent of *exoB*. Nevertheless, the increased survival of

expRm in mixed cultures with the wild type (≈200 CFU/million) could not match that of the wild type (≈8300 CFU/million) or the *exoB* mutant (≈1409 CFU/million). Thus, these results indicate that the advantages conferred by ExpR during desiccation are mostly private.

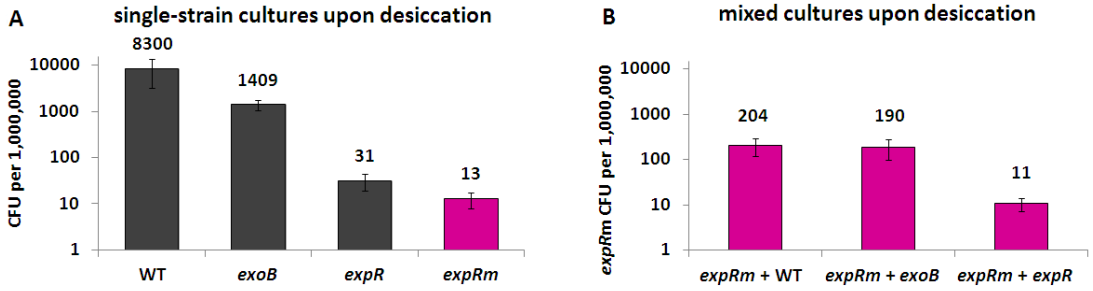

Fig. 6 *expR* is essential for dessication survival. (A) Survival rate (CFU / million CFU) was estimated by comparing the CFU of cultures exposed to desiccation with the CFU of comparable cultures without desiccation. (B) Survival rate of expRm after mixing 1:1 with WT, *exoB* or *expR* strains and exposure to desiccation. Error bars indicate standard deviation from three independent biological replicates.

The survival of rhizobia during desiccation is influenced by the presence and nature of protective media or solutes (Cliquet & Catroux, 1994), the rate of drying (Fouilleux *et al.*, 1994; Mary *et al.*, 1985), relative humidity and temperature (Mary *et al.*, 1993; Paul *et al.*, 1993), and rehydration (Kosanke *et al.*, 1992; Salema *et al.*, 1982). The physiological status of cells also influences the response of bacteria to a number of stresses. It is generally assumed that non-growing cells are more tolerant of desiccation than their actively-growing counterparts (Bale *et al.*, 1993). This assumption fits very well with the observations that the Sin QS restrains growth and enhances survival upon desiccation. However, the enhanced survival exhibited by the wild type might be the result of multiple QS-controlled processes. Although the mechanism(s) for an ExpR-dependent desiccation survival advantage are unknown, there are some plausible explanations, e.g., activation of metabolic dormancy and anhydrobiotic-like state (Billi & Potts, 2002), repair of DNA damage (Humann *et al.*, 2009), activation of trehalose production (Casteriano *et al.*, 2013; Cytryn *et al.*, 2007), and fatty acid composition alteration of bacterial membrane and cytosolic lipids (Kieft *et al.*, 1994; Teixeira *et al.*, 1996; Zikmanis *et al.*, 1982).

3.3.3 Competitive symbiotic potential is not strongly affected by *expR*

In free-living state, the QS-deficient *expR* mutant has a strong growth advantage under standard laboratory conditions, and a poor survival under desiccation. It is interesting to know how the mutant performs in a symbiotic relationship with its host plant *Medicago sativa*. To answer this question, a competitive nodule occupancy assay was performed using two pairs of *S. meliloti* strains: (1) the intensively studied *expR* mutant strain Rm1021 versus its spontaneous *expR*$^+$ derivative Rm8530, and (2) Sm2B3001, referred to as the wild type in the previously described growth competition assay, versus the *expR* mutant Rm2011. The exponentially growing cultures of the wild type and the corresponding *expR* mutant were mixed 1:1 and applied to roots of *M. sativa*. The *expR* mutant Rm1021 showed 20±2% higher nodule occupancy than its *expR*$^+$ counterpart, Rm8530 (Fig. 3A,

Chapter 9). Consistently, the *expR* mutant Rm2011 showed a 10±3 higher nodule occupancy than its *expR*$^{+}$ counterpart, Sm2B3001 (Fig. 3B, Chapter 9). Therefore, under these conditions, the *expR* mutant seems to have a slightly higher competitive symbiotic potential than the wild type.

A previous study has also reported that the nodulation efficiencies of single-strain cultures are comparable between the *expR*$^{+}$ wild type Rm8530, the *expR* mutant Rm1021, and the *expR/sinI* double mutant (Gurich & González, 2009). But the *sinI* mutant is less efficient than the wild type in nodule invasion. However, blocking flagella synthesis in the *sinI* strain completely restored its competency for establishing symbiosis to wild-type levels (Gurich & González, 2009). An interesting question for future studies would be how QS and flagella production contribute to symbiosis establishment by multi-strain cultures of *S. meliloti*.

Chapter 4: Conclusion

The Sin QS system of *S. meliloti* is a useful model for studies on cooperative bacterial responses and adaptation to environmental changes. The system consists of two basic LuxRI-type QS components, the autoinducer synthase SinI and a transcriptional regulator SinR, and an additional master transcriptional regulator ExpR. ExpR regulates all the three Sin QS genes (*sinI, sinR,* and *expR*) and many downstream genes in an AHL-dependent manner. The results of this study indicate that the direction of regulation by ExpR depends on the location of the ExpR-binding site with respect to the relevant transcription start within each promoter region.

In contrast to ExpR, SinR activates the expression of *sinI* independently of AHLs. At low population density, the AHL is produced at a basal level through SinR-activated *sinI* expression. When the AHL concentration reaches the threshold (5 nM), AHL-bound ExpR firstly induces the *sinI* expression, resulting in even higher AHL concentrations of around $\geq$ 50 nM, the minimum concentration at which many other genes are upregulated (Fig. 7). These include genes responsible for the production of symbiotically important EPS, succinoglycan and galactoglucan, genes encoding calcium-binding proteins and some transcriptional regulators. AHLs accumulate during growth of the bacterial population. When high AHL concentrations ($\geq$200 nM) are reached, ExpR/AHL represses the expression of genes involved in motility and nutrient uptake. The expression of *sinR* is also inhibited by ExpR/AHL at this stage. The results are a strong decrease of *sinI* expression and the correspondingly reduced AHL production. This probably indicates a self-restraint in response to nutrient limitation which prevents overcrowding.

AHL accumulation is not only controlled by the Sin QS itself, but also by an endonuclease, RNase E. This study shows that RNase E specifically targets the 5' UTR of *sinI* mRNA and prevents excess AHL production by degradation of *sinI* mRNA. The high turnover of *sinI* mRNA mediated by RNase E allows the Sin QS to respond rapidly to transcription control. The fact that the Sin QS regulates a multitude of genes involved in various cellular processes indicates its significance in *S. meliloti*. Despite this fact, the QS-efficient wild type cultures are typically invaded and eventually out-competed by the *expR* mutant when grown under laboratory conditions. The results show that the *expR* mutant has a growth advantage over the wild type under such conditions. This is partially because the mutant has superior motility and is relieved in the cost of galactoglucan production, but mainly because *expR* restrains growth.

The production of EPS had been thought to shield bacterial cells within biofilms from desiccation. On the contrary, the results in this study indicate that not the production of surface polysaccharides, but rather some other target of QS regulation is the major contributor to desiccation survival. One possibility is that the signal-blind mutant gains fitness advantage in nutrient rich environment through its superior, uncontrolled growth, while the self-restraining QS wild type is better-prepared for survival upon desiccation. Mechanisms of an ExpR-dependent restraint of growth are unknown, although recent studies in the rice pathogen *Burkholderia glumae*, describing the role of QS in metabolic slowing and controlling nutrient uptakes (An *et al.*, 2014), as well as stationary-phase survival (Goo *et al.*,

2012) may provide some hints. Desiccation is probably one of the most prevalent causes of death for soil bacterium in natural habitats, which makes QS a particularly important survival mechanism. This is, so far, the first study to find a definite link between desiccation resistance and QS in any bacterium. Correlation between specific environmental stimuli, the corresponding QS responses, such as the regulation of specific genes, and the impact of this regulation on survival could be one of the attractive focal areas for future studies.

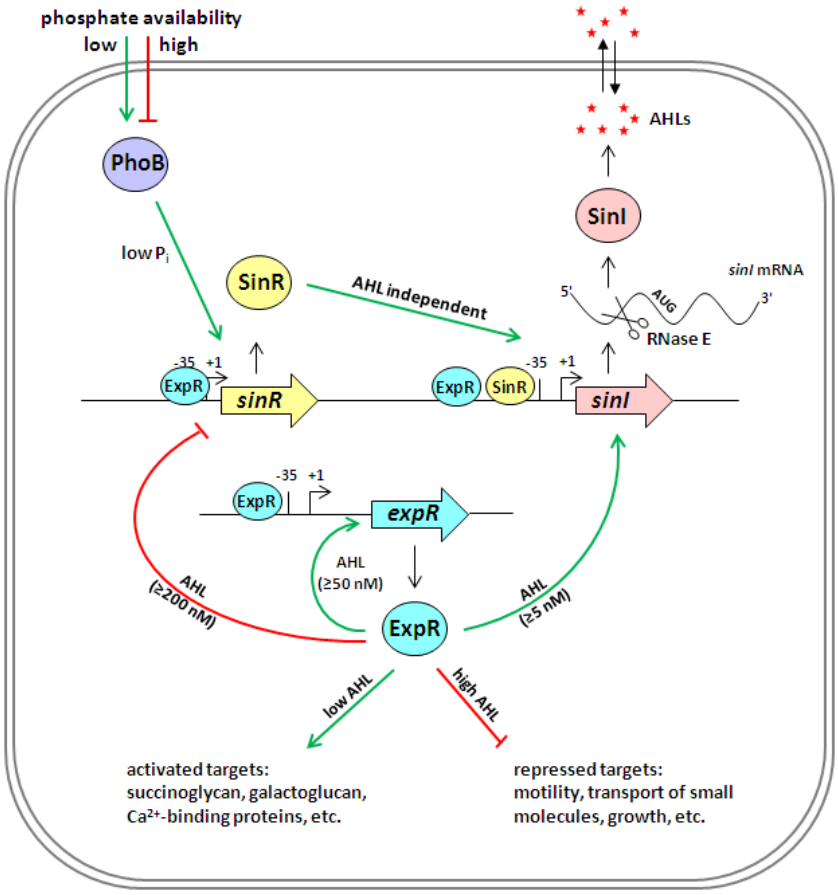

Fig. 7 Regulatory diagram of the Sin QS system in *S. meliloti*. AHL autoinducer molecules (represented by red stars) are produced by the AHL-synthase, SinI. The transcription of *sinI* is regulated by SinR and ExpR. SinR activates *sinI* transcription independently of AHLs. A predicted SinR binding site has been located in the *sinR-sinI* intergenic region close to the -35 region. ExpR regulates the three Sin genes, *sinI, sinR,* and *expR*, as well as many other target genes in an AHL-dependent manner. At low AHL concentrations, ExpR activates the expression of *sinI* and its own gene by binding to an ExpR site (upstream of the -35 region in both cases), generating a positive feedback. A negative feedback starts when the AHL concentration reaches the *sinR*-repression threshold (≥200 nM). At these concentrations, ExpR blocks *sinR* transcription by binding to the ExpR site between the *sinR* transcription start and the -35 region, which then results in a strongly reduced *sinR*-dependent expression of *sinI*. AHL accumulation is also controlled by RNase E, an endoribonuclease that is probably essential for growth. RNase E specifically targets the 5' UTR of the *sinI* mRNA. Overproduction of RNase E leads to a significant reduction of both *sinI* mRNA level and AHL accumulation. The Sin QS can be triggered by environmental stimuli, e.g. phosphate starvation. At low phosphate concentration, *sinR* expression is induced probably through PhoB, the response regulator of the PhoR/PhoB two-component system.

Chapter 5: Literature

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Chapter 6: Abbreviations

μm	micrometer
5'-UTR	5'-untranslated region
ACP	acyl carrier protein
AHL	*N*-acyl-homoserine lactone
bp	base pair
CFU	colony forming unit
cyclic-di-GMP	cyclic diguanosine monophosphate
DNA	deoxyribonucleic acid
EGFP	enhanced green fluorescent protein
EPS	exopolysaccharide
GPF	green fluorescent protein
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
mm	millimeter
mM	millimolar
mRNA	messenger RNA
nM	nanomolar
OD	optical density
PCR	polymerase chain reaction
qRT-PCR	quantitative real time PCR
QS	quorum sensing
RNA	ribonucleic acid
SAM	S-adenosylmethionine
sRNA	small regulatory RNA
TY	tryptone-yeart extract

Chapter 7: Temporal expression program of quorum sensing-based transcription regulation in *Sinorhizobium meliloti*

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Individual contributions:

- ExpR-binding site analysis using gel shift assay and DNA sequence alignment
- Construction of promoter-*egfp* reporter plasmids, including modified promoters of *expR*
- Analysis of promoter activity profile using EGFP fluorescent assay
- AHL sensitivity test
- Contributed to writing of the manuscript

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Temporal Expression Program of Quorum Sensing-Based Transcription Regulation in *Sinorhizobium meliloti*

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The Sin quorum sensing (QS) system of *S. meliloti* activates exopolysaccharide and represses flagellum production. The system consists of an *N*-acyl-homoserine lactone (AHL) synthase, SinI, and at least two LuxR-type regulators, SinR and ExpR. SinR appears to be independent of AHLs for its control of *sinI* expression, while ExpR is almost completely dependent upon AHLs. In this study, we confirmed 7 previously detected ExpR-DNA binding sites and used the consensus sequence to identify another 26 sites, some of which regulate genes previously not known to be members of the ExpR/AHL regulon. The activities of promoters dependent upon ExpR/AHL were titrated against AHL levels, with varied outcomes in AHL sensitivity. The data suggest a type of temporal expression program whereby the activity of each promoter is subject to a specific range of AHL concentrations. For example, genes responsible for exopolysaccharide production are activated at lower concentrations of AHLs than those required for the repression of genes controlling flagellum production. Several features of ExpR-regulated promoters appear to determine their response to AHLs. The location of the ExpR-binding site with respect to the relevant transcription start within each promoter region determines whether ExpR/AHL activates or represses promoter activity. Furthermore, the strength of the response is dependent upon the concentration of AHLs. We propose that this differential sensitivity to AHLs provides a bacterial colony with a transcription control program that is dynamic and precise.

The ability of bacteria to perceive population density has become known in the world of microbiology as quorum sensing (QS). This widespread bacterial mechanism facilitates the recognition of population density and an appropriate response (1). The best-studied QS signaling systems are based upon the employment of *N*-acyl-homoserine lactones (AHLs) as the signaling molecule. As might be expected, the genetic determinants of AHL production and perception are frequently integrated in complex regulatory networks and affect numerous aspects of the bacterial lifestyle (2). Typically, quorum-sensing systems operate as transcription networks regulated by a LuxR-type transcription regulator and its cognate AHL (reviewed in reference 3). While many studies on quorum sensing have focused on extending the known regulon (4–13), few have considered the temporary aspects of transcription regulation (12). Interestingly, studies on regulatory networks are revealing the dynamic behavior and precision timing of transcription control (14, 15). This raises the question as to whether quorum sensing-regulatory controlled networks exhibit similar characteristics in response to accumulating AHL levels.

In a previous study on a model bacterium for legume-rhizobium symbiosis, *Sinorhizobium meliloti*, we found a subset of genes whose expression was dependent not only on the presence of AHLs but also on the level of AHLs supplied to the growth culture of a strain incapable of producing AHLs (16). These genes, *sinI*, *sinR*, and *expR*, are all essential for quorum sensing regulation (see Fig. 1A for the regulatory scheme). *sinI* encodes an AHL synthase which catalyzes the synthesis of several long-chain AHLs, including oxo-C14-HL, oxo-$C_{16:1}$-HL, and $C_{16:1}$-HL (17–19). Upstream of the *sinI* gene and separated from it by 156 nucleotides is *sinR*, which encodes a transcription regulator controlling the activity of the promoter of *sinI* (16–18, 20). The promoter of *sinR* responds to environmental cues, such as nutrient limitation, by increasing the transcription of *sinR* (16). SinR protein is necessary for activation of the promoter of *sinI*, most likely through a SinR binding site immediately preceding the −35 position (21), and an increase in the production of SinR results in an increase in *sinI* expression (16).

The third gene of the Sin system, *expR*, is disrupted by an insertion element in the *S. meliloti* strains Rm1021 and Rm2011 (22), which are most intensively studied. A restoration of the gene confers QS capacity upon the bacterium and a strong increase in production of the symbiotically important exopolysaccharides galactoglucan and succinoglycan (11, 13, 20, 22–24). The DNA-binding activity of ExpR depends upon the presence of AHLs (21), and the same is true for its regulatory activity (16). The ExpR-AHL complex regulates a number of promoters throughout the genome. Exactly how many is unknown, but binding has been demonstrated for the promoters of genes controlling galactoglucan production (*wgeA* and *wgaA*), genes related to succinoglycan production (*exoI* and *exsH*), genes controlling flagellum production (*visNR*), and the Sin system genes *sinR* and *sinI* (16, 20, 25).

From the outset of this study, we suspected that the ExpR-AHL complex binds many more promoters in addition to the seven listed above. This is because evidence from both mRNA (12, 13) and protein (11) accumulation analyses suggests that the Sin/ExpR system regulates a multitude of genes (Fig. 1B). However, for the majority of these genes, the mechanisms underlying the regulation are unknown.

The purposes of this study were to extend the known Sin/ExpR regulon and to understand its mechanisms of regulation. Over 100

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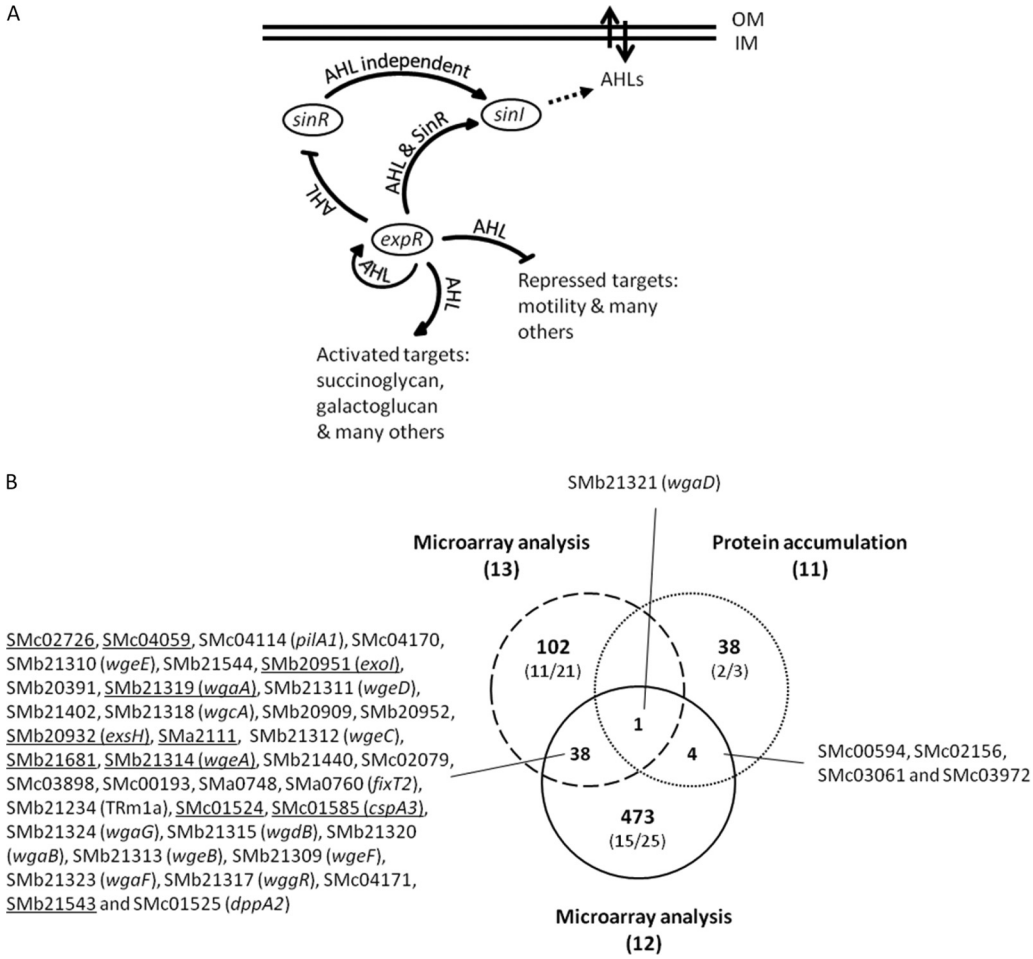

FIG 1 The Sin/ExpR quorum-sensing system regulates its own genes (*sinR*, *sinI*, and *expR*) and a multitude of other genes. (A) Regulatory diagram (based upon reference 16) showing the transcriptional control of *sinR* and *expR* by ExpR and AHLs and of *sinI* transcription by SinR and ExpR and AHLs. ExpR and AHLs also control the expression of genes important for motility, exopolysaccharide production, and many other processes that are less well known. Arrows indicate activation; flat-ended lines indicate repression. OM, outer membrane; IM, inner membrane. (B) A total of 570 genes have been identified as being regulated by ExpR and AHLs based on all three global approaches. The Venn diagram shows the overlap in genes determined to be regulated by both ExpR and AHLs in various studies (references 11 to 13, as indicated in parentheses). Bold type indicates the number of genes in each study identified as regulated. Ratios in parentheses are the number of genes immediately preceded by an ExpR site/number of genes either immediately preceded by an ExpR site or located within an operon-like structure downstream of an ExpR binding site. The numbers in the overlap are the numbers of genes identified in more than one study. Genes in the overlaps are listed; the underlined genes are situated immediately downstream of an ExpR binding site identified either in a previous study or in this study.

promoter regions were tested for binding of ExpR, and we analyzed the regions that were able to bind to ExpR for ExpR/AHL dependent expression activity. We show that the expression of each gene in the ExpR regulon is controlled by a specific AHL concentration range, resulting in a differential expression program which is sensitive to increasing levels of AHLs. Based on this information, we speculate on general regulatory mechanisms involved in the Sin/ExpR QS process and how these might be related to survival strategies.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains used in this work are shown in Table S1 in the supplemental material. Unless otherwise specified, *S. meliloti* strains were cultivated at 30°C on solid or liquid TY medium (26) or a modified MOPS (morpholinepropanesulfonic acid)-buffered minimal medium containing 48 mM MOPS (adjusted to pH 7.2 with KOH), 55 mM mannitol, 21 mM sodium glutamate, 1 mM $MgSO_4$, 250 mM $CaCl_2$, 37 mM $FeCl_3$, 48 mM H_3BO_3, 10 mM $MnSO_4$, 1.0 mM $ZnSO_4$, 0.6 mM $NaMoO_4$, 0.3 mM $CoCl_2$, 4.1 mM biotin and 0.1 mM K_2HPO_4 (27). *E. coli* strains were cultivated at 37°C in Luria-Bertani (LB) medium (28). When required, antibiotics were added at the following concentrations: 10 μg ml^{-1} nalidixic acid, 10 μg ml^{-1} tetracycline, and 200 μg ml^{-1} kanamycin for *S. meliloti* and 100 μg ml^{-1} ampicillin, 10 μg ml^{-1} tetracycline, and 50 μg ml^{-1} kanamycin for *E. coli*.

Plasmid construction. The construction of plasmids pLK64, pLK65, and pLK66, in which the promoters regions of *sinI*, *sinR*, and *expR* plus the translation start were fused to the gene encoding enhanced green fluorescent protein (*egfp*) using pPHU231, was described previously (16, 20). In similar manner, the other plasmids in the pLK series were generated using the primers listed in Table S2 in the supplemental material. Typically, the promoter regions included in the plasmid constructs contained the first 300 bp upstream of the target gene, the ATG of the target gene and ensuing ≤15 bp, and these regions were fused to the ATG of *egfp*.

Binding site cloning and DNA labeling. The promoter regions used in the electrophoretic mobility shift assays were prepared via PCR in two steps. The DNA fragments were first amplified from genomic DNA using a promoter-specific forward primer which contains a linker, GTGAGCG

GATAACAATTTCACACAGGA, together with a promoter-specific reverse primer. (These primers were also used for the construction of pLK plasmids.) The resulting PCR product was then used as a template for a second PCR using the Cy3-labeled primer GTGAGCGGATAACAATTT CACACAGGA (pUC18-unifwd-Cy3) together with an unlabeled promoter-specific reverse primer.

PCR-based mutation of the ExpR binding site upstream of *expR*. For the replacement of specific nucleotides within the ExpR binding site upstream of *expR*, internal complementary primers were designed based around the region of interest. In the first round of PCRs, each of the complementary primers was included with the matching peripheral primer carrying a specific DNA restriction site for cloning. In the second round of PCRs, both PCR products from the primary PCRs were combined and used as a template with the peripheral primers. The resulting product depended upon the annealing of the PCR products from the primary PCR round and resulted in a modified promoter region upstream of *expR*. The *wggR* promoter region was modified in a similar manner.

Expression and purification of His_6-ExpR. The expression and purification of recombinant His_6-ExpR were performed as described previously (21) with modifications. LB (250 ml) was inoculated with an overnight culture (1:100 dilution) and grown at 37°C until an optical density at 600 nm (OD_{600}) of 0.6 was reached. The *Escherichia coli* M15 culture was then induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and grown at 21°C overnight. The cell pellet was resuspended in 5 ml of lysis buffer containing 50 mM MOPS, 0.5 M NaCl, and 20 mM imidazole (pH 7.5). Cell breakage was performed by passing the cell suspension three times through a French pressure cell at 1,000 lb/in^2. Cell debris was removed by centrifugation at 10,000 × *g* for 10 min at 4°C. The supernatant was filtered with a 0.5-μm filter and then loaded onto a 1-ml nickel-nitrilotriacetic acid (Ni-NTA) affinity column. The column was washed with lysis buffer to remove nonspecifically bound proteins. Elution of the protein His_6-ExpR was achieved with an imidazole gradient (0.02 to 1.0 M). Fractions (0.5 ml) were collected and analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and protein concentration was measured using a NanoDrop1000 (PEQLAB). Purified protein was mixed with glycerol (1:1, vol/vol) and stored at −20°C for up to 2 weeks.

Electrophoretic mobility shift assay (EMSA). The EMSA protocol was as described previously (16, 21) with slight modifications. The Cy3-labeled DNA fragments were mixed with purified His_6-ExpR in a reaction buffer containing approximately 2.5 A_{260} units ml^{-1} of sonicated salmon sperm DNA (GE Healthcare) and 1.0 mg ml^{-1} of bovine serum albumin (Sigma) in a final volume of 10 μl of DNA binding buffer (10 mM Tris-HCl, pH 8.5, and 50 mM KCl). In the 10-μl reaction mixture, the Cy3-labeled DNA was included at 0.05 pmol (1 ng μl^{-1}); His_6-ExpR was included at 85 pmol (0.25 μg μl^{-1}); AHL ($C_{16:1}$-HL) was included at 100 pmol (10 μM). The reaction mixture was incubated at room temperature for 15 min. Loading buffer (2.5 μl; 20% TAE buffer, 80% glycerol) was added, and the reaction mixtures were loaded onto a 2% agarose gel. Following electrophoresis at 5 V cm^{-1} and room temperature for 1.5 h, gel images were scanned using a Typhoon 8600 variable-mode imager (Amersham Bioscience).

EGFP fluorescence assay. The EGFP fluorescence assay was modified from a previous study (16). *S. meliloti* strains were grown in MOPS minimal medium containing 0.1 mM phosphate at 30°C. Starter cultures were grown for 20 h in MOPS minimal medium and then diluted to an OD_{600} of 0.002 in fresh MOPS minimal medium. For measurement of promoter activity, bacteria were grown in 0.1-ml volumes (4 biological replicates) in a 96-well microtiter plate (Greiner) with shaking (800 rpm) at 30°C. Measurements were made at 8 h, 16 h, 24 h, and 40 h. Background fluorescence (≈600 to 700 fluorescence units/OD unit) was determined from strains carrying the pLK vector with a promoterless *egfp*. This background has not been removed from any of the promoter activity profiles (Fig. 5A). For all fluorescence assays, optical density (OD_{600}) and fluorescence (excitation, 485 nm; emission, 538 nm) were measured using the Tecan Infinite M200 reader (Tecan Trading AG, Switzerland). For measurement of promoter sensitivity to $C_{16:1}$-HL, the *sinI* mutant strain carrying one of the pLK series vectors was grown in MOPS minimal medium supplemented with various concentrations of $C_{16:1}$-HL (Cayman Chemical Company). Expression of *expR* is ≈1.5- to 2-fold higher in the wild-type strain than in the *expR* and *sinI* mutant strains (16). In order to compensate for this lower level of *expR* expression in the *sinI* mutant, a low level of ectopically expressed *expR* from the vector pBSexpR (16) was induced via the addition of 0.1 mM IPTG. We have tested the level of IPTG needed to induce ectopic expression of *expR* from pBSexpR (in which *expR* expression is controlled by IPTG-inducible *lacp*). This estimation was based upon the concentration of IPTG which restored *sinIp* activity in a *sinI* mutant strain supplemented with 1 μM AHL to those observed in the wild type (see Fig. S2 in the supplemental material). We selected this concentration of $C_{16:1}$-HL because AHL concentrations in cultures of the wild-type strain were previously estimated at ≈1.3 μM (16). $C_{16:1}$-HL and IPTG were added at the point of culture inoculation. Measurements were made at 24 h and 40 h.

Determination of *expR* transcription start site. Rapid amplification of cDNA 5′ ends (5′-RACE) PCR experiments were performed using tobacco acid pyrophosphatase (Epicentre Biotech) treatment of purified mRNA followed by ligation to the 3′end of the RNA primer GUAUGCG CGAAUUCCUGUAGAACGAACACUAGAAGAAA using T4-RNA ligase (Fermentas). After reverse transcription, the forward primer GCGC GAATTCCTGTAGAACG (based on the RNA primer above) and the *expR* specific reverse primer GTCCGGCCAGAAGAAGTCTC were used to amplify cDNA fragments in a PCR. These fragments were then cloned into the TOPO-TA vector (Invitrogen) and sequenced.

RESULTS

SinR activation of *sinIp* is independent of AHLs. LuxR-type proteins, such as SinR and ExpR, typically contain a C-terminal domain which binds to DNA and affects transcription activity, plus an N-terminal domain which binds to a ligand (3). Previous studies have shown that the loss of *sinR* results in a loss of detectable promoter activity of *sinI*, both in the presence and absence of *expR* or AHLs. Thus, SinR is necessary for a basal activity of *sinIp*, and this activity is modulated in the presence of both ExpR and AHLs (16, 20, 22, 24). Furthermore, the *expR* mutant produces significant levels of AHLs (17–20). However, the question of whether SinR requires AHLs for its activation has not been rigorously addressed. Since SinR is encoded immediately upstream of *sinI*, it has been typically assumed to be dependent upon AHLs, but this has not been demonstrated. The results in this study show that in an *expR* mutant, *sinIp* remained active in the absence of *sinI* (Fig. 2). Thus, neither *expR* nor *sinI* is necessary for the basal activity of *sinIp*. Furthermore, addition of AHLs to the double mutant (*expR sinI*) did not alter *sinIp* activity (Fig. 2). This indicates that while ExpR is dependent upon AHLs for its activation of the *sinIp*, SinR-dependent activation is unaffected by the presence of AHLs. Also, ExpR/AHLs cannot activate *sinIp* in a *sinR* mutant, consistent with previous observations (16, 20), even with ectopic expression of *expR* from pBSexpR (data not shown). However, ectopic expression of *sinR* strongly activates *sinIp* activity, even in the absence of AHLs or ExpR (16). Therefore, SinR is necessary for the AHL-independent basal activity of *sinIp*, and ExpR enhances this activity in an AHL-dependent manner (see Fig. 1A for the regulatory scheme). Since ExpR, but not SinR, is dependent upon AHLs for its regulatory activity, we focused upon the regulatory targets of ExpR.

ExpR binds to multiple binding sites. ExpR binds to a DNA sequence of imperfect or degenerate dyad symmetry, as noted previously (16). From this alignment of seven previously detected

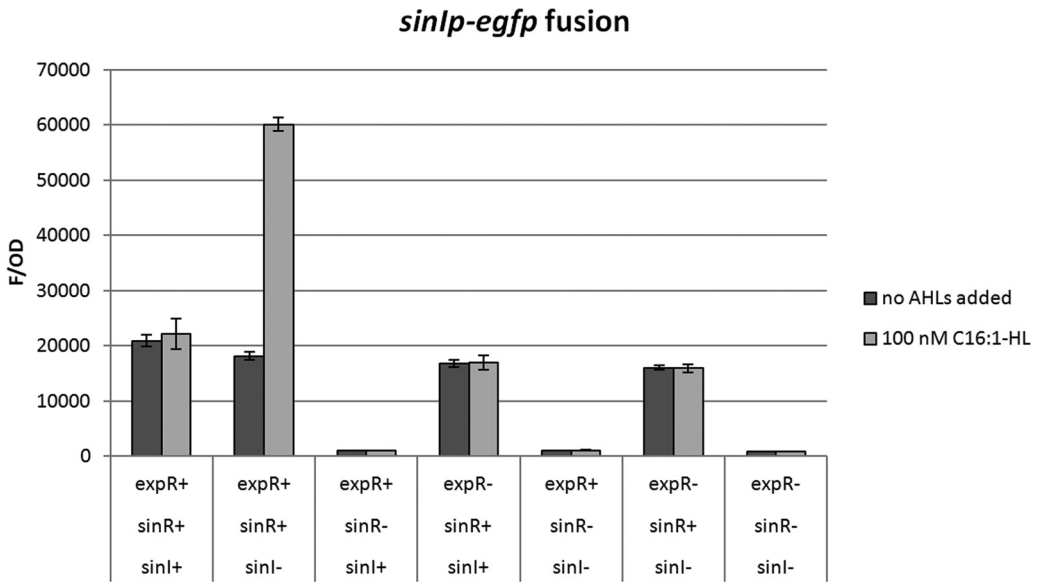

FIG 2 SinR activates promoter activity of *sinI* independently of AHLs. Strains carrying the vector pLK64, in which the promoter of *sinI* is fused to *egfp*, were measured after 24 h growth in phosphate-limiting MOPS minimal medium. The promoter activity of *sinI* is dependent upon the presence of SinR and ExpR/AHLs but in the absence of ExpR is unaffected by the presence of SinI or AHLs. The measurements were made at least three times. Error bars show errors from 4 biological replicates.

ExpR DNA-binding regions delimited to 20 nucleotides (nt), we deduced a consensus sequence which was then used with PatScan (29) at http://iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi to identify additional sites in the genome of the *S. meliloti* strain 1021. A list of hits was compiled using the following criteria: a hit must consist of 18 to 20 nucleotides with some similarity to the ExpR consensus sequence (CCCANNATTNTATTGGGG) and be located within a promoter region (≈250 bp upstream of the translation start) of a gene. Particular attention was paid to genes that were previously identified as being regulated by ExpR/AHLs, by either protein or mRNA accumulation (11–13). To test for ExpR-DNA binding, the DNA surrounding the hit was included in a gel shift binding assay together with purified His_6-ExpR and AHLs (Fig. 3). Table 1 lists the binding site sequences and the genes located downstream of each ExpR binding site.

To increase the confidence in the correct identification of each ExpR binding site, we sequentially reduced the size of the original 300-bp fragment from both ends to either include or exclude the hit and tested each derivative fragment in a gel shift assay (data not shown). In this way, the relevance of the hit for the binding between ExpR and the 300-bp fragment was established. Using this method, 26 novel binding sites were confirmed in this study. We also tested some 93 additional promoter regions in gel shift assays, selected on the basis of containing some similarity to the ExpR binding site consensus or having promoter activity which was dependent upon ExpR and AHLs. However, these did not bind to His_6-ExpR under our conditions. As negative controls, the promoters of *glgP*, *mucR*, and *pstS*, which do not contain any similarity to the ExpR binding site in their promoter regions, were included. Their lack of binding to His_6-ExpR also indicates the specificity of the ExpR-DNA interaction (Fig. 3C).

Previous attempts to identify an ExpR binding site in the promoter region of *expR* were unsuccessful, despite a weak but clear positive regulatory effect of ExpR and AHLs on this promoter (16). However, several factors prompted us to reexamine this. First, we identified a single transcription start for *expR* at 88 bp upstream of the ATG (see Fig. S1 in the supplemental material). Assuming a classical promoter structure, this places the −35 region immediately downstream of a DNA sequence that weakly resembles the ExpR binding site consensus. Second, improvements in the protein-purifying procedure provided a more active fraction of His_6-ExpR. Thus, when the improved purified His_6-ExpR was included with the 300-bp fragment upstream of the *expR* gene in an EMSA, a clear shift was observed (Fig. 3B). When a single nucleotide was exchanged (T→C) within the binding site sequence to improve resemblance to the ExpR binding site consensus, a stronger binding to ExpR was not apparent (Fig. 3D). However, when another nucleotide within this sequence was modified (C→T) to decrease similarity to the ExpR binding site consensus, the result was an almost complete lack of binding to ExpR (Fig. 3D). Together with corresponding changes in *expR* promoter activity associated with the nucleotide exchanges (see below), these experiments increase the confidence in the location and function of this ExpR binding site.

In each gel shift assay, the same amount of His_6-ExpR and target DNA was used. This allows a comparison of relative binding strengths encoded by the DNA fragments, which is apparent in Fig. 3. Addition of 10 μM $C_{16:1}$-HL to the assay (which represents a saturation concentration in our assay) did increase shift strength in some cases, particularly for the weaker binding sites. Additions of >10 μM $C_{16:1}$ did not increase shift strength (data not shown). However, the majority of the promoter regions bound to His_6-ExpR even in the absence of AHLs. This is perhaps best explained by the amount of His_6-ExpR included in the assay, which was set at a concentration that was optimal for the observance of the weakest shifts but was in excess for the stronger binding sites. Generally, a closer resemblance to the ExpR binding consensus (Table 1) correlates positively with a stronger shift. For example, binding sites

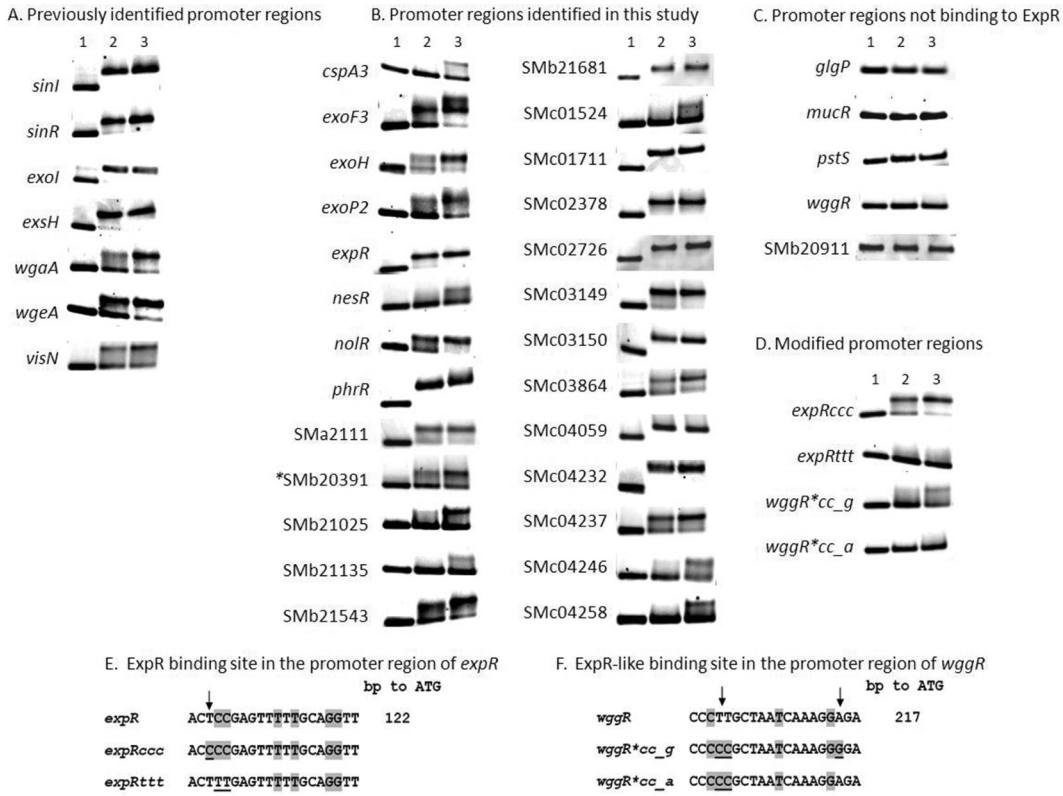

FIG 3 Electrophoretic mobility shift assays (EMSA) using purified His_6-ExpR and Cy3-labeled DNA fragments derived from the promoter regions, except for SMb20391 (asterisk), which contains an ExpR site inside the coding region. For each set of three lanes, the first lane is the negative control, which includes only the Cy3-labeled DNA from the promoter region. The second contains both the Cy3-DNA and His_6-ExpR. The third contains Cy3-DNA, His_6-ExpR, and AHLs (10 μM, $C_{16:1}$-HL). (A) EMSAs of promoter regions known to bind to ExpR from previous studies. (B) Promoter regions identified as binding to ExpR in this study. (C) Promoter regions that do not bind to ExpR, included here as negative controls. (D) Promoter regions with modifications depicted in panels E (*expR* upstream region) and F (*wggR* upstream region). The gel shift assays were carried out at least three times.

located upstream of *sinI*, *exoI*, SMb21681, SMc01711, SMc02378, SMc02726, SMc03149, SMc03150, SMc04232, and *phrR* are among those exhibiting the stronger shifts and are therefore better indicators of the binding consensus. In contrast, binding sites upstream of *cspA3*, *nesR*, SMb21135, SMc01524, SMc04246, and SMc04258 are among the weakest. For the SMc04258 and *nesR* promoters, a weak shift is observable upon addition of His_6-ExpR and AHLs (Fig. 3), although we could not detect any expression activity from these promoters. Conversely, such a weak shift was also observed with the promoter region of SMc01524 (Fig. 3), and yet this appears to be adequate for a ≥2-fold reduction in promoter activity (see Fig. S2 in the supplemental material). This was not the case with the control promoters (*mucR*, *pstS*, and *glgP*) and those of *wggR* and SMb20911, where addition of His_6-ExpR and AHLs clearly did not induce a shift (Fig. 3). Typically, sites with 3 or 4 Cs on the gene-distal side and 3 or 4 Gs on the gene-proximal side separated by 10 to 12 nucleotides rich in A and T are better suited for binding (Table 1). The presence of Gs or Cs in the A/T-rich regions appears to weaken the shift, e.g., SMb21543 and SMb21135. Similarly, the presence of As or Ts in the G or C regions may weaken the shift, e.g., *wgeA* and *wgaA*. A new 19-nucleotide consensus sequence was derived from all 33 binding sites tested in this study. It can be represented as CCCCAAAAATTTT TTGGGG (Table 1). This is comparable to a recent study on the *Pseudomonas aeruginosa* LuxR-type regulator, LasR (5), where the consensus sequence is based upon binding sites in 14 promoters.

Promoter-*egfp* reporter assays reveal effects of ExpR and AHLs on promoter activity. ExpR in the presence of AHLs regulates the promoter activity of *sinI*, *sinR*, *expR*, *wgaA*, *wgeA*, *wggR* (12, 16, 20, 31), *exoI*, *exsH*, *exoH* (23), and *visN* (12, 25), as determined by real-time PCR and promoter-reporter fusions. Good congruence between promoter activities reported here (Fig. 4 and 5; also, see Fig. S2 in the supplemental material) and those in other studies (12, 13, 16, 20) provides for a high confidence in these results, despite variation in the use of strains and culture conditions. Three strains, SM2B3001 (wild type, with a functional *expR*), SM2B4001 (*sinI*) and Rm2011 (*expR*), were used to establish the role of ExpR and AHLs in the regulation of promoter activity. All promoter regions which bound to His_6-ExpR in the gel shift assay were fused to *egfp* in the plasmid pPHU231. As controls, promoter regions which do not bind to His_6-ExpR were also fused to *egfp*. Two examples of these are those of *glgP* (glycogen production/breakdown) and *pstS* (phosphate transport during phosphate limiting growth), selected as examples of active promoters that are not affected by QS.

The *expR* promoter (Fig. 5) responds to ExpR and AHLs, as was previously reported (16). Nucleotide replacements in the binding sequence correlated well with changes in promoter activity. The effect of the T→C change was not obvious in a gel shift assay (Fig. 3B and D), but it did result in a significant promoter activity increase in response to ExpR and AHLs (Fig. 5). Furthermore, the C→T change almost completely removed not only the binding to

TABLE 1 ExpR binding sites and their relevant surrounding features

Direction of regulation by AHL-ExpR	Gene[a]	Gene product description	% amino acid homology[b]		ExpR binding site			% nucleotide homology[f]	
			S. medicae	*S. fredii*	DNA sequence[c]	No. of bp to translation start[d]	Position relative to transcription start[e]	*S. medicae*	*S. fredii*
Up-regulated	**SMb20932** or ***exsH***	Glucan endo-1,3-beta-D-glucosidase, succinoglycan production	93	89	TCCCCCAAACTCTTGGGGGT	106	−43†	90	70
	SMb20951 or ***exoI******	Succinoglycan biosynthesis protein			CACCCATAGTTTTGAAGGAT	69			
	SMb20954 or *exoH**	Succinyltransferase protein, succinoglycan production	94		TGCCCCAAAATATTGCGGGG	257	−181†	70	
	SMb21314 or ***wgeA****	Putative Ca^{2+}-binding protein, galactoglucan biosynthesis	98		CTCCTAAAATTATATTGTAC	185	−102†	95	
	SMb21319 or ***wgaA****	Putative membrane protein, galactoglucan biosynthesis	93		GCCCCAAGAATCTATTGTTG	135	−43†	95	
	SMb21543	Putative hemolysin-adenlyate cyclase, Ca^{2+}-binding protein	95	93	CACCCCCGCCTTTGTGGGGG	150		80	85
	SMc00168 or ***sinI***	AHL autoinducer synthesis	96	91	CCCCCACAAATCTATTGGGA	106	−78†‡	90	90
	SMc03150	Uncharacterized transcriptional regulator	89	74	AACCCGTAAAAACCAAGGGG	102		100	
	SMc04237	Uncharacterized protein	93	80	TCCCCTTAAATGGCGGGGTT	60	−42†		
	SMc05026 or *expR*	LuxR-type transcriptional regulator	99	98	ACTCCGAGTTTTTGCAGGTT	129	−41†¶	100	95
	SMa2111	Putative hemolysin-type calcium-binding protein	93		ATCCAGTGAGAATTGGGAGT	64	−46†		
Down-regulated	SMb21135*	Periplasmic solute-binding protein, transport of sugar amines	96	96	GCCCAAGGCATTTGATGGGT	81	−11†	85	80
	SMb21245 or *exoF3*	Exopolysaccharide export outer membrane protein	86	72	CTCTAAAAATTCAATAAGTT	188	−150†	90	
	SMc00170 or ***sinR***	LuxR-type transcriptional regulator of *sinI*	96	86	AACCATGTTTATTATAGGGG	29	−5†§	100	95
	SMc01110 or *phrR*	DNA-binding protein, pH-regulated regulator	100	93	CCCCTAAAATTGCATAGTGG	121	+2†	90	100
	SMc01524*	Dipeptidase	95	90	TTCCCGGATATTCATTGAAA	111	−39†	100	85
	SMc02378	Probable glycine betaine ABC transporter	97	94	AACCCATCAATTATTTGTGT	238		100	
	SMc03015 or ***visN****	LuxR-type transcriptional regulator of motility genes	93	80	CGCCCTGTCTTTTTGGGGGC	147		90	85
	SMc03864	Putative amino acid-binding periplasmic ABC transporter	96	93	TCCCCATAGATTTTATGACA	42	−19†	100	75
	SMc04246	Hypothetical transmembrane signal peptide protein	83	75	TGCCGTACGTTTATGAGGGA	116		70	
	SMc04059	Uncharacterized protein			TTCCCATTAGAAGCCACGGG	151	−112†		
Regulation unclear	SMc01585 or *cspA3*	Cold shock transcriptional regulator	100	97	AACCCATCACTCTTACTGTG	227		100	85
	SMc05009 or *nolR*	Nodulation protein, negative regulator of the *nod* regulon	93	89	TACCCCCATTTCTTGCAGGG	96		95	90
Regulation not detected	SMb21025	Uncharacterized protein			GCCCCTAAGATGAAACGGGG	146			
	SMb21070 or *exoP2*	MPA1 family auxiliary surface saccharide export protein		74	AACCTACTCCATTTGGGGGC	227			
	SMc01711	Uncharacterized protein			CCCCTAAAATTGTTTTGGTT	172			
	SMc03149	Uncharacterized protein			ATCCTATAAAAGATGAAGGG	47			
	SMc04032 or *nesR*	LuxR-type transcriptional regulator			CGCCCAGCTATAAGAAGTGA	42			
	SMc04232	Putative glycine-rich transmembrane protein			ACCCCCTTTTTTTAGTGGTA	113			
	SMc04258*	ABC transporter, permease	98	94	AGCCCCGCACTTTGACGGGG	169		85	
	SMc02726	Hemin-binding membrane protein (ShmR)			ACCCCTAGATTTATATTGAC	145			
	SMb21681	Uncharacterized protein			ACCATTAAAACGTAGGGTAG	35			
	SMb20391	Cellulose synthase (UDP forming)	95	88	GGCCCCAATTTCTATTGGGT	197**		95	95

[a] Asterisks indicate genes that are the first gene in an operon-like structure, whereas the other genes are probably monocistronic. Gene names in bold indicate those which contain ExpR binding sites reported in previous studies.
[b] Similarity (≥70% identical amino acids) between the predicted protein in *S. meliloti* Rm1021 and those in *S. medicae* and *S. fredii*.
[c] The frequency of each nucleotide within the ExpR consensus sequence is indicated at the bottom.
[d] Number of nucleotides between the middle of the ExpR binding site (indicated at the top by the inverted triangle) and the ATG of the gene. **, the ExpR site is 186 bp after the ATG.
[e] Position of the middle of the ExpR binding site with respect to the transcription start, where known. Data are from references 16 (§), 20 (‡), and 30 (†) and from this study (¶).
[f] Identity (≥70%) between the ExpR binding sites found in *S. meliloti* and those detected in *S. medicae* and *S. fredii*. See Table S3 in the supplemental material for names of homologous genes in *S. medicae* and *S. fredii*.

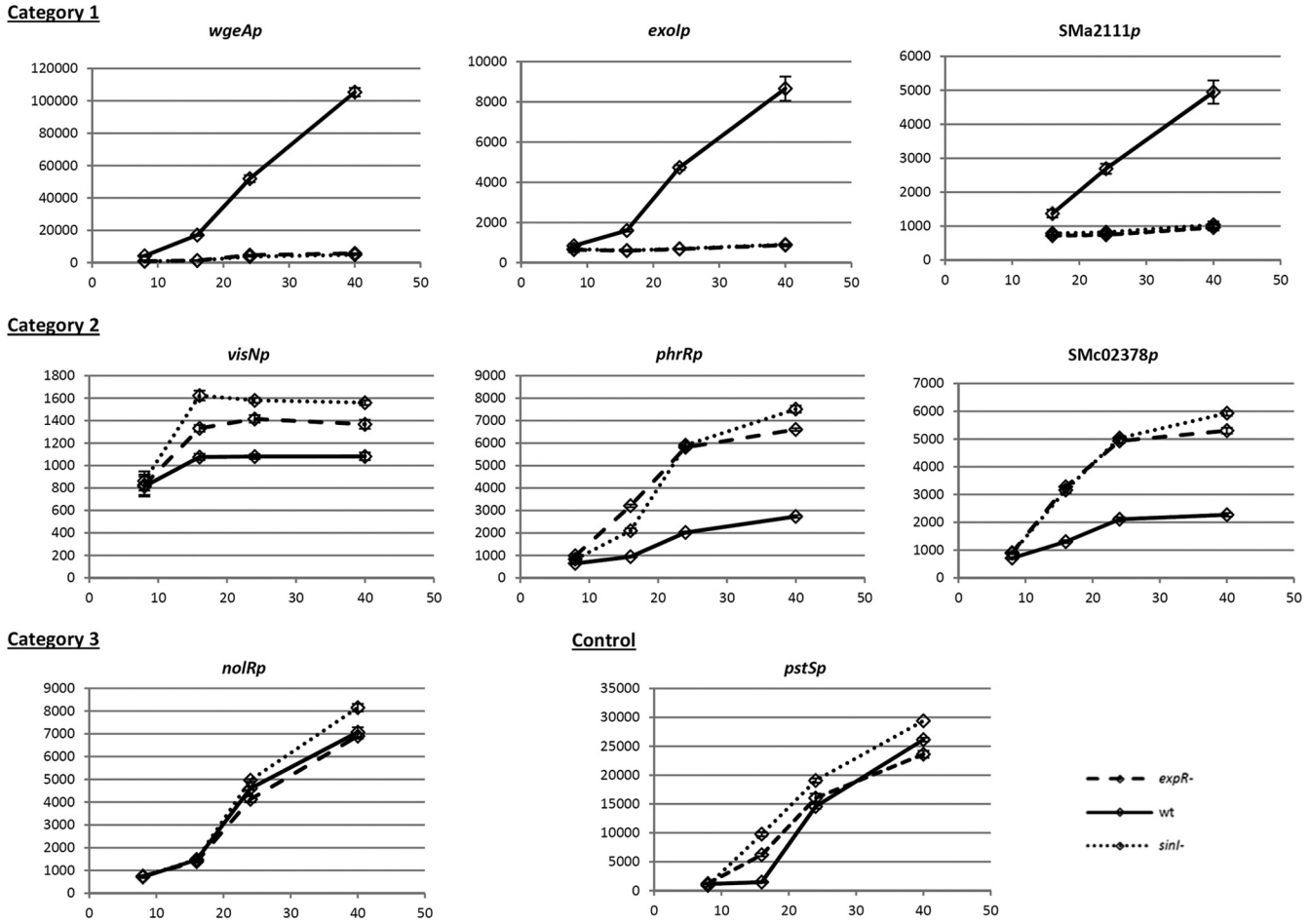

FIG 4 Selected examples of promoters with various dependence upon the presence of ExpR and AHLs. Promoter regions were fused to *egfp* in plasmid pPHU231 and grown in three *S. meliloti* strains: SM2B3001 (wild type), SM2B4001 (*sinI*), and Rm2011 (*expR*). Fluorescence units per unit of optical density (F/OD; *y* axis) were measured for each promoter at the indicated time points (hours; *x* axis). Category 1, ≥2-fold-upregulated promoters in the presence of ExpR and AHLs which bind to His_6-ExpR; category 2, ≥2-fold-downregulated promoters in the presence of ExpR and AHLs which bind to His_6-ExpR; category 3, promoter region (*nolR*) which binds to His_6-ExpR but is not regulated by ExpR and AHLs; control, promoter region (*pstS*) which neither binds to His_6-ExpR and is not regulated by ExpR and AHLs. The measurements were made at least three times. Error bars show errors derived from 4 biological replicates.

ExpR (Fig. 3D) but also the activating effect from ExpR and AHLs (Fig. 5A).

Other promoter regions containing ExpR binding sites showed a variety of responses to the presence of ExpR and AHLs (see Fig. S2 in the supplemental material). These fall into several categories. In the first are those promoters which are upregulated in the presence of ExpR and AHLs ≥2-fold. In this category are the promoters of *sinI*, *wgeA*, *wgaA*, *exoI*, and *exsH*, to which we add those of *expR*, *exoH*, SMb21543, SMc04237, SMc03150, and SMa2111. For these promoters, maximal activation under such growth conditions requires the presence of both ExpR and AHLs (Fig. 4 and 5; also, see Fig. S2). For example, the *exoH* promoter responds to a combination of ExpR and AHLs by a 3- to 4-fold increase in activity, but not if either ExpR or AHL is lacking. In the case of SMc03150, promoter activity (see Fig. S2 in the supplemental material) was observable only when a ribosome binding site from another gene (*sinI*) was inserted between the promoter of SMc03150 and *egfp*. This may be because our methods of detection are not sensitive enough, or because the native ribosome binding site and translation start were misidentified.

In the second category are those promoters where ExpR and AHLs downregulate activity ≥2-fold. In this category are the promoters of *sinR* and *visNR* plus the promoters identified in this study which include those of SMc01110 (*phrR*), SMc01524, SMc02378, SMc03864, and SMc04059 (see Fig. S2 in the supplemental material). As was the case of the ExpR/AHL activated promoters, repression of these promoters requires the presence of both ExpR and AHLs.

In the third category are promoters which contain a binding site but whose activity is affected by ExpR and AHLs, either negatively or positively, <2-fold. In this category are the promoters of SMc05009 (*nolR*), SMb21135, SMc04246, SMc01585 (*cspA3*), and SMb21245 (*exoF3*) (see Fig. S2 in the supplemental material). In the case of the promoter region of *nolR*, expression activity was observed only when a downstream (+24 bp) sequence (which included a likely ribosome binding site and alternative ATG) was included in the promoter-*egfp* fusion. In the *S. meliloti* strains Rm1021 and Rm2011 (used in this study), *nolR* is inactivated by a frameshift mutation (32). In the presence of a functional copy of *nolR*, activity from this promoter region was reduced (data not

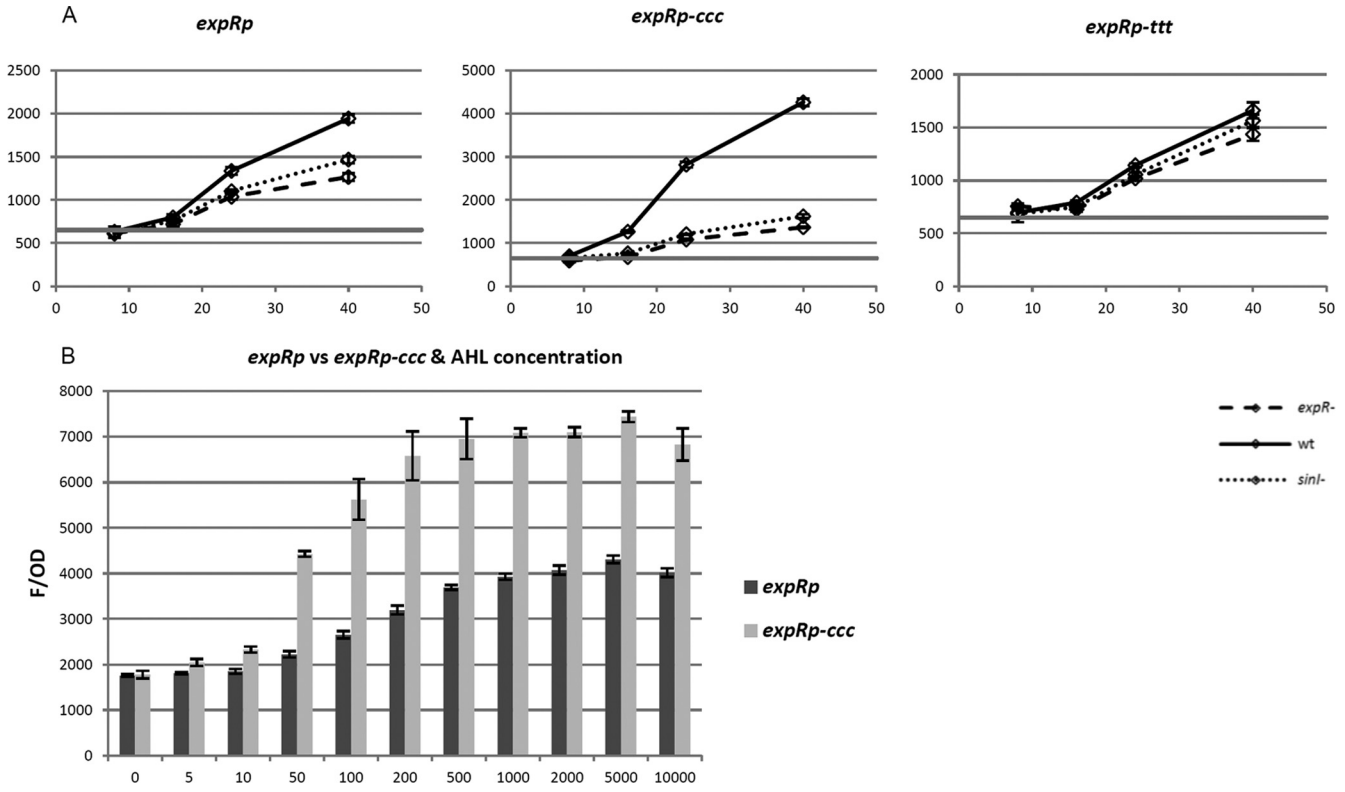

FIG 5 Response of *expRp* to ExpR and AHLs. The *expR* promoter region was fused to *egfp* in plasmid pPHU231. Growth conditions were comparable to those used in the experiment whose results are shown in Fig. 4. The gray line indicates background fluorescence (see Materials and Methods). The effects of the CCC and TTT alterations (see Fig. 3E for details) are shown (A); also, the effects of various concentrations of supplemented AHL are shown (B). The measurements were made at least three times. Error bars show errors derived from 4 biological replicates.

shown), consistent with the negative autoregulation properties of NolR as reported previously (33). Despite a previous study reporting variation in *nolR* expression with increasing population density in *S. meliloti* AK6321 (34) and the presence of an ExpR binding site upstream of *nolR* in S. meliloti 2011, ExpR did not greatly affect the activity detected from the *nolR* promoter region in our study. One possibility is that this region of DNA carries multiple promoters, which may depend upon some interaction between ExpR and AHLs and signals not present in our growth conditions. In *S. meliloti* AK631, *nolR* expression was affected by a number of environmental stimuli, such as nutrients, pH, and oxygen (34).

In a fourth category are DNA regions located upstream of an annotated gene which contain a binding site but do not contain detectable promoter activity under our conditions. These binding sites are located upstream of the genes SMb21025, SMb21071 (*exoP2*), SMc01711, SMc03149, SMc04032 (*nesR*), SMc04232, and SMc04258. For regions containing an ExpR binding site but lacking detectable promoter activity, it is possible that their downstream genes have falsely annotated translation starts (as was the case for *nolR*) or that mutations accumulating in our lab strain Rm2011 have rendered their promoters inactive. Another possibility is that these promoters are dependent upon external signals not present in these growth conditions. Yet another possibility is that these binding sites are components of ExpR/AHL-regulated promoters but that their activity is too weak for our methods of detection. For example, Patankar and González (35) reported a phenotype associated with *nesR* (SMc04032) disruption. However, we were unable to detect any activity from the *nesR* promoter, in either the presence or absence of ExpR or AHLs.

In a fifth category is the occurrence of an ExpR binding site located inside a coding region. The gene SMb20391 is annotated as a cellulose synthase. Using PatScan, we located an ExpR binding site downstream of the annotated ATG translation start for this gene. This site binds to His_6-ExpR in a gel shift assay, but we have not tested for promoter activity surrounding the binding site. In one other case, the ExpR binding site controlling activity of the promoter of *exoH* is located inside the coding region of the upstream gene SMb20953.

Several promoter regions do not appear to bind to His_6-ExpR in our gel shift assays despite a significant dependence upon ExpR/AHL for expression activity. These include the promoters of *wggR*, SMc04171, and SMb20911 (see Fig. S2 in the supplemental material). Expression of these genes was previously reported to be controlled by ExpR and AHLs (12), and we have reproduced those results. (See also a recent study by Gao et al. [36] for the dependence of *wggRp* on various concentrations of AHLs.) We do not know why these promoters do not bind to His_6-ExpR in our gel shift assays (see Fig. 3C for *wggR* and SMb20911) despite strong promoter activation (>10-fold for *wggR* and >3-fold for SMc04171) and repression (>30-fold for SMb20911) dependent upon ExpR and AHLs. Nonetheless, we selected the *wggR* promoter region as a case study and looked for any possible location in the sequence that might contain a weak ExpR binding site. Similarly to the approach taken for *expRp*, we replaced selected

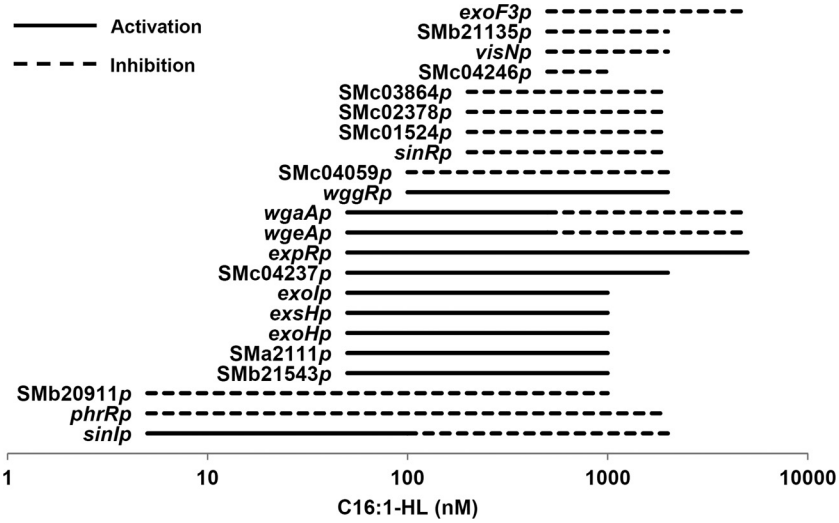

FIG 6 Overview of the sensitivity of ExpR-regulated promoters in the *sinI* mutant supplemented with different concentrations of AHL. The source data from which this overview is derived are shown in Fig. S3 in the supplemental material. Solid lines indicate a positive response of a promoter to AHL. Broken lines indicate a negative response. Line beginnings and endings indicate the range of AHL concentrations to which the promoter responded, indicated by asterisks in Fig. S3. The following concentrations (nM) were used: 0, 5, 10, 50, 100, 200, 500, 1,000, 2,000, 5,000, and 10,000.

nucleotides at two of the best-fitting locations to increase similarity to the ExpR binding site consensus. While these alterations did allow binding between His_6-ExpR and the altered promoter region of *wggR* (Fig. 3D), they did not affect the activity of *wggRp* (data not shown). Therefore, for the promoters of *wggR*, SMc04171, and SMb20911, it is possible that the control exerted by ExpR/AHLs is through an intermediate(s) or that their binding to ExpR is too weak to be detected in our assay or dependent on factors absent from the assays.

Correlating promoter response to AHL concentrations in growth medium. In a previous study, it was shown that the ExpR-dependent promoter activities of *sinR*, *sinI*, and *expR* are not only dependent upon the presence of AHLs but also sensitive to the level of AHLs (16). In that study, it was demonstrated that the promoter of *sinI* responds to very low concentrations (<5 nM) of supplemented AHLs in a culture of a mutant incapable of producing its own AHLs. In contrast, the promoters of *expR* and *sinR* required substantially higher concentrations (50 nM and 200 nM, respectively) of supplemented AHLs before a response was observable. In this study, we applied the same approach to all of the promoters that respond to the presence of ExpR and AHLs. A strain incapable of producing AHLs (SM2B4001, *sinI* mutant) was grown in the presence of various concentrations of supplemented $C_{16:1}$-HL. The resulting change in promoter activity relative to the concentration of $C_{16:1}$-HL, as determined by promoter-*egfp* fusions, is presented in Fig. S3 in the supplemental material and summarized in Fig. 6, showing the minimal and maximal AHL concentrations that induced a change in promoter activity after 40 h. Interestingly, each promoter responded to a specific range of AHL concentrations. For example, similar to *sinIp*, the promoters of *phrR* and SMb20911 responded to very low levels of AHLs (5 to 10 nM). However, unlike *sinIp*, their response to the presence of AHLs was negative. All other promoters required higher levels of AHLs for a response. Addition of 50 to 100 nM was sufficient to induce regulation of the promoters of *expR*, SMc04237, SMb21543, SMa2111, and genes controlling exopolysaccharide production (*exoH*, *exsH*, *exoI*, *wgeA*, and *wgaA*) (see Fig. S3 in the supplemental material). Interestingly, all of these promoters respond positively to the addition of AHLs. In contrast, most of the promoters that are repressed by AHLs required a higher level (100 to 1,000 nM) before a response was observable (see Fig. S3). These include promoters of SMc04059, *sinR*, SMc01524, SMc02378, and SMc03864, which responded at 100 to 200 nM, and SMc04246, *visN*, SMb21135, and *exoF3*, which responded at 500 nM. Thus, from these data, a fascinating pattern emerges: promoters repressed by AHLs tend to require higher levels of AHLs for this response, while promoters activated by AHLs tend to begin responding at lower levels. Three exceptions to this pattern are the promoters of *phrR*, SMc04059, and SMb20911, which respond negatively to AHLs.

Interestingly, the majority of promoters did not respond to changes in AHL levels above 2,000 nM. This suggests that most promoters have a specific lower and a common upper limit in their sensitivity to AHLs. Between these limits, promoter activity varies with AHL concentrations.

Another interesting observation regards the opposing effects on promoter activity from lower versus higher levels of AHL. For example, the promoter of *sinI* is activated by low levels of AHL while higher levels of AHL reduced activity (16). In a similar fashion, the activity profiles of the promoters of *wgaA* and *wgeA* were almost inactive in the absence of AHLs (Fig. 4; also, see Fig. S2 in the supplemental material) and increasingly active with increasing levels of AHL to a maximal activity at 500 nM (see Fig. S3 in the supplemental material). Intriguingly, as the levels of AHL were further increased to 2,000 nM, these promoters responded by decreasing in activity, so that both exhibited a lower activity (*wgaAp*, 5-fold; *wgeAp*, 3.5-fold) at 2,000 nM compared to that at 500 nM. Of all the promoters measured in this study, only those controlling the *wga* and *wge* operons and *sinI* showed such clear double-response effects that depend on AHL levels. In the case of *sinIp*, the second (negative) response is mediated not by an ExpR binding site located upstream of *sinI* itself but by another site upstream of

sinR. Binding of ExpR to the site upstream of *sinR* results in a decrease of *sinR* expression and thus a decrease in SinR-dependent *sinI* expression (16). Likewise, in the case of *wga* and *wge* promoters, the second response may be due to the ExpR/AHL-dependent regulation of other genes related to the activity of these promoters.

DISCUSSION

QS is a social strategy that facilitates the detection of population density and the appropriate regulation of the production of common goods and other survival-related strategies. The interest in defining AHL-based QS regulons is evident from the number of studies that have focused on regulon determination and include organisms such as *Burkholderia* (4), *Pseudomonas* (5), *Vibrio* (6–9), *Agrobacterium* (10), and *Sinorhizobium* (11–13). *S. meliloti* contains genes which code for at least 8 LuxR-type proteins but only one AHL synthase, SinI. ExpR, SinI, and SinR, together with the long-chain AHLs produced by SinI, are essential for the Sin QS system. To date, only one of the LuxR-type proteins, ExpR, has been shown to be dependent upon the long-chain AHLs produced by SinI. The results in this study strongly indicate that SinR is independent of the long-chain AHLs for its activation of the promoter of *sinI* (Fig. 2). Likewise, VisN and VisR are also LuxR-type regulators whose activities are independent of AHLs (25). Interestingly, the N-terminal receiver domains of 11 other LuxR-type proteins, including TraR of *A. tumefaciens*, LasR of *P. aeruginosa*, and LuxR of *V. fischeri*, have been aligned and the conserved AHL-interacting residues highlighted (3). Compared to that analysis, ExpR contains most of the conserved AHL-interacting residues, while SinR, VisN, and VisR appear to differ from the consensus at the most conserved region (residues 58 to 77) upon alignment using CLUSTALW (data not shown). This fits with our observations that ExpR binds to an AHL and controls expression of the genes coding for VisN, VisR, and SinR, placing them under QS regulation, while the SinR, VisN, and VisR proteins have activities that are independent of AHLs. VisN, VisR, and SinR have not been characterized with respect to the presence or nature of an activating ligand. This is partly due to the highly insoluble nature of these proteins upon overexpression (our unpublished data). ExpR, on the other hand, is soluble upon overexpression, even in the absence of AHLs, making it suitable for this study.

Regulatory targets of ExpR. A variety of methods have been used to detect *S. meliloti* genes that are regulated by QS, including transcriptomics and proteomics (Fig. 1B). Microarray approaches found over 100 genes (13) and almost 500 genes (12) regulated by ExpR in the presence of AHLs. A protein two-dimensional gel separation approach found 38 proteins affected at least 2-fold in their accumulation (11) by ExpR in the presence of AHLs. Curiously, the overlap of genes identified as being regulated by ExpR in different studies is surprisingly low (Fig. 1B), prompting some speculation over differences in bacterial strains and culture conditions (37). Furthermore, each promoter differs in its response to QS, both in degree and in timing (Fig. 4; also, see Fig. S2 in the supplemental material). Some genes respond early, for example, because of a higher sensitivity to AHL accumulation, while others require higher levels of AHLs before a response is observable (Fig. 6) (see also Table S1 in reference 12). Therefore, genes detected as controlled by ExpR in the presence of AHLs will depend upon the point in the growth phase at which the mRNA or proteins are harvested and the method used for the harvest.

The total number of ExpR binding sites discovered thus far in *S. meliloti* is 33, dispersed among all three replicons. Most of the ExpR binding sites in *S. meliloti* also appear in *S. medicae* and *S. fredii* (see Table 1; also, see Table S3 in the supplemental material), although to our knowledge these have never been tested. In *S. meliloti*, eight sites are located upstream of operon-like arrangements. When genes within operon-like arrangements are taken into account, these 33 sites appear to control 66 to 71 genes. The study by Gurich and González (12) is the most sensitive study to date, revealing a total of 473 genes whose expression is dependent upon ExpR in the presence of AHLs, slightly less than 8% of the genome (38). Within this group of 473, only 25 genes are located downstream of an identified ExpR binding site, either within an operon-like structure or monocistronic. But perhaps this should be expected given that some genes directly regulated by ExpR are themselves characterized as transcription regulators.

One prominent example is *phrR* encoding a global transcription regulator. Expression activity of this gene was previously identified as responding to various stress conditions, such as low pH and high concentrations of ethanol, Zn^{2+}, Cu^{2+}, or H_2O_2 (39). More recently, in *Rhizobium leguminosarum*, a gene highly similar to *phrR*, *praR*, was shown to be integrated in QS regulation (40), including repression of *praR* expression by the *R. leguminosarum* ExpR homologue. According to our data, the *S. meliloti* ExpR also negatively regulates the promoter of *phrR* in the presence of AHLs.

Perhaps the most important novel ExpR site is located upstream of *expR*, which presumably confers the property of self-regulation. Together with the other previously reported sites located upstream of *sinR* (16) and *sinI* (20, 21), these three sites may explain the positive feedback loop (at low AHL levels) and the negative feedback loop (at high AHL levels) by which the Sin/ExpR system appears to control AHL levels. Most genes preceded by a novel ExpR binding site are not yet characterized for their function, such as SMc04237 (unknown function), SMc03150 (uncharacterized transcription regulator), SMb21543 (putative adenylate cyclase), and SMa2111 (putative hemolysin-type Ca^{+}-binding protein).

Characteristics of promoter regulation by ExpR. Of considerable interest are the molecular mechanisms by which QS regulates its target genes. A theoretical model of bacterial transcription found regulatory logic functions of plausible complexity by varying only two factors: strength of interaction between regulatory proteins and the relative positions of the relevant protein-binding DNA sequences in the *cis*-regulatory region (41). One example of this is the TyrR protein of *E. coli* (reviewed in reference 42), which can act as a repressor or activator of transcription for its eight known target promoters. Transcription activation and repression by TyrR are effected by binding to its TyrR box, and the direction of regulation is determined by the location of the TyrR box relative to the promoter. Tyrosine is the most important ligand which controls multimerization states of TyrR and affects binding to the TyrR box. TyrR boxes are present in two basic classes. Strong TyrR boxes can bind to TyrR even in the absence of tyrosine, but weaker-affinity boxes require the presence of tyrosine. The mechanism for repression can involve the exclusion of RNA polymerase from the promoter or interference with the ability of bound RNA polymerase to form open complexes or to exit the promoter. For transcription activation, TyrR can bind upstream of a promoter and interact with the α-subunit of the RNA polymerase. Finally, intra-

cellular levels of TyrR protein are thought to be critical for determining regulatory outcomes.

Somewhat analogous to the *E. coli* TyrR paradigm, there are at least three factors that determine the strength of the regulatory effect of the *S. meliloti* ExpR/AHL combination on its regulon: (i) the abundance of ExpR, (ii) the abundance of AHLs, and (iii) the DNA sequence in and around each ExpR binding site. Evidence for factor 1 was reported in a previous study (16), where levels of ExpR were controlled via expression from an IPTG promoter. In that study, various levels of ExpR intensified or weakened the promoter responses correspondingly.

Evidence for the abundance of AHLs as a determinant of gene expression was revealed in this study when AHL levels were varied in cultures carrying a promoter-*egfp* fusion (see Fig. S3 in the supplemental material). Promoter activity clearly depends upon the concentration of AHLs. However, in many cases, the effect of AHL addition on the ExpR-induced shift was only weakly apparent, if at all (Fig. 3). Furthermore, a previous study using atomic force spectroscopy found that the strength of interaction between ExpR and its DNA binding site upstream of *sinI* was significantly increased upon the addition of AHLs (21). Evidence for the DNA sequence within and surrounding the ExpR binding site as one determinant of gene expression is suggested by the banding patterns in Fig. 3. We have not presented data in this study showing the titration of His_6-ExpR against target DNA in the gel shift assays or any other measurement of ExpR-DNA binding strength. However, we believe that such data could provide important and interesting verifications of the conclusions drawn from this study. ExpR-DNA binding is only one step in a multistep process of transcription activation and is therefore not necessarily a good indication of the strength of transcription activation. Examples of this are the ExpR binding sites upstream of the genes *wgeA* and *wgaA*. These promoters are strongly activated by ExpR in their transcription activity but do not appear to produce strong shifts in the gel shift assay. However, in both transcription activation and repression, the strength of the ExpR-DNA interaction is arguably one of the most critical steps in the regulation. This is supported by our study of *expRp*, in which alterations in the ExpR binding site in *expRp* affected not only the strength of the shift in a gel assay (Fig. 3D) but also promoter activity in the presence of ExpR (Fig. 5). Based on these data, we propose a testable hypothesis: at least one determinant of varying promoter sensitivity to AHLs is the DNA sequence to which ExpR binds, in which binding strength is stronger for sites that are more similar to the ExpR consensus.

Also relevant is the location of the binding site with respect to the promoter and transcription start, which may determine whether the regulation is positive or negative (Table 1; also, see Fig. S1 in the supplemental material), as is the case for TyrR (42). For our analysis, the experimentally determined transcription starts were reported previously (30) and additionally independently determined in the case of *sinR* (20), *sinI* (16), and *expR* (this study). All of the ExpR-binding promoter regions which were activated by ExpR contained a binding site either covering or upstream of the −35 region (see Fig. S1). An example of this is *sinIp*, where the ExpR site is not at −35 but at −75 (see Fig. S1). Activation of this promoter via ectopic expression of *sinR*, although dependent upon an intact −35 region, does not require either ExpR (16) or the ExpR binding site (unpublished data). This fits with the current model, in which SinR binds at or close to the −35 region and is necessary for a basal activity of *sinIp*, while ExpR binds at −75 and enhances *sinIp* activity. In contrast to the ExpR-activated promoters, if the ExpR binding site is downstream of the −35 region, ExpR represses promoter activity. It is likely that the mechanism of repression is via ExpR covering the −10 region or the transcription start (+1). Two exceptions to these generalizations are the promoters of SMc04059 and SMc01524. In the case of SMc04059, the ExpR binding site is upstream of the −35 region. We cannot at this stage exclude the possibility of an alternative promoter with a transcription start that is closer to this binding site. In the case of SMc01524, the ExpR binding site covers the −35 region in a manner similar to the positively regulated promoters. We do not know why repression occurs in this case. One possibility is that ExpR represses activity via competition with the RNA polymerase, or that the binding site covers the transcription start of an alternative promoter.

Biological function of a quorum sensing program. Although the long-chain AHLs are suspected not to cross the double membrane barrier of Gram-negative bacteria via diffusion (43–45), the presumptive transport systems and processes involved remains elusive. An individual which relies solely upon imported AHLs might be expected to have a weaker response to QS than an individual that engages in both AHL importation and production. Therefore, mutant cultures incapable of producing their own AHLs cannot be directly compared to wild-type cultures. However, the use of an AHL mutant strain in detecting the sensitivities of selected promoters to supplemented AHLs reveals a variety of AHL sensitivities and suggests that these promoters are organized in a program of QS regulation. Data from this study suggest that as AHLs accumulate in a growing colony, positively regulated promoters are programmed to respond prior to the negatively regulated promoters. The clearest example of this is the activation of the expression of genes controlling exopolysaccharide production and the repression of genes controlling motility. Such inverse regulation appears to be a general feature of many bacteria (46), including *Pseudomonas* (47), where regulation is achieved via the signal molecule cyclic di-GMP (c-di-GMP). Not only does the quorum sensing program in *S. meliloti* fit nicely with the pattern of inverse regulation, it also provides a fascinating glimpse of how the QS regulation exhibits dynamic behavior and precision timing. For example, individuals on the periphery of a colony might be expected to encounter lower levels of AHLs than those nearer the center, depending upon parameters such as rates of AHL diffusion and degradation. The lower level of AHLs (e.g., <500 nM) encountered by the peripheral individuals may be insufficient to repress the promoter for the *visNR* master regulator of the motility regulon (48). Our results indicated that *visNR* expression is not repressed until AHL concentrations are ≥500 nM (Fig. 6; also, see Fig. S3 in the supplemental material). This would allow these individuals to continue producing more flagella and so enhance mobility. Furthermore, these individuals would encounter sufficient AHLs to activate exopolysaccharide production (50 to 500 nM), which also enhances sliding mobility that is not necessarily dependent upon the presence of flagella (49). In contrast to the peripheral individuals, those near the center of the colony may encounter higher levels (≥500 nM) of AHLs and respond by reducing flagellum production. In a similar way, galactoglucan production appears to be reduced in response to higher levels of AHLs (Fig. 6; also, see Fig. S3 in the supplemental material). Thus, lower levels of AHLs stimulate an increase in exopolysaccharide production but do not affect flagellum production. Conversely, higher

AHL levels would result in a simultaneous reduction in the production rates of galactoglucan and flagella. This is presumably a finely tuned solution to avoid unnecessary and costly productions by individuals in the center of a colony with limited access to nutrients. Thus, the rates of galactoglucan and flagellum production may be tightly bound to local AHL concentrations, which are in turn determined by such factors as the shape and size of the colony. This may serve to ensure the production of sufficient levels of galactoglucan and flagella at the appropriate localities within a colony.

In this study, we have explored the mechanisms of the *S. meliloti* ExpR QS transcriptional regulatory network. The data suggest a type of quorum-sensing program whereby variation in a single input, AHL concentration, is sufficient to generate a tremendous diversity in the sensitivity, direction, and extent of promoter response. Although the details required to account for all the variation in this network appear to be multiple and complex, there are at least two features of the ExpR regulon which could at least partially account for the variation: variations within the DNA sequence of the ExpR binding site, which suggest different binding strengths, and the location of the ExpR binding site with respect to the promoter and transcription start. Both explanations could be tested and provide attractive focal areas for future studies. It would also be interesting to see if QS regulatory networks in other bacteria make use of similar regulatory strategies.

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Chapter 8: RNase E affects the expression of the acyl-homoserine lactone synthase gene *sinI* in *Sinorhizobium meliloti*

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Individual contributions:

- Construction of all promoter-*egfp* recombinant and IPTG-inducible *rne* overexpressing plasmids
- Analysis of the effect of *rne* on promoter activities
- Contributed to data analysis and manuscript preparation

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RNase E Affects the Expression of the Acyl-Homoserine Lactone Synthase Gene *sinI* in *Sinorhizobium meliloti*

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Quorum sensing of *Sinorhizobium meliloti* relies on *N*-acyl-homoserine lactones (AHLs) as autoinducers. AHL production increases at high population density, and this depends on the AHL synthase SinI and two transcriptional regulators, SinR and ExpR. Our study demonstrates that ectopic expression of the gene *rne*, coding for RNase E, an endoribonuclease that is probably essential for growth, prevents the accumulation of AHLs at detectable levels. The ectopic *rne* expression led to a higher level of *rne* mRNA and a lower level of *sinI* mRNA independently of the presence of ExpR, the AHL receptor, and AHLs. In line with this, IPTG (isopropyl-β-D-thiogalactopyranoside)-induced overexpression of *rne* resulted in a shorter half-life of *sinI* mRNA and a strong reduction of AHL accumulation. Moreover, using translational *sinI-egfp* fusions, we found that *sinI* expression is specifically decreased upon induced overexpression of *rne*, independently of the presence of the global posttranscriptional regulator Hfq. The 28-nucleotide 5′ untranslated region (UTR) of *sinI* mRNA was sufficient for this effect. Random amplification of 5′ cDNA ends (5′-RACE) analyses revealed a potential RNase E cleavage site at position +24 between the Shine-Dalgarno site and the translation start site. We postulate therefore that RNase E-dependent degradation *of sinI* mRNA from the 5′ end is one of the steps mediating a high turnover of *sinI* mRNA, which allows the Sin quorum-sensing system to respond rapidly to changes in transcriptional control of AHL production.

Quorum sensing (QS) is a communication system enabling bacteria to coordinate gene expression relative to population density (1). Important cellular functions, such as biofilm formation and production of virulence factors, depend on QS (2, 3). In Gram-negative bacteria, the autoinducers are frequently of the acyl-homoserine lactone (AHL) class, and the paradigm for studying AHL-based QS is the LuxRI system of *Vibrio fischeri* (1, 4). Typically, transcriptional regulators belonging to the LuxR-type family recognize AHLs, and the resulting protein/AHL complex alters expression of multiple target genes, including that of the AHL synthase gene. This perception of appropriate AHL concentrations happens when AHLs are initially produced at a low basal rate. With increasing population density, the AHL concentration reaches a critical level, whereupon the LuxR/AHL complex dramatically stimulates the expression of the gene coding for the LuxI AHL synthase. This is the basis of a positive feedback which generates a burst in AHL production. The increased number of LuxR/AHL complexes then coordinates changes in global gene expression in the bacterial population.

Throughout the phylum *Proteobacteria*, many factors have been found to control AHL production and accumulation at the levels of transcription, translation, and protein activity. Some examples of such factors are the transcriptional repressors of *luxI* in *Vibrio* and *Pseudomonas* (1, 5). In *Agrobacterium tumefaciens*, an anti-activator protein binds to the LuxR-type transcriptional activator and increases its proteolysis (6, 7). QS can also be quenched by enzymes such as lactonases, which degrade the AHL. Two different lactonases (encoded by *attM* and *aiiB*) were found in *A. tumefaciens* (8). Small regulatory RNAs (sRNAs) have also been found to regulate QS (9, 10). Typically, sRNAs interact with mRNAs with the help of the RNA chaperone Hfq and influence the translation rate and/or half-life of the mRNA targets. Usually both the sRNA and the mRNA are degraded in an RNase E-dependent manner (11–13). However, the direct role of RNases in QS had not been explored so far.

In this study, we were interested in the role of RNase E in QS in *Sinorhizobium meliloti*, a soil alphaproteobacterium performing nitrogen fixation in symbiosis with leguminous plants. Features important for the interaction between *S. meliloti* and its host plant, such as motility, the ability to form a biofilm, and production of exopolysaccharides are regulated by QS (14–16). *S. meliloti* produces at least five different AHLs with long carbon chains (containing 12, 14, 16, and 18 C atoms) via a single LuxI-type synthase, SinI (17), although only those with 14 to 16 carbons can complement the disruption of *sinI* (18, 19). Transcription of *sinI* is controlled by the LuxR-type transcriptional regulators SinR and ExpR (Fig. 1A) (20–22). With increasing population density, the concentration of AHLs reaches a threshold value of 1 nM, leading to the activation of ExpR, which induces strong expression of *sinI*. This positive feedback rapidly generates an elevated production rate of AHLs (23). A second feedback mechanism is activated at higher AHL concentrations (~40 nM), which appear to cap production of AHLs to the μM range (23, 24). Both feedback mech-

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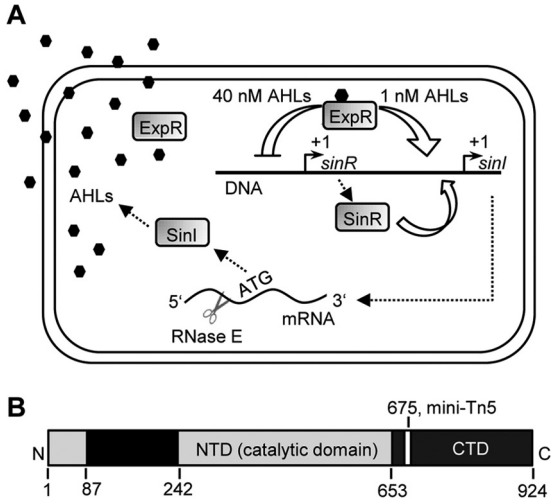

FIG 1 Quorum sensing and RNase E in *S. meliloti*. (A) Schematic representation of the Sin quorum-sensing system in *S. meliloti*. The role of the transcription factors SinR and ExpR on the expression of the autoinducer (AHL) synthase SinI was elucidated previously (20–24). SinR and SinI are expressed from the same locus on the chromosome. SinR is necessary for the efficient expression of the *sinI* gene and is independent of AHLs. ExpR senses the AHLs (octagons). At AHL concentrations of approximately 1 nM, ExpR activates the expression of SinI, leading to a strong increase in the AHL concentration (positive feedback loop). At 40 nM AHLs, ExpR negatively influences the expression of *sinR*, leading to low *sinI* expression (negative feedback loop) (23). The results of this work show that RNase E (scissors) specifically targets the 5′ UTR of *sinI* mRNA. (B) RNase E domains (NTD, N-terminal domain; CTD, C-terminal domain) and mini-Tn*5* insertion position in *S. meliloti* 2011. The *rne* gene of *S. meliloti* encodes a protein comprising 924 amino acid residues. Gray bars represent regions with homology to the catalytically active, N-terminal half of RNase E of *E. coli* (26). Black bars represent regions without homology to RNase E of *E. coli*. These regions are most probably involved in the formation of the *S. meliloti* degradosome (41). The mini-Tn*5* insertion in strain 2011rne::Tn*5* is in codon 675 of *rne* (44).

anisms are sensitive to specific AHL levels and depend upon the AHL receptor ExpR, which acts as a transcriptional activator of *sinI* expression (positive feedback) and repressor of *sinR* expression (negative feedback) (Fig. 1A). The ExpR-DNA binding sites enabling this transcriptional control have been identified, along with another 30 binding sites throughout the *S. meliloti* genome (24). To date, the regulation of QS in *S. meliloti* has been studied mainly at the level of transcription, and little is known about factors acting posttranscriptionally. Recently, it was found that *sinI* mRNA levels are higher in an *hfq* mutant of *S. meliloti* (25), strongly suggesting the involvement of an sRNA and possibly of RNase E in the Hfq-dependent regulation of this gene (11, 12). To address this question, we decided to study the impact of RNase E on AHL accumulation.

RNase E is an endoribonuclease with major importance for the decay of mRNA in bacteria (most recently reviewed in reference 26). In *E. coli*, its catalytic activity is located in the N-terminal domain. The C-terminal, unstructured domain serves as a scaffold for the assembly of a multiprotein complex, the degradosome, which contains the 3′-5′ exoribonuclease polynucleotide phosphorylase (PNPase), an RNA helicase, enolase, and other minor proteins (27). Hfq also interacts with the C-terminal domain of RNase E and thereby participates in the sRNA-based regulation of gene expression in *Escherichia coli* (12). In *Rhizobium leguminosarum*, Hfq is also associated with RNase E and the degradosome, where it is necessary for the RNase E-dependent activation of the translation of NifA, the major transcriptional regulator of nitrogen fixation (28).

Different bacteria contain various compositions of RNA-degrading multiprotein complexes. However, some common characteristics, such as the association of exo- and endoribonucleases with RNA helicases and a specific subcellular localization, seem to be important for bacterial RNA metabolism (27, 29, 30). RNase E and the degradosome are bound to the cytoplasmic membrane in *E. coli* (31). The degradosome of *Bacillus subtilis*, which lacks RNase E, is also bound to the membrane. This degradosome contains PNPase, an RNA helicase, enolase, and other proteins and is organized by the endoribonuclease RNase Y (32–34). RNase E-containing degradosomes were also isolated from three alphaproteobacteria, *Rhodobacter capsulatus*, *Caulobacter crescentus*, and *R. leguminosarum* (28, 35, 36). In addition to RNase E, the degradosome of *R. capsulatus* contains two RNA helicases, the transcriptional terminator factor Rho, and substoichiometric amounts of PNPase. The degradosome of *C. crescentus* contains PNPase, an RNA helicase, and aconitase, while an RNA helicase and Hfq were found in the degradosome of *R. leguminosarum* together with other proteins. RNase E and the degradosome of *S. meliloti* have not yet been studied.

The N-terminal domain of RNase E is highly conserved and essential for growth of *E. coli* under most conditions, while mutants lacking the C-terminal domain are viable (37–40). In *Streptomyces coelicolor*, however, RNase E is nonessential and structurally shuffled: the catalytic domain is located in the central part of the polypeptide, while regions at the termini are involved in the interaction with PNPase (41). Bioinformatic analyses revealed an insertion of a putative degradosome-scaffold region into the putative catalytic N-terminal domain of RNase E in *S. meliloti* (41) (Fig. 1B). The availability of a *S. meliloti* Rm2011 *rne* mutant with a mini-Tn*5* transposon insertion (44) (Fig. 1B) prompted us to analyze the role of RNase E in QS. In this study, we show that RNase E affects the production of AHLs in *S. meliloti* and provide evidence that the 5′ untranslated region (UTR) of *sinI* mRNA is a specific target of RNase E independent of Hfq.

MATERIALS AND METHODS

Strains and cultivation methods. In this work we used the laboratory strain *S. meliloti* Rm2011 (referred to here as 2011), which is closely related to the first sequenced *S. meliloti* strain Rm1021 (42, 43). Its isogenic RNase E mutant 4.07.G10 originates from a mini-Tn*5* library (44). The transposon is inserted downstream of the putative catalytic domain, in the 675th codon of the gene *rne* (SMc01336) (41, 42) (Fig. 1B). Since strain 2011 is a wild type in respect to *rne* but is an ExpR-deficient mutant with an insertion element in the *expR* gene (21, 23), it is referred to as parental strain 2011 in this study. The mini-Tn*5* RNase E mutant is referred to as 2011rne::Tn*5*. To mimic the mini-Tn*5* insertion, we used the pK18mob2 suicide vector carrying a 902-bp internal fragment from position 1123 to 2024 of the 2,775-nucleotide (nt) *rne* gene. Following homologous recombination between the plasmid and the *S. meliloti* chromosome, the *rne* gene was disrupted by the insertion of pK18mob2 at nucleotide position 2025 (675th codon). This mutant was named 2011rne675.

S. meliloti was cultivated on tryptone-yeast (TY) plates or in liquid TY cultures (45) with appropriate antibiotics (streptomycin, 250 $\mu g\ \mu l^{-1}$; neomycin, 120 $\mu g\ \mu l^{-1}$; gentamicin, 20 $\mu g\ \mu l^{-1}$; and tetracycline, 20 $\mu g\ \mu l^{-1}$). Routinely, 50 ml *S. meliloti* culture was grown semiaerobically in 100-ml Erlenmeyer flasks at 140 rpm and 30°C. For the experiments whose results are shown in Fig. 5 to 7, 100-μl cultures in a 96-well microtiter plate (Greiner) were grown at 30°C and 200 rpm in modified MOPS

TABLE 1 Strains and plamids used in this study

Strain or plasmid	Description	Reference or source
Strains		
E. coli JM109	*endA1 recA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *relA1 supE44* Δ(*lac-proAB*)	75
E. coli DH5α	F^- *endA1 supE44 thi-1 recA1 gyrA96 relA1 deoR* Δ(*lacZYA-argF*)*U169*	76
E. coli S17-1	*E. coli* 294; Thi RP4-2-Tc::Mu-Km::Tn*7* integrated into the chromosome	47
E. coli MT102(pJBA89)	pUC18Not-luxR-P_{luxI} -RBSII-gfp(ASV)-T_0-T_1; expresses EGFP upon addition of AHLs; Ap^r	56
S. meliloti 2011	Contains insertion sequence within *expR* gene; Nx^r Sm^r	43
S. meliloti 2011rne::Tn*5*	2011 derivative, RNase E mutant 4.07.G10 with mini-Tn*5* inserted in the 675th codon of *rne* (SMc01336); Sm^r Nm^r	44
S. meliloti 2011rne675	2011 derivative; RNase E mutant with a suicide vector pK18mobII inserted in the 675th codon of *rne*; Sm^r Km^r	This study
S. meliloti Sm2B3001	2011 derivative with restored *expR* gene on the chromosome	77
S. meliloti Sm2B4001	*sinI* mutant of Sm2B3001	23
S. meliloti Sm2011dhfqGmLR	2011 derivative, Δ*hfq* mutant, Gm^r	54
A. tumefaciens NTL4(pZLR4)	Expresses beta-galactosidase upon addition of AHLs; Gm^r	58
Plasmids		
pK18mob2	Suicide vector; *mob lacZ* Km^r	78
pK1123-2024	pK18mobII carrying an internal fragment of rne, nt 1123–2024; Km^r	Stefan Meyer
pK79-926	pK18mobII carrying an internal fragment of rne, nt 79–926; Km^r	Stefan Meyer
pPHU231	pRK290 with a 388-bp HaeII insert containing pUC18 polylinker; Tc^r	79
pLK01	pPHU231 with a promoterless *egfp*; Tc^r	50
pLK60	pLK64 derivative without *sinI* codons; Tc^r	This study
pLK61	pPHU231 containing *sinIp-sinI′-egfp* translational fusion; allows expression of full-length SinI fused to EGFP; Tc^r	This study
pLK64	pPHU231 containing *sinIp-sinI-egfp* translational fusion, allows the expression of a SinI′-EGFP containing the first 9 amino acid residues of SinI; Tc^r	50
pLK65	pPHU231 containing *sinRp-sinR′-egfp* translational fusion; Tc^r	23
pLK002	pPHU231 containing *cspA3p-cspA3′-egfp* translational fusion; Tc^r	This study
pLKrec01	pLK64 derivative containing *cspA3* promoter instead of the *sinI* promoter; Tc^r	This study
pLKrec02	pLK002 derivative containing *sinI* promoter instead of the *cspA3* promoter; Tc^r	This study
pSRK-Km and -Gm	Broad-host-range expression vectors with tightly regulated, IPTG-inducible *lac* promoter; Km^r or Gm^r	49
pWBrne	pSRK-Gm containing *rne*; Gm^r	This study
pWBrne675	pSRK-Gm containing *rne* (codons 1–675); Gm^r	This study
pBSrne	pSRK-Km containing *rne*; Km^r	This study
pRK415	Tc^r broad-host-range expression vector; the *lac* promoter is constitutive in *S. meliloti*	48
pRKrne	pRK415 containing *rne* with a C-terminal streptavidin tag-coding sequence; Tc^r	This study
pDrive	PCR cloning kit	Qiagen

(morpholinepropanesulfonic acid)-buffered minimal medium containing 48 mM MOPS (adjusted to pH 7.2 with KOH), 55 mM mannitol, 21 mM sodium glutamate, 1 mM $MgSO_4$, 250 mM $CaCl_2$, 37 mM $FeCl_3$, 48 mM H_3BO_3, 10 mM $MnSO_4$, 1.0 mM $ZnSO_4$, 0.6 mM $NaMoO_4$, 0.3 mM $CoCl_2$, 4.1 mM biotin, and 0.1 mM K_2HPO_4. *Escherichia coli* was grown in LB broth. *E. coli* JM109 and *E. coli* DH5α were used for standard cloning methods (46). Plasmids were transferred from *E. coli* S17-1 to *S. meliloti* by diparental conjugation (47). Bacterial strains and their relevant characteristics are listed in Table 1.

Plasmid construction. The plasmids used in this work are listed in Table 1, and the primers used for cloning are listed in Table S1 in the supplemental material. For disruption of the C-terminal region of *rne*, see the strain descriptions above. For disruption of the N-terminal region of *rne*, an 848-bp region of *rne* from positions 79 to 926 was cloned into the suicide vector pK18mob2. No mutants were obtained following the homologous recombination procedure with this construction.

For complementation, the *rne* gene (excluding UTRs) was cloned between the HindIII and KpnI restriction sites of the broad-host-range vector pRK415 (48). The resulting plasmid, pRKrne, allows the expression of RNase E-streptavidin from a *lac* promoter, which is constitutively active in *S. meliloti*. Furthermore, the pSRK plasmids (49) were used for construction of pBSrne (Km^r) and pWBrne (Gm^r), which allow induced overexpression of *rne* in *S. meliloti*.

Construction of plasmids pLK64 and pLK65 with *sinI′-egfp* and *sinR′-egfp* translational fusions were previously described (23, 50). The plasmid pLK64 contains the promoter region of *sinI*, the 5′ UTR, and the first 9 codons of *sinI* fused to *egfp*. Two derivatives of pLK64 were also constructed in which the *sinI* codons were omitted (pLK60) and in which all *sinI* codons were included in the fusion to *egfp* (pLK61). Similarly, pLK002 was constructed, which contains the promoter and the 5′ UTR of *cspA3* in a translational fusion to *egfp* (24). Synthetic derivatives of the pPHU231-based plasmids pLK64 and pLK002 were constructed by swapping the 5′ UTR of the *sinI* mRNA with the 5′ UTR of the *cspA3* mRNA in each of the translational fusions (pLKrec01 and pLKrec02).

Isolation and analysis of nucleic acids. Total DNA was isolated by the method of Masterson et al. (51). To isolate RNA for RT-PCR analysis or 5′-RACE, 1 ml of *S. meliloti* cultures grown to an optical density at 600 nm (OD_{600}) of 1.3 was added to 1 ml of RNAprotect bacterial reagent (Qiagen). Cells were harvested by centrifugation (6,000 × *g* for 10 min at 4°C) and resuspended in the lysis buffer provided with the RNeasy minikit (Qiagen). After the addition of acid-washed glass beads (Sigma), cells were disrupted in a Tissuelyser (Retsch) for 50 s. Glass beads were re-

moved by centrifugation. RNA was isolated with the RNeasy minikit (Qiagen), treated with RNase-free DNase (Invitrogen), and resuspended in water.

The primers employed for analyzing relative mRNA amounts of genes using real-time quantitative reverse transcription-PCR (qRT-PCR) are listed in Table S2 in the supplemental material. For normalization of mRNA levels, the *rpoB* gene, which encodes the beta subunit of RNA polymerase of *S. meliloti*, was used. Conditions for qRT-PCR were as previously described (52). We used a one-step RT-PCR kit (Qiagen) and added 4 ng μl^{-1} of total RNA into the reaction mixture. SYBR green I (Sigma) was diluted at 1:100,000 in the master mix to detect double-stranded DNA. Relative expression of a gene in the mutant strain was calculated relative to expression in the parental strain and relative to *rpoB* (53). Similarly relative mRNA levels were calculated before and after addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to cultures. PCR efficiencies of primer pairs were determined using serial dilutions of RNA (see Table S2 in the supplemental material). At least two biologically independent experiments were performed, each with two technical replicates.

mRNA half-lives were determined as previously described (54), with the following modifications. A *S. meliloti* culture was grown to an OD_{600} of 0.5 and split into two flasks, and to one of the flasks 1 mM IPTG was added to induce ectopic expression of RNase E. No IPTG was added to the flask with the control culture. Transcription was stopped 60 min later by the addition of rifampin (500-$\mu g\ ml^{-1}$ final concentration; stock concentration, 30 mg ml^{-1} in methanol). Cells were harvested at time points of 0, 3, and 6 min by adding 1 ml of the culture to 1 ml of RNAprotect bacterial reagent (Qiagen). RNA was isolated with an RNeasy minikit (Qiagen) as described above and treated with Turbo DNA-free (Ambion). mRNA levels were determined by qRT-PCR as described above, using 16S rRNA as the reference (55). Half-lives were calculated from linear-log graphs of time after rifampin addition against relative mRNA amounts.

AHL and eGFP detection. AHLs were extracted 10 min from 1 ml bacterial culture supernatant with 0.3 ml chloroform. Extracts were evaporated, and the remaining pellet was resuspended in 30 μl of acetone. The detection of AHLs was done with two different systems. First, *E. coli* MT102 (pJBA89) expressing enhanced green fluorescent protein (eGFP) upon addition of AHLs and detecting a range of AHLs from C_6-HSL to oxo-C_{14}-HSL was used (56). Reporter bacteria were grown on LB medium with specific antibiotics. Ten μl of acetone extract was dropped on the bacterial lawn. Fluorescence was observed 4 h after incubation using filter with an excitation wavelength of 480/40 nm and an emission wavelength of 510 nm. The second method for detection of AHLs is based on *A. tumefaciens* NTL4(pZLR4) expressing beta-galactosidase from an AHL/TraR-dependent promoter (57, 58). *A. tumefaciens* grown with 40 $\mu g\ ml^{-1}$ gentamicin was mixed with MGM agar containing 11 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g glutamate, 10 g mannitol, 1 μg biotin, 0.25 mM $CaCl_2$, and 1 mM $MgSO_4$ per liter without gentamicin. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added to a final concentration of 80 $\mu g\ ml^{-1}$. Two μl of the AHL extracts were spotted onto the agar, and the plate was incubated at 32°C overnight. A blue color indicated the detection of AHLs.

Rapid amplification of 5′ cDNA ends. For the determination of 5′ ends of RNA by rapid amplification of 5′ cDNA ends (5′-RACE), cells were grown in TY medium to an OD_{600} of 1.0. Ectopic expression of RNase E was induced by addition of 1 mM IPTG. Cells were harvested 20 min, 40 min, and 60 min after induction. No IPTG was added to the control cultures. 5′-RACE was performed as described previously (59) with primers described by McIntosh et al. (50).

RESULTS

The C-terminal region of RNase E is nonessential. Loss of the *rne* gene in *E. coli* is lethal under most conditions (37, 40). However, insertion mutations in the C-terminal coding region of *rne*, which codes for the nonessential macromolecular-interaction domain, are growth permissive (39). The availability of a *S. meliloti* 2011rne::Tn*5* mutant, in which a mini-Tn*5* transposon is inserted in the C-terminal region (675th codon) of the *rne* gene (44) (Fig. 1B), suggests an arrangement similar to that in *E. coli*. In this study, we created another RNase E mutant which carries the suicide vector pK18mobII, also inserted in the 675th codon of *rne*. This mutant strain, 2011rne675, was viable, like the mini-Tn*5* mutant, confirming that the C-terminal domain of *S. meliloti* RNase E is nonessential. However, attempts to insert pK18mobII into the N-terminal coding region of *rne* (309th codon) failed to produce any colonies. Therefore, we used the 2011rne::Tn*5* and 2011rne675 mutants to study the effect of RNase E on AHL production. In addition, plasmids bearing *rne* with a constitutive (pRKrne) and an IPTG-inducible *lac* promoter (pWBrne and pBSrne) were used to study the effect of ectopic expression of *rne* on AHL production.

Overexpression of *rne* affects AHL accumulation. To address the question of whether RNase E regulates quorum sensing in *S. meliloti*, AHLs harvested from the 2011rne::Tn*5* mutant and the mutant containing pRKrne were compared to AHLs from the 2011 parent strain using an AHL sensor system with a GFP reporter in *E. coli* (Fig. 2B). AHLs were extracted from supernatants of cultures at OD_{600} of 1.3 (Fig. 2A) and added to the *E. coli* reporter strain, and fluorescence was measured. Similar fluorescence levels were observed for the AHL extracts from the transposon mutant and the 2011 parent strain, while constitutive ectopic expression of the *rne* gene from pRKrne resulted in a dramatic reduction of fluorescence (Fig. 2B). We postulated that this extremely low fluorescence reflects a strongly reduced AHL production in strain 2011rne::Tn*5* (pRKrne) due to overproduction of RNase E.

To test whether ectopic expression of the *S. meliloti rne* gene results in an elevated accumulation of *rne* mRNA, qRT-PCR analysis of *rne* was performed. Two primer pairs annealing to different *rne* regions (downstream and upstream of the mini-Tn*5* insertion in strain 2011rne::Tn*5*) were used to analyze total RNA isolated from cultures at an OD_{600} of 1.3. Consistent with the location of the primer annealing sites with respect to the mini-Tn*5* insertion (Fig. 1B), the level of the C-terminal coding region of the mRNA from downstream of the mini-Tn*5* insertion was lower in the 2011rne::Tn*5* mutant than in the parental 2011 strain (Fig. 2C). However, the N-terminal coding region of *rne* mRNA upstream of the mini-Tn*5* insertion was not changed in the mutant compared to the parental strain 2011. The level of *rne* mRNA increased in the mutant carrying the plasmid pRKrne compared to the parental strain 2011 (Fig. 2C). We conclude that ectopic expression of *rne* does indeed lead to increased *rne* mRNA accumulation and that the mini-Tn*5* insertion does not greatly alter the abundance of mRNA from the N-terminal region of *rne*.

To confirm that *rne* overexpression leads to changes in AHL accumulation in *S. meliloti*, we used pWBrne, allowing IPTG-inducible ectopic expression of full-length *rne* in the parental strain 2011 and in the mutant 2011rne::Tn*5*. AHLs were extracted and detected with an *A. tumefaciens* reporter system (Fig. 2D). The experiment was also performed with pWBrne675, bearing *rne* codons 1 to 675 under the control of an IPTG-inducible *lac* promoter with similar effects: a reduction of AHLs to nondetectable levels in strains 2011 and 2011rne::Tn*5* in the presence of IPTG (Fig. 2D). Similar results were obtained with the mutant strain 2011rne675 when full-length RNase E from pWBrne or the trun-

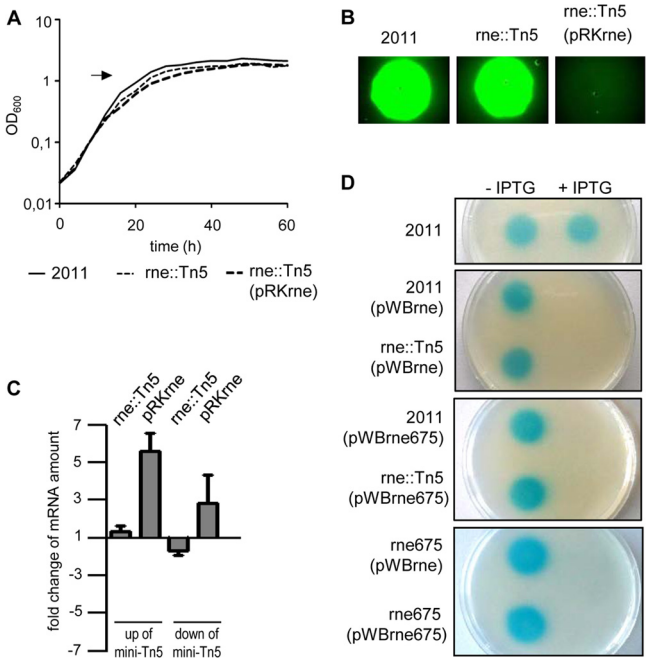

FIG 2 Overexpression of *rne* dramatically decreases AHL accumulation. (A) Growth curves of the *expR*-deficient parent strain *S. meliloti* 2011, the RNase E mutant 2011rne::Tn*5*, and the RNase E mutant containing pRKrne. AHLs were extracted from supernatants of cultures grown to an OD_{600} of 1.3 (arrow). (B) The extracted AHLs were detected with a GFP reporter system in *E. coli* MT102 (pJBA89). Shown is the fluorescence of the reporter strain grown with AHLs extracted from the indicated *S. meliloti* cultures. The spots were on the same plate. (C) Real time RT-PCR analysis of the *rne* gene, encoding RNase E. Levels of *rne* mRNA in the mutant 2011rne::Tn*5* and in the mutant containing pRKrne were compared to the levels in the parental strain, 2011. Two primer pairs targeting *rne* gene at locations upstream and downstream of the mini-Tn*5* insertion in the RNase E mutant were used. Results are from three independent experiments with two technical replicates. Error bars indicate the standard deviations (SDs). (D) Detection of AHLs in cultures of strain 2011 and its isogenic mutants 2011rne::Tn*5* and 2011rne675 grown without (−) or with (+) 1 mM IPTG. The strains contain pWBrne or pWBrne675 as indicated, with *rne* or truncated *rne* under the control of an inducible *lac* promoter. Detection was performed with the *A. tumefaciens* reporter strain NTL4(pZLR4).

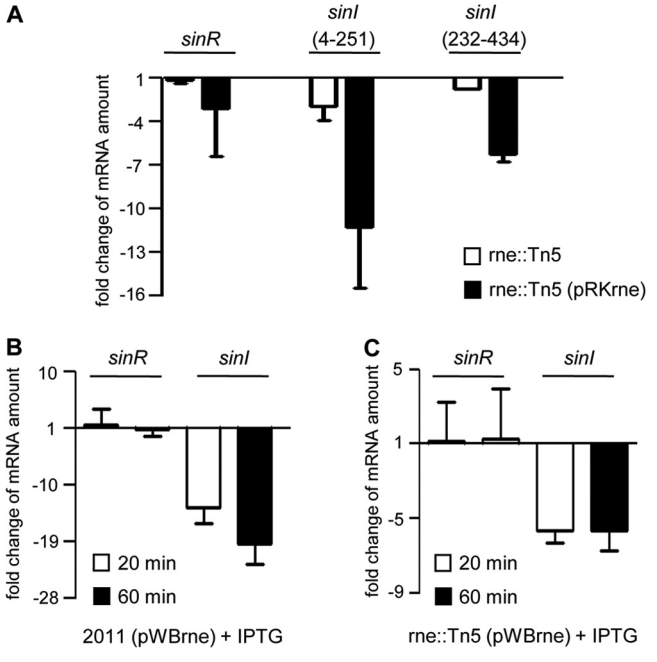

FIG 3 Real-time RT-PCR reveals a strong decrease in levels of *sinI* mRNA in strains overexpressing *rne*. (A) Real-time RT-PCR of *sinR* and *sinI* was performed with total mRNA isolated from the parental strain 2011, the RNase E mutant 2011rne::Tn*5*, and the RNase E mutant containing pRKrne. Two primer pairs amplifying nucleotides 4 to 251 and 232 to 434 of the *sinI* open reading frame (ORF) were used. The mRNA levels in strain 2011rne::Tn*5* and strain 2011rne::Tn*5* (pRKrne) were compared to the levels in the parental strain. Results are from two independent experiments with two technical replicates. An exception was the analysis of strain 2011rne::Tn*5* (pRKrne), for which three biological experiments with two technical replicates were performed. Data are means and SDs. (B) Real time RT-PCR of *sinR* and *sinI* in strain 2011 (pWBrne). Samples were harvested at 20 and 60 min after addition of 1 mM IPTG to cultures grown to an OD_{600} of 1.3, and mRNA levels were compared to the levels before IPTG addition. (C) Real-time RT-PCR of *sinR* and *sinI* in strain 2011rne::Tn*5* (pWB*rne*), as described for panel B.

cated RNase E from pWBrne675 was overexpressed (Fig. 2D). Altogether, these results show that overexpression of only the region encoding the N-terminal part of RNase E is sufficient for the disruption of AHL accumulation. This is consistent with the assumption that like in RNase E of *E. coli*, the N-terminal part of *S. meliloti* RNase E contains the catalytically active domain (41) (Fig. 1B).

Overexpression of *rne* diminishes *sinI* mRNA accumulation. To determine the mechanism for the dependence of AHL accumulation on *rne*, real-time RT-PCR (qRT-PCR) analysis was performed on the genes encoding SinI (the AHL synthase) and SinR (the AHL-independent transcriptional activator of *sinI*) (24). We found that in comparison to *S. meliloti* 2011, the *sinR* mRNA levels were not changed significantly in the mutant 2011rne::Tn*5* or in the pRKrne-containing mutant, which constitutively overexpresses *rne* (Fig. 3A). A slight decrease in the amount of *sinI* mRNA was detected in 2011rne::Tn*5*, and a strong decrease was detected in the overexpressing strain. To exclude artifacts due to stable mRNA fragments, the *sinI* analysis was performed with two different primer pairs with similar results (Fig. 3A). Even 20 min after induction of IPTG-induced overexpression of full-length *rne* in both the 2011rne::Tn*5* mutant and the parental strain 2011, the levels of *sinI* mRNA but not of *sinR* mRNA were decreased (Fig. 3B and C). These data correlate with the observed decrease in AHL accumulation upon overexpression of *rne* (Fig. 2B and D) and fit with the hypothesis that RNase E specifically degrades *sinI* mRNA but not *sinR* mRNA. This is also consistent with the results shown in Fig. 2C, where ectopic expression of *rne* results in an increase in *rne* mRNA accumulation.

The reduction of the steady-state amount of *sinI* mRNA upon overexpression of *rne* is most probably due to decreased *sinI* mRNA stability. To test whether overexpression of *rne* affects the half-life of *sinI* mRNA, mRNA stability was measured in strain 2011 (pWBrne) grown without IPTG and compared to mRNA stability in the same strain following the addition of IPTG. Relative mRNA amounts were determined 0, 3, and 6 min after the addition of rifampin, which stops RNA transcription in bacteria. We did not obtain signals for *sinI* mRNA in Northern blots, probably due to the small amount of this messenger (data not shown). Therefore, qRT-PCR analysis was performed to determine the relative amounts of *sinI* mRNA. As an internal reference, the stable 16S rRNA was used. To test the specificity of the *rne* effect on *sinI* mRNA stability, half-lives were also determined for *sinR* and *rpoB*

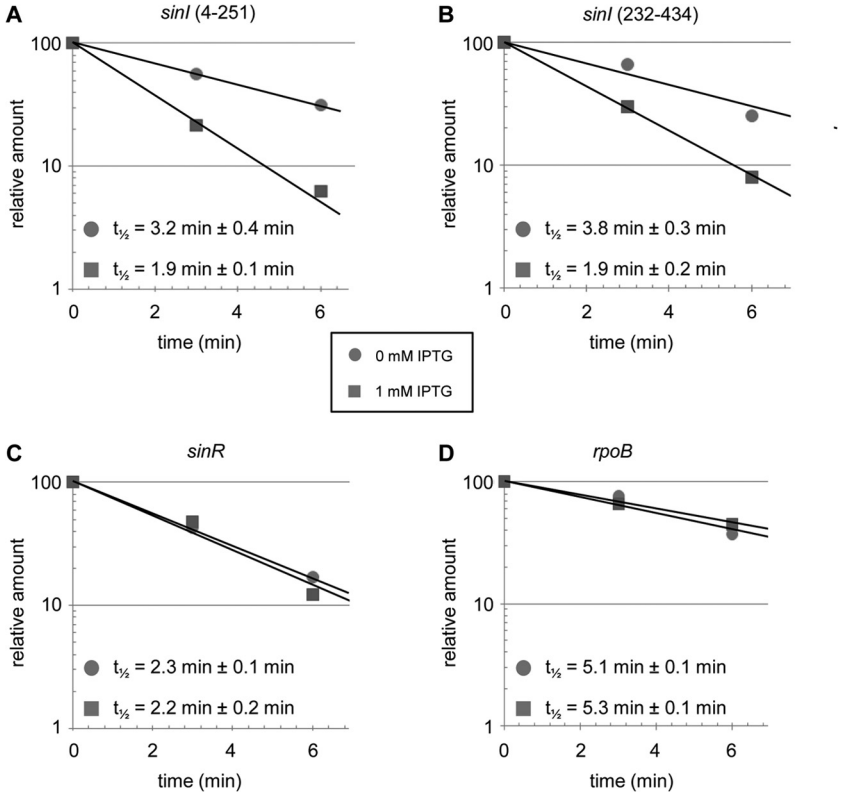

FIG 4 Overexpression of *rne* specifically decreases the stability of *sinI* mRNA. The graphs show results of representative experiments. Unless differently stated, half-lives ($t_{1/2}$) were calculated from two independent experiments, each with two technical replicates. Cells were harvested 0, 3, and 6 min after rifampin addition to 2011 (pWBrne) cultures at an OD_{600} of 0.5 grown with and without IPTG. Total RNA was isolated and relative mRNA levels were determined by qRT-PCR. (A) Stability of *sinI* mRNA was determined with the primer pair targeting nt 4 to 251 in the *sinI* ORF. Measurements without IPTG were performed in two independent experiments with two technical replicates at three ODs (0.5, 1.0, and 1.3) with very similar results, and the half-lives (at 0 mM IPTG) were calculated from a total of 12 measurements. (B) Stability of *sinI* mRNA was determined with the primer pair targeting nt 232 to 434. (C) Stability of *sinR* mRNA. (D) Stability of *rpoB* mRNA.

mRNA, which was the internal reference in the qRT-PCR experiments whose results are shown in Fig. 3.

The results of the mRNA stability measurements are shown in Fig. 4. The half-life of *sinI* mRNA was determined with the two different primer pairs with very similar results (3.2 ± 0.4 min and 3.8 ± 0.3 min). As expected, the stability of *sinI* mRNA was significantly reduced upon overexpression of *rne* (1.9 ± 0.1 and 1.9 ± 0.2 min with each of the primer pairs, respectively) (Fig. 4A and B). In contrast, the stability of *sinR* and *rpoB* mRNAs was not affected (Fig. 4C and D). Using one of the primer pairs and cultures without IPTG, *sinI* mRNA stability was determined in two independent experiments at ODs of 0.5, 1.0, and 1.3. It is noteworthy that the *sinI* mRNA stability was comparable at all three ODs (Fig. 4A, 0 mM IPTG). Based on these data, we conclude that there is no differential regulation of *sinI* expression at the level of mRNA stability in strain 2011 at these three ODs and that overexpressed *rne* specifically decreases the stability of *sinI* mRNA, leading to lower steady-state amounts.

Overexpression of *rne* lowers *sinI* expression. Overexpression of *rne* in *S. meliloti* leads to both low AHL levels and low *sinI* mRNA levels, as determined by AHL extraction and detection, and by qRT-PCR. To better understand the mechanism of *rne*-dependent reduction in *sinI* mRNA levels, we used a plasmid-based reporter system with *sinI-egfp* fusions. The plasmid pLK64 has been used previously to study the control of the *sinI* promoter (23, 24, 50). In this construct, the *sinI* promoter region (287 bp), the region corresponding to the 5′ UTR of *sinI* mRNA (28 bp), and the first 27 bp of the *sinI* coding sequence are fused to the ATG of *egfp*. In addition, two other constructs were designed: pLK61, in which the *sinI* promoter, the 5′ UTR, and the full-length *sinI* coding region were fused to *egfp*, and pLK60, in which only the promoter region and the 5′ UTR of *sinI* were fused to *egfp* (Fig. 5). We tested pLK61 in a *sinI* deletion strain, and this plasmid restored AHL production (data not shown), indicating that the SinI-EGFP fusion protein is functional. Fluorescence detected from each of the three reporter plasmids was measured in the parental strain 2011 (Fig. 5B) and in the 2011rne675 mutant (Fig. 5C) containing the empty vector control pSRK-Gm or one of the IPTG-inducible plasmids pWBrne or pWBrne675. Background fluorescence was measured using the vector pLK01, which contains a promoterless *egfp*.

Plasmid pLK64 produced the highest fluorescence (8,000 to 9,000 fluorescence units per unit of optical density [F/OD]), followed by pLK60 (5,000 to 6,000 F/OD). Plasmid pLK61 produced the lowest fluorescence (2,000 to 3,000 F/OD), indicating that the *sinI-egfp* mRNA and/or the fusion protein has lower stability. All three plasmids produced a significantly lower fluorescence upon overexpression of either *rne* (pWBrne) or the truncated *rne*

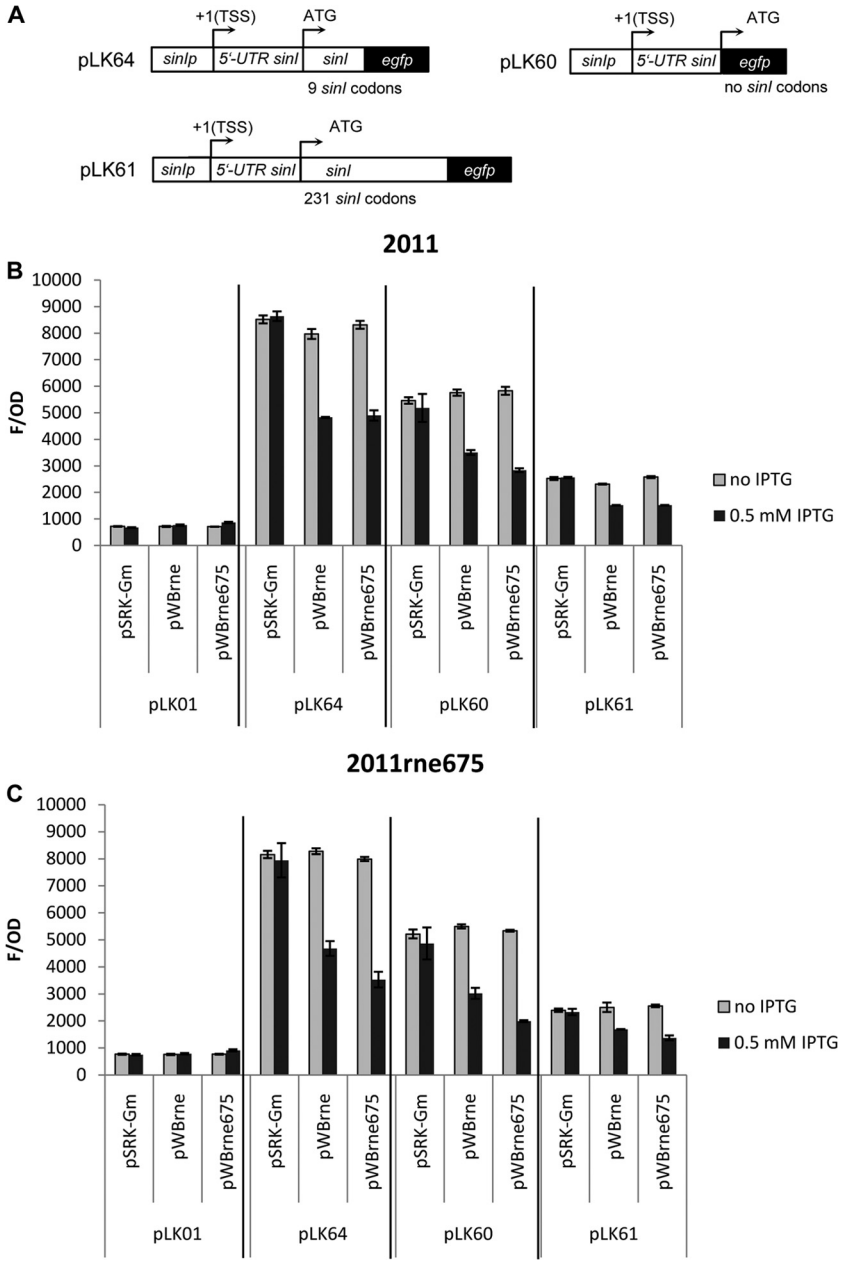

FIG 5 Induced overexpression of either *rne* or truncated *rne* leads to low SinI expression. (A) Schematic of the translational *egfp* fusions in the plasmids pLK64, pLK60, and pLK61 (not to scale). Included are the plasmids containing the *sinI* promoter, the 5′ UTR, and the *sinI* coding regions of the indicated lengths followed by the *egfp* ORF. The transcriptional start site (+1 TSS) and the start codon (ATG) are marked. For further descriptions, see Table 1 and the text. (B) Fluorescence from the parental strain 2011 carrying pLK01 (promoterless *egfp*, background fluorescence), pLK64, pLK60, or pLK61 was measured in the presence and absence of IPTG-induced overexpression of *rne* (pWBrne) or truncated *rne* (pWBrne675). Included as a control is the empty vector pSRK-Gm lacking *rne*. All three *sinI-egfp* fusions (pLK64, pLK60, and pLK61) produced less fluorescence in response to overexpression of either *rne* or truncated *rne*. (C) As for panel B, except that strain 2011rne675 was used. Once again, all three *sinI-egfp* fusions produced less fluorescence in response to either *rne* or truncated *rne* overexpression. Error bars indicate variations from 4 cultures.

(pWBrne675). This effect was similar in both the parental strain 2011 and the 2011rne675 mutant. These results are similar to those obtained via AHL extraction and qRT-PCR and are consistent with an RNase E-dependent degradation of *sinI* mRNA. Also notable is that this effect requires only the N-terminal part of the coding region of *rne*. The results also strongly suggest that as a target for RNase E, the minimal requirement is the 5′ UTR of *sinI* mRNA. This is remarkable in that the length of the 5′ UTR of *sinI* is only 28 nucleotides.

Rne influence on sinI expression is independent of *expR*. The sequenced laboratory strain Rm1021 (42) and the closely related strain 2011 have incomplete Sin QS systems due to an interruption of the *expR* gene by an IS element (21, 23). The lack of ExpR renders the *sinI* promoter insensitive to the presence of AHLs, but

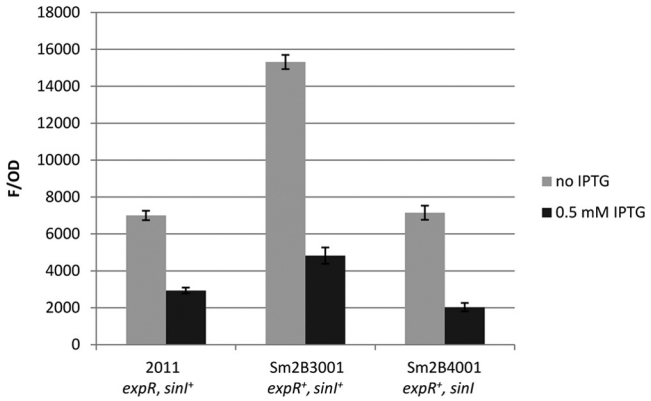

FIG 6 *rne* overexpression effect is independent of *expR* and AHLs. Fluorescence from the *sinI-egfp* fusion in pLK64 was used to determine whether the *rne* effect on *sinI* expression was dependent upon either the presence of *expR* or AHLs. Upon IPTG-induced overexpression of *rne* from plasmid pBSrne, a decrease in fluorescence was observed not only in the parental *expR* mutant strain 2011 but also in a derivative strain carrying a functional copy of *expR* (Sm2B3001) and in a second derivative strain with a functional copy of *expR* but without *sinI* (Sm2B4001, no AHLs). Error bars indicate variations from 4 cultures.

sinI expression and AHL production continue in a SinR-dependent manner (see Fig. 1 for the regulatory scheme). Thus, in the absence of ExpR, *sinI* expression and AHL production are detectable, albeit ~3-fold weaker (23). This is because the activation of the *sinI* promoter by SinR is independent of AHLs (24). One advantage of using an *expR* mutant strain, such as 2011, is that it enables the study of the regulation of genes underlying QS in a simplified genetic background. To check whether the *rne* effect on *sinI* expression is dependent upon *expR* or AHLs, fluorescence from the plasmid pLK64 was compared in three genetic backgrounds: (i) in strain 2011 (*expR sinI*$^+$), (ii) in its derivative Sm2B3001 (*expR*$^+$ *sinI*$^+$) with a restored *expR* gene located at its native site on the chromosome, and (iii) in strain Sm2B4001, a *sinI* mutant derivative of Sm2B3001 (*expR*$^+$ *sinI*) (Fig. 6). Strains carrying pBSrne, in which *rne* was placed under the control of an IPTG-inducible *lac* promoter in plasmid pSRK-Km, were grown with and without IPTG. In all three strains, fluorescence was significantly reduced upon overexpression of *rne*, indicating that the *rne* effect on *sinI* expression was not dependent upon either *expR* or AHLs.

The 5′ UTR of *sinI* mRNA is a specific target of RNase E. The influence of RNase E on *sinI* is related to the region encompassing the promoter, the 5′ UTR, and the first nine codons of *sinI* (Fig. 5). To better understand the underlying mechanisms, additional fusions with the reporter *egfp* gene were used (Fig. 7A), and fluorescence was measured after cultivation of the strains with and without overexpression of *rne* (Fig. 7B). Overexpression of *rne* did not reduce fluorescence from the vector pLK65, which contains the promoter and 5′ UTR of *sinR* fused to the translation start of *egfp*. Therefore, *rne* does not appear to control the expression of *sinR*. As an additional control, we used the plasmid pLK002, which contains a fusion of the *cspA3* promoter and 5′ UTR to *egfp* (Fig. 7A). The gene *cspA3* encodes a cold shock protein, and its promoter is QS independent (24). The expression of the *cspA3′-egfp* fusion was also almost unaffected by *rne* overexpression (Fig. 7B).

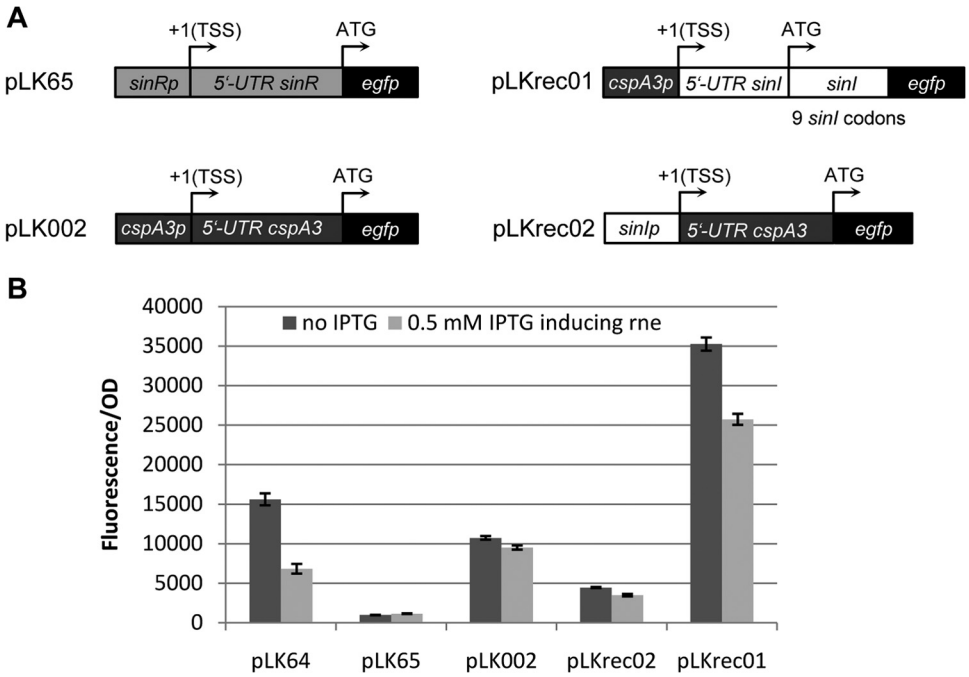

FIG 7 The 5′ UTR of *sinI* is sufficient for the decrease in *sinI* expression when *rne* is overexpressed. (A) Schematic of the translational *egfp* fusions in the plasmids pLK65, pLK002, pLKrec01, and pLKrec02. Indicated are the plasmids along with the *egfp* fusions containing *sinI*-, *sinR*-, or *cspA3*-specific promoters, 5′ UTRs, and/or coding regions. For additional descriptions, see Fig. 5. (B) Measurement of fluorescence in strain Sm2B3001 (*sinI*$^+$ *expR*$^+$) from the indicated plasmids with and without IPTG-induced overexpression of *rne* from pBSrne. A comparison between the two synthetic constructs, pLKrec01 and pLKrec02 (see Fig. 4 for an explanatory scheme), shows that *rne* overexpression does not greatly affect *cspA3′-egfp* expression from the *sinI* promoter (pLKrec02) but the *sinI′-egfp* expression from the *cspA3* promoter (pLKrec01) does show a clear decrease upon *rne* overexpression. Error bars indicate variations from 4 cultures.

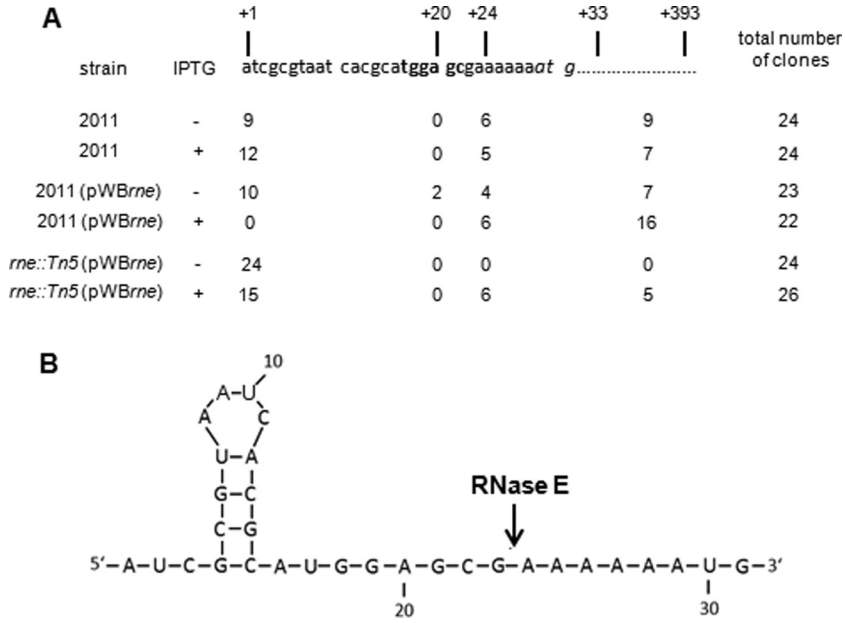

FIG 8 RNase E is necessary for occurrence of processed 5′ ends in the first half of the *sinI* mRNA and probably cleaves at position +24 in the 5′ UTR of the transcript. (A) Mapping of 5′ ends by RACE. Results are from at least two biologically independent 5′-RACE experiments with strains 2011, 2011 (pWBrne), and the RNase E mutant 2011rne::Tn*5* (pWBrne). 5′ ends were mapped for cultures with (+) and without (−) IPTG. The total number of the sequenced clones (experimentally determined 5′ ends), the number and the positions (indicated above the mRNA sequence; +1 is the TSS) of 5′ ends in the 5′ UTR of *sinI* mRNA, and the number of 5′ ends at variable positions in the coding region of the transcript are shown. The Shine-Dalgarno sequence is in bold; the start codon is in italics. For detailed information, see Table S1 in the supplemental material. (B) Proposed secondary structure (Mfold [74]) of the 5′ UTR of *sinI* mRNA with the RNase E cleavage site at position +24.

Altogether, these experiments are in agreement with the conclusion that *sinI* mRNA is a specific target of RNase E.

To confirm that RNase E specifically targets the 5′ UTR of *sinI* mRNA, we used a synthetic approach and fused the *cspA3* promoter to the 5′ UTR of *sinI* followed by *egfp* (pLKrec01). Additionally, the promoter of *sinI* was fused to the 5′ UTR of *cspA3* followed by *egfp* (pLKrec02) (for a fusion scheme, see Fig. 7A). Fluorescence in strain Sm2B3001 (*sinI*$^+$ *expR*$^+$) bearing these plasmids was measured with and without IPTG-induced expression of *rne*. Upon *rne* overexpression, fluorescence from pLKrec02 essentially did not decrease, while fluorescence from pLKrec01 did decrease (Fig. 7B). These results, together with data in Fig. 5 demonstrating the *rne*-dependent decrease of fluorescence from pLK60 lacking *sinI* codons, show that the 5′ UTR of *sinI* mRNA is sufficient for downregulation of *sinI* expression upon overexpression of *rne*. This confirms that the 5′ UTR of *sinI* is a specific target of RNase E.

RNase E is expressed as a streptavidin-tagged fusion protein from pRKrne. However, we were not able to isolate the protein for an *in vitro* determination of the putative cleavage site in the 5′ UTR of *sinI*. Therefore, we decided to use an *in vivo* approach. The 5′ ends of *sinI* mRNA were detected via 5′-RACE analysis in strains 2011 and 2011rne::Tn*5* and following overexpression of *rne* from pWBrne. The results are summarized in Fig. 8 and also in Table S3 in the supplemental material. A total of 24 5′ ends were detected in RNA extracted from strain 2011 without pWBrne and without IPTG. Nine of these were mapped to the previously determined transcriptional start site (TSS; +1) of *sinI* (50), six to position +24 in the 5′ UTR of the transcript (between the Shine-Dalgarno sequence and the start codon), and nine within the coding region of *sinI*, mostly at position +359. The 5′ ends downstream of the TSS probably correspond to degradation intermediates. Similar results were obtained from the control strain 2011, which was grown with IPTG but lacked pWBrne. Ten of the 23 mapped 5′ ends from strain 2011 with pWBrne but without IPTG corresponded to the TSS of *sinI*, four were found at position +24, and the rest mapped to different internal positions in the *sinI* mRNA (Fig. 8; also, see Table S3 in the supplemental material). However, upon IPTG-induced overexpression of *rne* (2011 with pWBrne and IPTG), the number of 5′ ends detected at position +1 dropped to zero, six were at position +24, and the remainder were at positions within the coding region of *sinI*.

The comparison between strains 2011 and 2011(pWBrne) without IPTG suggests that even in the absence of IPTG, RNase E is slightly expressed from pWBrne. This leaky expression without IPTG does not block the AHL production (Fig. 2D) but possibly leads to the occurrence of multiple internal 5′ ends at various positions in the *sinI* transcript (Fig. 8; also, see Table S3 in the supplemental material). A stronger scattering of internal 5′ ends was observed in the corresponding IPTG-induced culture of strain 2011 (pWBrne), supporting the view that overexpression of *rne* leads to their occurrence. These results are consistent with strong degradation of *sinI* mRNA upon overexpression of *rne*. When analyzed temporally (see Table S3), the data show that the proportion of 5′ ends downstream of +1 increased with increasing exposure to IPTG-induced *rne* expression. Thus, increased expression of *rne* leads to an increased degradation of *sinI* mRNA in the 5′-3′ direction.

For the 2011rne::Tn*5* mutant carrying pWBrne, a total of 24 5′ ends corresponding to the TSS (+1) of *sinI* were detected in the

absence of IPTG (Fig. 8). This indicates that our method for detecting 5′ ends does not include premature stops during cDNA synthesis from mRNA and supports the conclusion that the internal 5′ ends represent RNase E-mediated degradation intermediates of *sinI* mRNA. In IPTG-induced 2011rne::Tn*5* (pWBrne) cultures, internal 5′ ends were detected in addition to the TSS. Six out of 11 internal 5′ ends mapped to position +24 in the 5′ UTR of *sinI* mRNA (from a total of 26 analyzed clones) (Fig. 8).

In summary, results presented in Fig. 8 show that overexpression of *rne* leads to a faster degradation of *sinI* mRNA, and that position +24 in the 5′ UTR of *sinI* mRNA is a potential RNase E cleavage site. Furthermore, full-length RNase E is necessary for occurrence of processed 5′ ends in the region analyzed by 5′-RACE.

RNase E acts on the 5′ UTR of *sinI* independently of Hfq. An RNase E cleavage in the 5′ UTR of bacterial mRNAs is often mediated by trans-encoded sRNAs, and the sRNA-mRNA interaction is usually Hfq dependent (12). Hfq-dependent RNase E cleavage in the 5′ UTR of *nifA* mRNA was also found in *R. leguminosarum* (28). Thus, a similar mechanism may operate at the 5′ UTR of *sinI*. To check for the involvement of Hfq, we used a 2011Δ*hfq* mutant (54). We detected larger AHL amounts in cultures of this mutant than in the parental strain, 2011 (data not shown), in agreement with the previously reported higher *sinI* mRNA and AHL levels in the absence of a functional *hfq* gene in *S. meliloti* (25). The plasmids pLK64 (containing the *sinI′-egfp* translational fusion) and pWBrne (containing *rne* under the control of an inducible *lac* promoter) were introduced into the 2011Δ*hfq* mutant. The strain was grown with and without IPTG in two independent experiments, and fluorescence was measured. In the presence of IPTG, fluorescence was reduced 2.4-fold. This is comparable to the reduction of fluorescence in the presence of IPTG in the parental strain 2011 containing the same plasmids (Fig. 5B, data for pLK64). We conclude that overexpression of *rne* negatively influences *sinI* expression in an Hfq-independent manner.

DISCUSSION

RNase E is an endoribonuclease with major importance for the decay of mRNA in bacteria (26). RNase E is essential for growth of *E. coli* and *M. smegmatis* under standard laboratory conditions (27, 40, 60). The role of RNase E in *S. meliloti* has not been analyzed so far, although it is likely to be essential as well, since our attempts to insert pK18mob2 in the *rne* region encoding the N-terminal domain of RNase E were not successful, while insertions into the region encoding the C-terminal domain were. Mutants 2011rne::Tn*5* and 2011rne674 are not strongly impaired in their growth (Fig. 2A, growth of 2011rne::Tn*5*). This can be explained by the assumption that these mutants express a truncated, catalytically active RNase E lacking (a part of) the domain responsible for the interaction with other components of the degradosome (26, 38). Both insertions are at codon 675 of *rne*, downstream of the region encoding the putative catalytic RNase E domain (Fig. 1B). This view is supported by the qRT-PCR analyses of *rne* regions upstream and downstream of the mini-Tn*5* insertion and by the fact that the mutant strains 2011rne::Tn*5* and 2011rne674 are both viable and capable of AHL production (Fig. 2).

Studies on the RNase E of *E. coli* have revealed RNase E as a potent autoregulator (reviewed in reference 26). When RNase E activity exceeds the demands for RNA processing and turnover, the *rne* mRNA becomes a target for degradation. The 5′ UTR of *rne* mRNA and a functional C-terminal domain of RNase E are important for this autoregulation (26, 61). The ectopic expression of the *S. meliloti rne* coding region led to elevated *rne* mRNA levels (Fig. 2C) and increased degradation of *sinI* mRNA (Fig. 4). Ectopically expressed *rne* may escape a potential autoregulation due to the lack of native nontranscribed regions with regulatory functions.

Previous studies revealed the involvement of sRNAs in the control of translation and mRNA levels of transcriptional regulators of QS in *Vibrio* and *Pseudomonas* species. These sRNAs influence the expression of *luxR*-like transcriptional regulators or other QS-dependent genes, but not directly the autoinducer synthase (62, 63). Although it can be assumed that endoribonucleases such as RNase E and RNase III contribute to the adjustment of mRNA levels by QS sRNAs, so far this was not demonstrated experimentally. Indeed, there is little experimental evidence for a role for ribonucleases in the control of bacterial QS systems. An exception is the work by Luo and Farrand (64) showing that an RNase D homolog is important for the expression of TraR, a LuxR-type transcriptional factor in *A. tumefaciens*.

Overexpression of *rne* results in enhanced degradation of *sinI* mRNA. Our data show that this negative effect is (at least partly) due to a specific, Hfq-independent cleavage of RNase E in the 5′ UTR of *sinI* mRNA. The Hfq-independent status of this cleavage does not exclude the involvement of an sRNA, since a trans-encoded, Hfq-independent sRNA was shown to regulate the expression of photosynthesis genes in the alphaproteobacterium *Rhodobacter sphaeroides* (65).

Although overexpression of *rne* specifically destabilizes *sinI* mRNA, no differences in the stability of *sinI* mRNA at different points of the growth curve were detected when *rne* was not overexpressed. This shows that RNase E cleavage in the 5′ UTR is an important factor in the turnover of *sinI* mRNA but is not modulated under the tested conditions. The importance of RNase E for the turnover of *sinI* mRNA is demonstrated by the lack of processed 5′ ends in the 5′ half of this mRNA in the 2011rne::Tn*5* mutant (Fig. 8; also, see Table S3 in the supplemental material). Despite the reduction in degradation events in the 5′ half of *sinI* mRNA in this mutant, the total level of *sinI* mRNA was not increased in comparison to the wild type (Fig. 3A). This is suggestive of an unknown, alternative degradation pathway(s) which also contributes to the degradation of *sinI* mRNA in the mutant.

Generally, mRNA degradation in bacteria is triggered by dephosphorylation of the primary transcript or by an internal endonucleolytic cleavage (66). Since many bacterial RNases, including RNase E, RNase G (which shows homology to the N-terminal part of RNase E and exhibits similar substrate specificity), RNase J, and RNase Y, prefer monophosphorylated substrates, dephosphorylation by a pyrophosphatase or an endonucleolytic cleavage strongly destabilizes the target transcripts, which are then degraded in a concerted action by endo- and exoribonucleases (reviewed in reference 66). While the exoribonucleolytic degradation proceeds only in the 3′-5′ direction in *E. coli*, 5′-3′ degradation by RNase J takes place in *B. subtilis* (67). *S. meliloti* harbors RNase E but not RNase G (42). In addition, it harbors RNase J, which is responsible for the 5′-end maturation of rRNA (68), and the 3′-5′ exoribonucleases RNase R and PNPase (42). The 5′ ends which we detected by 5′-RACE resulted from either endonucleolytic cleavages or exoribonucleolytic decay in a 5′-3′ direction. Based on our 5′-RACE data, we suggest that *sinI* mRNA decay includes endonucleolytic

cleavages at positions +24 and +359, the two internal positions at which 5′ ends were found in independent experiments. It is not clear whether the scattered 5′ ends, represented by the detection of single (nonduplicated) events at multiple positions within the *sinI* mRNA (see Table S3 in the supplemental material), result directly from increased RNase E activity in the cell or from increased accessibility of mRNA for exoribonucleolytic 5′-3′ degradation.

One question that arose in the course of this study was whether the overexpression of *rne* somehow regulates gene expression that leads to AHL degradation independently of its effect on *sinI* expression. However, when we added synthetic AHLs to cultures overexpressing *rne* and lacking the *sinI* gene (and therefore incapable of producing endogenous AHLs), we saw no difference in the amount of AHLs recovered compared to cultures not overexpressing *rne* (our unpublished data). This is consistent with our conclusions that RNase E affects AHL accumulation through targeting the mRNA of *sinI*. However, we cannot rule out other mechanisms by which RNase E affects AHL accumulation, since multiple pathways of mRNA degradation and interdependence of RNases are known for other bacteria (69–71).

In this study, we have shown that RNase E specifically targets the 5′ UTR of *sinI* mRNA at position +24. This seems to be an efficient cleavage site for regulation of *sinI* expression, since it is located immediately after the Shine-Dalgarno sequence, preventing translation of the mRNA and destabilizing the transcript. This fits very well with previous observations. For example, a mathematical model of the *S. meliloti* Sin system has been described which correlates predicted and observed behavior of the Sin system using the activity of the *sinI* promoter as the output and the relative abundance of ExpR, SinR, and AHLs as various inputs (72). In that study, one basic assumption necessary for a workable model of the Sin system is that the gene products of both *sinR* and *sinI* should be rapidly degraded, allowing a finely tuned transcriptional control of AHL production that is sensitive to AHL levels. Consistent with this, the half-lives of both *sinR* and *sinI* mRNAs are in the range of typical mean chemical half-lives of RNA measured in bacteria (between 2.4 min in *Prochlorococcus* and 6.8 min in *E. coli*) (73). With RNase E, we have identified and reported the first factor which is specifically involved in the turnover of *sinI* mRNA.

In summary, our data strongly suggest that RNase E is essential in *S. meliloti*. It can be assumed that in this species, as in other bacteria, RNase E influences many cellular processes. We show that RNase E is one of the factors involved in the degradation of the AHL synthase transcript *sinI* and that the 5′ UTR of *sinI* is a specific target of RNase E. These findings open the door to understanding the posttranscriptional mechanisms influencing the expression of QS-related genes in *S. meliloti*.

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Chapter 9: Quorum sensing restrains growth and is rapidly inactivated during domestication of *Sinorhizobium meliloti*

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Individual contributions:

- Consecutive cultivation of *S. meliloti* under standard laboratory conditions
- Sequence analysis of spontaneous loss-of-function *expR* mutations
- Characterisation of ExpR mutant variants using Western blot analysis, overexpression and purification of recombinant proteins, SDS-PAGE, gel shift assay, and EGFP fluorescent assay
- Competitive growth assay on solid surface
- Competitive growth assay in liquid cultures
- Contributed to writing of the manuscript

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Quorum sensing restrains growth and is rapidly inactivated during domestication of *Sinorhizobium meliloti*

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Summary

Microbial cooperative behaviours, such as quorum sensing (QS), improve survival and this explains their prevalence throughout the microbial world. However, relatively little is known about the mechanisms by which cooperation promotes survival. Furthermore, cooperation typically requires costly contributions, e.g. exopolysaccharides, which are produced from limited resources. Inevitably, cooperation is vulnerable to damaging mutations which results in mutants that are relieved of the burden of contributing but nonetheless benefit from the contributions of their parent. Unless somehow prevented, such mutants may outcompete and replace the parent. The bacterium *Sinorhizobium meliloti* uses QS to activate the production of copious levels of exopolysaccharide (EPS). Domestication of this bacterium is typified by the appearance of spontaneous mutants incapable of EPS production, which take advantage of EPS production by the parent and outcompete the parent. We found that all of the mutants were defect in QS, implying that loss of QS is a typical consequence of the domestication of this bacterium. This instability was traced to several QS-regulated processes, including a QS-dependent restraint of growth, providing the mutant with a significant growth advantage. A model is proposed whereby QS restrains population growth to prevent overcrowding and prepares the population for the survival of severe conditions.

Received 4 September, 2014; accepted 11 December, 2014. *For correspondence. E-mail matthew.mcintosh@synmikro.uni-marburg.de; Tel. +49 (0) 6421 – 28 24444; Fax ••. Subject category: Microbial population and community ecology.

Introduction

Many bacteria enhance their survival by population density-dependent regulation of gene expression, a form of cooperation that is often referred to as quorum sensing (QS). Perhaps the best-studied examples of QS can be found in the acyl-homoserine lactone (AHL)-mediated QS of the *Proteobacteria* (Parsek and Greenberg, 2005; Waters and Bassler, 2005; Ng and Bassler, 2009). The fact that QS-based cooperation is so prevalent in the bacterial world is evidence of its success as a survival strategy. However, a major threat for cooperation is the damaging effects of mutation. Mutants can arise which are incapable of QS and are relieved of the cost of contributing extracellular factors, otherwise known as common goods if they benefit each member of the population (Schuster *et al.*, 2013). Thus these mutants are sometimes referred to as 'cheaters' or 'free-riders' and might be expected to dominate a population, leading to a rapid loss in fitness of the whole population. Yet nature abounds with microbes capable of cooperation, which implies that cooperative behavior greatly enhances survival. While QS-based cooperation has generally received much attention regarding its mechanisms of control of gene expression, relatively little has been focused on its costs and benefits to the organism (Schuster *et al.*, 2013).

We are interested in characterizing the cooperative behavior of *Sinorhizobium meliloti*, a soil-dwelling bacterium capable of a free-living state (reviewed by Vriezen *et al.*, 2007; Rinaudi and Giordano, 2010) and nitrogen fixing symbiosis (reviewed by Jones *et al.*, 2007). *Sinorhizobium meliloti* is a particularly attractive model to study cooperation since one of its most striking features is a heavy production of exopolysaccharides (EPS) in response to a single QS system known as the Sin QS system. *Sinorhizobium meliloti* produces at least two EPS: succinoglycan (EPS I) and galactoglucan (EPS II) (reviewed by Janczarek, 2011). Both are upregulated by the Sin QS system 2-fold and 20-fold, respectively, contributing to a distinct mucoid phenotype on agar (Pellock *et al.*, 2002; Glenn *et al.*, 2007; Charoenpanich *et al.*, 2013). The Sin system consists of SinI, the AHL synthase and SinR, a LuxR-type transcription regulator that controls *sinI* expression but whose activity is not affected by

the presence of AHLs (Charoenpanich *et al.*, 2013). Instead, a second LuxR-type regulator, ExpR, is responsible for the QS response. Interestingly, most of the studies on *S. meliloti* to date have relied on *expR* mutants (e.g. see Janczarek, 2011). The spontaneous recovery of a functional *expR* in the laboratory strain Rm1021 and a corresponding extremely mucoid phenotype was reported (Pellock *et al.*, 2002). Since that time, the importance of ExpR as the master regulator of cooperation in *S. meliloti* has been established. The ExpR regulon has been relatively well characterized, with over 30 DNA binding sites biochemically identified (Charoenpanich *et al.*, 2013; Zatakia *et al.*, 2014) and differential expression of at least 570 genes, almost 9% of the genome (Gurich and González, 2009). The presence of AHLs is necessary for most of the regulatory targets of ExpR, including EPS production, so that the disruption of either *sinI* or *expR* results in a dry phenotype. *expR* mutants also produces significant levels of AHLs (Marketon *et al.*, 2002; Teplitski *et al.*, 2003), but the presence of these AHLs affects the expression of very few genes (Hoang *et al.*, 2004; Glenn *et al.*, 2007), consistent with the conclusion that ExpR is the major regulator of AHL-controlled gene expression.

In this study, we observed that serial cultivations of mucoid isolates of *S. meliloti* grown as a bacterial lawn on agar appeared to gradually decrease in mucoid levels with each serial cultivation, and eventually gave rise to a dry colony phenotype. We suspected that mutations were occurring that blocked EPS production. One possibility was that EPS production incurred a heavy cost, providing the mutant with a distinct growth advantage. Intriguingly, we found that all of the dry phenotype mutants contained a loss-of-function mutation in *expR* and not in any other genes essential for EPS production. This challenged the notion that the cost of EPS production drives mutant invasions and led to the discovery of a novel function for the Sin QS system. We report here that the Sin QS system restrains growth, providing a significant advantage for the non-cooperator during domestication.

Results

Spontaneous loss of mucoid phenotype is caused by random mutations in expR

To learn more about the loss of mucoidy by *S. meliloti*, we performed three independent experiments based on the serial subculturing of three mucoid *S. meliloti* strains: Rm8530, Sm2B3001 and Rm41. In each case, the starting point was a glycerol stock prepared from a single colony. Bacteria were streaked out on agar using a glass pipette. Because mucoid strains of *S. meliloti* spread rapidly via copious EPS production, the inoculation typically formed a bacterial lawn. After a number of subcultivations, spontaneous non-mucoid mutant colonies were observable following serial dilution and growth to single colonies (supporting information, Fig. S1A). The proportion of non-mucoid colonies increased with each cultivation, eventually resulting in the absence of mucoid colonies (data not shown). Loss of the mucoid phenotype occurred in all three strains and in all three experiments. Generally, this loss was more rapid with a higher incubation temperature and longer incubation between subcultures, which is consistent with a mutant invasion rate that increased with the number of generations.

Previously characterized dry phenotypes of *S. meliloti* strains were associated with mutations at the *expR* locus (Pellock *et al.*, 2002). Therefore, we sequenced the *expR* locus from colonies randomly selected during the consecutive subculturing experiments. Each of the 30 mutants selected contained either a single nucleotide polymorphism (SNPs) or an insertion/deletion (indel) in the *expR* gene (supporting information, Fig. S1C, Table S1). The mucoid phenotype was restored via a plasmid-based copy of *expR* (supporting information, Fig. S1B).

There are many genes essential to EPS production, so why does only *expR* mutate? Either the mutations are somehow specific to the *expR* locus (e.g. directed), or that they are random and under a strong selection pressure. We conclude that the latter is more likely for the following reasons. First, the nature of the mutations (SNPs and indels) is consistent with random mutation. Second, the locations of the mutations are relatively evenly dispersed over the sequence of *expR* (738 bp). Third, changing the genomic position of the *expR* locus did not protect against mutations. To show this, a single copy of the functional *expR* gene together with its native promoter was inserted into the megaplasmid pSymA (between SMa0175 and SMa0179). *expR* at this locus was even more susceptible to mutation (strain Sm2B3001A, supporting information, Fig. S1A, experiment 3), and this indicated that mutations in *expR* are not related to its native chromosomal location in the genome. Last, when sequencing *expR* from dry phenotype colonies, we routinely selected several colonies from each invaded culture. Only one to three *expR* sequence variants were found per culture, implying that mutation of *expR* is rare. Altogether, the data are consistent with a scenario in which the *expR* mutations are the product of rare and random genetic events that disrupt ExpR function, thereby providing the mutant with a strong selection advantage.

Dry phenotype is because of a loss-of-function alteration in ExpR

The majority of loss-of-function mutations in *expR* were caused by frameshifts or indels (supporting information,

Table S1). To see if mutations in the *expR* gene always correlate with ExpR protein activity, 10 *expR* mutants from dry colonies with single amino acid changes were selected for further characterization. These variants were analysed for their activity and stability (supporting information, Figs S1D and S2) and were demonstrated to be defect. In conclusion, each of the *expR* mutant variants associated with a dry phenotype was either a nonsense or missense mutation causing a loss of function.

Invasion of agar cultures by the spontaneous expR *mutants is conditional upon galactoglucan and flagella*

To study the invasion of mucoid cultures by the *expR* mutants, we used a selection of mutants labelled with mCherry and observed their growth under standard laboratory conditions. Mutant strains included those with disruptions in *exoB*, *wgeB*, *exoY* and *visN*. Production of galactoglucan and succinoglycan requires *exoB*, encoding the UDP-glucose 4′-epimerase, an enzyme responsible for the production of UDP-galactose from UDP-glucose (Buendia *et al*., 1991). *wgeB* and *exoY* are essential structural genes in the production of galactoglucan and succinoglycan respectively (Reuber and Walker, 1993; Becker *et al*., 2002). *visN* is a master regulator that is necessary for expression of genes related to flagella production and chemotaxis, so that a loss of *visN* results in a loss of flagella-dependent motility (Sourjik *et al*., 2000; Nogales *et al*., 2012).

After inoculation as single-strain cultures, *exoB*, *wgeB* and *expR* mutants exhibited a dry colony phenotype on agarose, while *exoY* and *visN* mutants appeared mucoid like the wild type (WT) (Fig. 1A). The mucoid strains, WT, and *visN* and *exoY* mutants exhibited vigorous colony expansion (Ø = 14 ± 1 mm), whereas strains with the dry phenotype showed reduced expansion (Ø = 5 ± 1 mm) and remained mostly at the site of the inoculation droplet. This shows that mobility under these conditions is dependent upon galactoglucan but not succinoglycan. Furthermore, in strains with a functional *expR*, a loss of *visN* did not greatly affect mobility (Fig. 1A), likely because flagella production is repressed by ExpR (Bahlawane *et al*., 2008; Hoang *et al*., 2008). This is also consistent with a previous report dealing with motility on a 0.6% agar surface (Nogales *et al*., 2012).

How do the mutations in *exoB*, *exoY*, *wgeB* and *visN* affect the *expR* mutant invasion? Colonies from the WT and *expR*$^+$ strains lacking *exoB*, *exoY*, *wgeB* or *visN* were harvested from the solid medium assay after 24 days, and the colony-forming unit (cfu) of the spontaneous *expR* mutants was estimated as a percentage of the total cfu (Fig. 1A, below photo). In the WT and *exoY* mutant colonies, *expR* mutant invasions were relatively high, at 22 ± 8 and 17 ± 4 cfu per 100 cfu. Invasions by *expR* mutants were severely restricted, however, in the absence of galactoglucan production (*exoB* and *wgeB* mutants, < 1%) or flagella (*visN* mutant, < 2%), suggesting that both were essential for the invasions under these conditions.

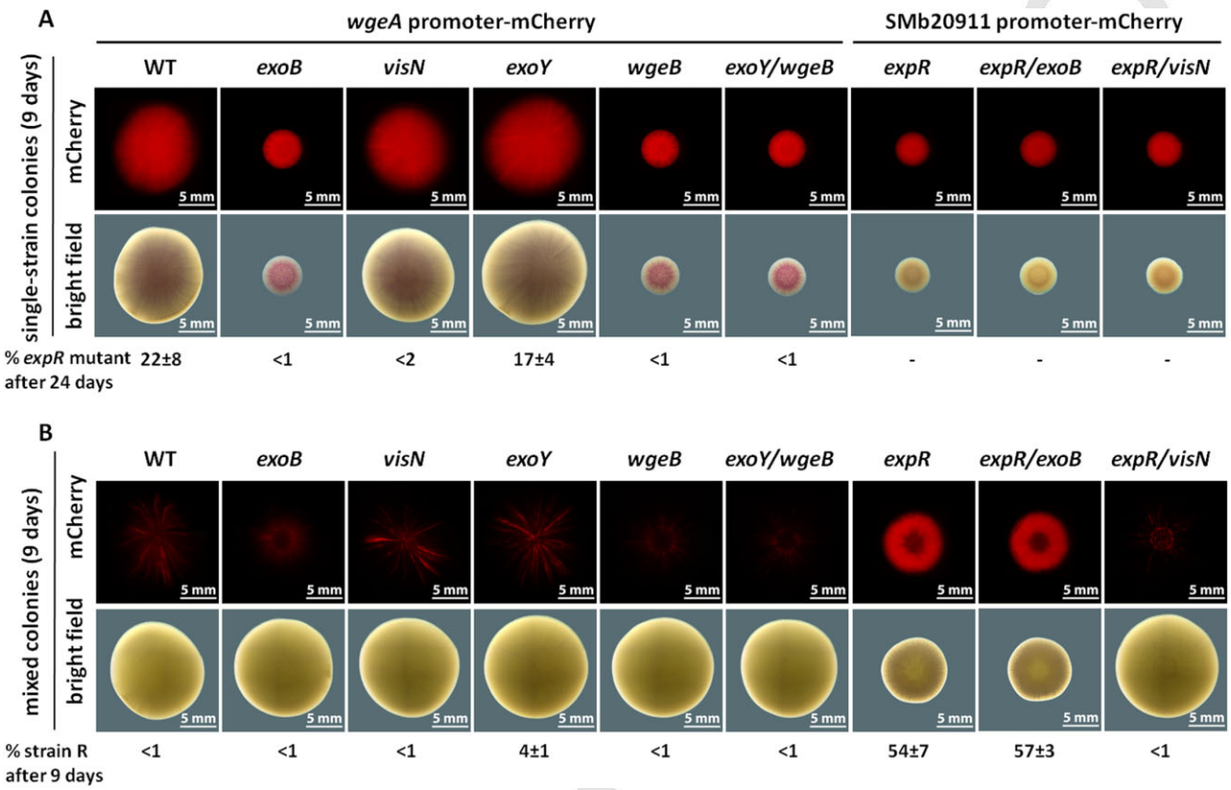
Competitive growth assay confirms expR *mutant with enhanced motility and growth*

To learn more about how the *expR* mutant invades WT cultures, we developed a two-strain competitive solid medium culture assay in which the WT (strain G) was labelled with *egfp* and the second (strain R) with *mCherry*. After 9 days, the mixed colonies appeared mucoid. All colonies were 14 ± 1 mm in diameter, except for the colonies with the WT (G) mixed with the *expR* or *expR/exoB* mutants (R). These were also mucoid but were significantly smaller (8 ± 1 mm). When the WT was included as strain R, red lines radiating from the centre of the colony indicate lines of expansion. When any of the mutants incapable of galactoglucan production (*exoB*, *wgeB*, *wgeB*/*exoY*) but with a functional *expR* was included as strain R, their expansion was significantly reduced. In contrast, the loss of *visN* or *exoY* alone had no effect on expansion of *expR*$^+$ strains (Fig. 1B). This fits perfectly with previous observations that succinoglycan and flagella do not affect mobility of *expR*$^+$ strains. Conversely, when the *expR* mutant was included as strain R, it revealed an invasion in progress, appearing as a red ring of outward-migrating *expR* mutants. Here, domination by the *expR* mutant was so strong that the mobility of strain G was impeded, despite a 9(G) : 1(R) ratio at inoculation.

To confirm the numerical dominance by the *expR* mutant, strains were recovered from the mixed culture assays by re-suspension, serial dilution and plating to observe single colonies. Only the *expR* and *expR*/*exoB* mutants (as strain R) achieved > 50% of the cfu. In contrast, the WT and all other mutants, including the *expR*/*visN* double mutant, remained at ≤ 4% of the cfu (Fig. 1B, below photo).

ExpR *restrains growth in liquid cultures*

Experiments described above show that the invasion by *expR* mutants in agar cultures is achieved through its superior mobility in a galactoglucan matrix. However, is superior mobility alone sufficient to explain the invasion? If so, growth in well-stirred liquid cultures should prevent this invasion. Therefore, we applied the competitive assay to shaking flask cultures. Under these conditions, stationary phase is reached at ≈ 2 days (Fig. S3). When the stationary phase mixed cultures were diluted and plated to observe single colonies, the *expR* mutant displayed a



Colour

Fig. 1. Phenotypes of single-strain and mixed-strain colonies on solid surface reveal mutant invasions.
A. Phenotypes of single-strain colonies labelled with mCherry. Strains are WT (Sm2B3001), *exoB* (Sm2B3001ΔexoB), *visN* (Sm2B3001visN), *exoY* (Sm2B3001ΔexoY), *wgeB* (Sm2B3001wgeB), *exoY/wgeB* (Sm2B3001exoY/wgeB), *expR* (Rm2011), *expR/exoB* (Rm2011ΔexoB) and *expR/visN* (Rm2011visN). Strains with *expR*$^+$ (WT, *exoB*, *visN*, *exoY*, *wgeB* and *exoY/wgeB*) were labelled with the *wge* promoter-mCherry fusion. Strains with a disrupted *expR* (*expR*, *expR/exoB* and *expR/visN*) were labelled with the Smb20911 promoter-mCherry fusion. Photos were taken after 9 days growth. After 24 days, *expR*+ strain colonies were recovered from the agarose, suspended and plated to observe single colonies. Numbers below each photo represents the percentage of *expR* mutants in cfu which arose spontaneously and invaded their colony in the 24 day incubation.
B. Phenotypes of mixed-strain colonies competition, in which strain R (red, labelled with mCherry) was mixed with strain G (labelled with *egfp*, green channel not shown) in a 1(R) : 9(G) ratio before spotting on TY agarose. Strain G is the WT in each case. As for Fig. 1A, photos were taken after 9 days growth. The percentage of strain R colony-forming individuals recovered from the mixed colony after 9 days is shown below each colony photograph. For both A and B, all numbers are the average of three experimental replicates, and standard deviation, where relevant, is indicated.

clear numerical dominance over all *expR*$^+$ strains (66–97%, Fig. 2A, pink bars), regardless of their galactoglucan-producing status. Although *S. meliloti* has previously been shown to produce galactoglucan at high levels in liquid cultures (Sorroche *et al.*, 2010), the genetic disruption of galactoglucan production (*exoB* and *wgeB*) did not prevent *expR* mutant invasions in liquid cultures. Furthermore, when both strains carried a functional *expR*, they maintained a G : R ratio of ≈ 9:1, regardless of the presence of *wgeB* or *exoB*. When the mixed stationary phase cultures were used to start a second culture which was also grown to stationary phase, the superior growth by the *expR* mutant compared with the WT was even more obvious, forming 97–99% of the cfu (Fig. 2A, red bars). In contrast, strains carrying a functional *expR* performed poorly against the WT, remaining at 14–22%.

These results suggested that the invasions by the *expR* mutant were supported by an ExpR-dependent restraint of growth. To see if ExpR required AHLs for this effect, we submitted a *sinI* mutant and an *expR*/*sinI* double mutant to the competitive liquid assay in the presence of increasing concentrations of supplemented AHL (Fig. 2B). In the absence of AHL, the *expR*/*sinI* double mutant did not outcompete the *sinI* mutant, but decreased from an initial cfu of 10% to < 1%. However, growth of the double mutant improved in the presence of 50 nM AHL (≈ 20% cfu). Increasing AHL up to 500 nM strengthened the growth of the double mutant even further (70–80% cfu). Additional increases in AHL concentration up to 10 mM did not further improve the double mutant growth, suggesting that 500–2000 nM represents a saturating point.

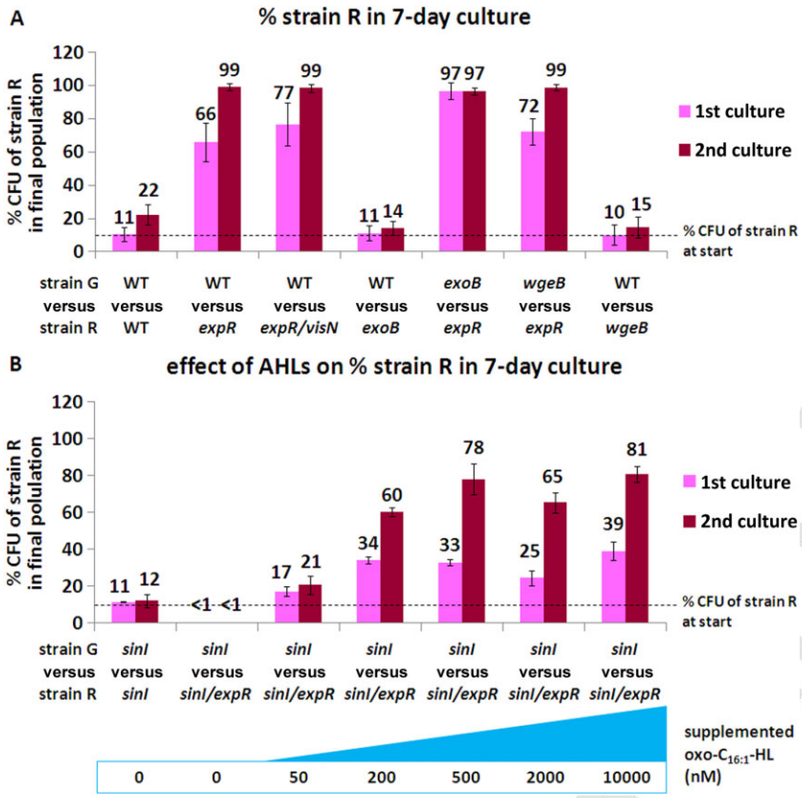


Fig. 2. Competitive growth in liquid culture reveals superior growth by strains lacking *expR*. In each competitive assay, two bacterial strains, R (labelled with mCherry) and G (labelled with egfp), were mixed at a ratio of 1(R) : 9(G).
A. Strains were included as indicated: WT (Sm2B3001), *exoB* (Sm2B3001ΔexoB), *wgeB* (Sm2B3001wgeB), *expR* (Rm2011) and *expR/visN* (Rm2011visN).
B. Strains used were *sinI* (Sm2B4001) and *sinI/expR* (Sm2B4011). AHLs were included at the concentrations indicated below the graph. Both A and B, cfu were determined both at inoculation (indicated by dashed line) and after 7 days inoculation (indicated by bars and numbers above bars). Pink bars indicate cfu at the end of the 1st culture. The 1st culture was used to inoculate a 2nd culture, and cfu from this culture was also estimated after 7 days (indicated by red bars). Error bars indicate standard deviation from three independent biological replicates.

expR *does not strongly affect symbiotic potential*

Compared with the WT, the *expR* mutant has a strong growth advantage under standard laboratory conditions. How does the mutant perform in a symbiotic relationship with its host plant *Medicago sativa?* To answer this question, we tested the competitive ability for nodule occupancy by mixed cultures of WT and the *expR* mutant applied to roots of *M. sativa*. The *expR* mutant showed 10–20% higher nodule occupancy than the WT (Fig. 3). Therefore, under these conditions, the *expR* mutant seems to have a slightly higher symbiotic potential than the WT.

Discussion

Sinorhizobium meliloti is an optimal model for understanding the advantages and costs of QS cooperation and how

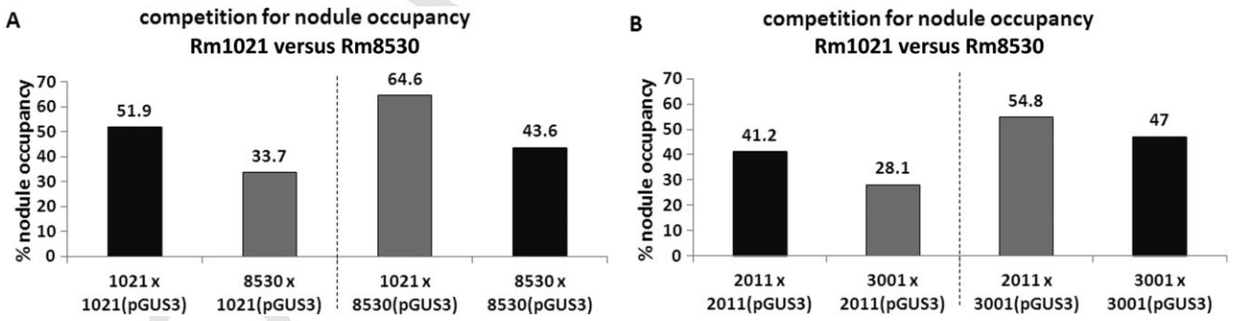


Fig. 3. Competitive nodulation assays reveals that the *expR* mutant has a slight (10–20%) advantage over the wild type. A, mucoid WT strain Rm8530 was mixed with *expR* mutant strain Rm1021, and B, mucoid WT strain Sm2B3001 was mixed with *expR* mutant strain Rm2011 at a ratio of 1:1. After applying these mixtures to roots of alfalfa (*Medicago sativa*), the number of nodules occupied by a single strain was estimated (see materials and methods for details).

this might be stabilized against damaging mutations. Copious galactoglucan production by *S. meliloti* is dependent upon QS, allowing easy detection of distinct phenotypes associated with parent and mutant. Galactoglucan is a common good that improves survival through bacterial autoaggregation and biofilm formation (Rinaudi and González, 2009; Sorroche *et al*., 2012), colony expansion (Gao *et al*., 2012; Nogales *et al*., 2012; Dilanji *et al*., 2014) and protection against predation (Pérez *et al*., 2014). Yet QS-activated galactoglucan production implies heavy carbon consumption and thus likely incurs a significant metabolic cost. The loss of *expR* removes this cost, although some of it might be offset by the cost of flagella and pili production, since these are downregulated by ExpR. In our experiments, a 1.5% agar or agarose surface likely presents a formidable motility barrier that is overcome by copious galactoglucan production. During invasions, the mutant requires its own flagella and galactoglucan contribution by the parent strain, moves rapidly through the mucoid matrix to the expanding frontier of the colony and thereby benefits from fresh nutrients. Assisting the invasions is the ExpR-dependent restraint of growth. Thus, the regulation of multiple targets by ExpR (i.e. pleiotropy) helps explain the rapid invasions by the *expR* mutant, where the disruption of *expR* is a single event with multiple benefits. Alternative mutations to achieve the same outcome are significantly less probable since they would require multiple genetic events. Interestingly, pleiotropy has also been invoked as a mechanism that stabilizes cooperation in the social amoeba *Dictyostelium discoideum* (Foster *et al*., 2004). We suspect that pleiotropic trade-offs also stabilize QS cooperation in *S. meliloti*, and that this is through ExpR-dependent regulation of one or many of the genes whose functions are not beneficial during growth under standard laboratory conditions. This situation highlights the importance of the genotype–environment interplay in stabilizing the cooperative genotype. Changes in the environment, such as domestication, create new selection criteria and render the 'wild type' genotype unstable (reviewed by Barrick and Lenski, 2013).

Interest in the costs and benefits of QS-based cooperation has been growing (Schuster *et al*., 2013). For example, recent studies in the rice pathogen *Burkholderia glumae* revealed that QS anticipates and counters ammonia-mediated alkaline toxicity during stationary phase (Goo *et al*., 2012) and restrains growth by slowing metabolism and controlling the uptake of nutrients (An *et al*., 2014). Restraint of growth is a particularly interesting aspect of cooperation, because it simultaneously provides the organism with a cost and an advantage. Although control of population growth may prevent overcrowding and population collapse, the cost is present because any non-cooperative mutant in the population is not restrained and can therefore outcompete the parent, rendering the population vulnerable to invasions.

Restraint of growth may be a widely distributed aspect of QS. Other hints of this can be found in various bacteria, such as *Sinorhizobium fredii* (He *et al*., 2003), *P. aeruginosa* (You *et al*., 1998), *Rhodospirillum rubrum* (Carius *et al*., 2013) and *Bacillus subtilis* (Lazazzera, 2000). Furthermore, an increasing number of studies reveal correlations between nutrient limitation and the stringent response and QS thresholds (Lazazzera, 2000; van Delden *et al*., 2001; Erickson *et al*., 2004; Moris *et al*., 2005; Duan and Surette, 2007; Schuster and Greenberg, 2007; McIntosh *et al*., 2009; Schafhauser *et al*., 2014). The observation here that ExpR restrains growth in *S. meliloti* fits with the notion that QS is useful for coping with starvation and population control. ExpR is well positioned to restrain population increase at an appropriate population density relative to nutrient availability. Under such conditions, ExpR restrains growth and flagella-based motility and diverts resources towards the sessile lifestyle in anticipation of harsh conditions. In contrast, the mutant is blind to the QS signal and continues growing until impeded only by the utter depletion of nutrients, ill-prepared for hard times ahead.

How is QS cooperation stabilized in *S. meliloti*? Unlike the *lasR* mutant of *P. aeruginosa* which is dependent upon its parent for the production of extracellular proteases during growth on casein (Dandekar *et al*., 2012), the *expR* mutant under standard laboratory conditions eventually outcompetes and eliminates the parent. Thus, single-strain cultures of the *expR* mutant grow unimpeded under standard laboratory conditions. However, we emphasize that this is conditional upon the specific environmental conditions. Other growth conditions may reveal additional parent dependencies of the *expR* mutant.

Based on the results of this study, the loss of QS capacity is a typical consequence of the domestication of *S. meliloti*, but this does not directly reduce symbiotic potential. Future work will be directed at understanding how QS in *S. meliloti* is stable in the natural environment. Additionally, the mechanism(s) for an ExpR-dependent restraint of growth are unknown, although a recent study on *B. glumae* describing a QS-induced metabolic switch may provide some hints (An *et al*., 2014). Finally, many regulatory targets of ExpR remain uncharacterized (Gurich and González, 2009; Charoenpanich *et al*., 2013). These will provide foci for future studies aimed at understanding the benefits and mechanisms of QS cooperation.

Experimental procedures

Bacterial strains, plasmids and growth conditions

Strains and plasmids used in this work are listed in Table S2. *Sinorhizobium meliloti* strains were grown at 30°C

on solid or liquid tryptone-yeast (TY) medium (Beringer, 1974). Antibiotics were routinely included at the following concentrations: 10 μg ml^{-1} nalidixic acid, 20 μg ml^{-1} gentamicin, 10 μg ml^{-1} tetracycline, 120 μg ml^{-1} neomycin and 200 μg ml^{-1} kanamycin.

Plasmid construction

For the construction of pWBPwgeAmCherry, the *wgeA* promoter region was PCR-amplified from genomic DNA using forward primer 5′-tgccaagctttcgggaggactgacctgt and reverse primer 5′-caaatctagattccaaagtggccatctgctt, generating a fragment with *Hin*dIII and *Xba*I restriction sites (underlined) fused to the promoter fragment. mCherry (Clontech) was PCR amplified using forward primer 5′-atctctagaatggtgagcaaggg cgagga and reverse primer 5′-catgtcgacttacttgtacagctcgtc catg, which fused *Xba*I and *Sal*I restriction sites. Vector pSRK-Gm^{R} was digested with *Hind*III and *Sal*I, the *wgeA* promoter region with *Hind*III and *Xba*I, and mCherry with *Xba*I and *Sal*I. pWBP20911mCherry was created similarly. For the PCR amplification of the Smb20911 promoter, forward primer 5′-tacaagcttcgtagatctgaggaggagagc and reverse primer 5′-tagtctagatctgatcatcgagcgccctt were used.

pPHUPexpRexpR was obtained by cloning the *expR* promoter and coding region into vector pPHU231 (*Hind*III/*Bam*HI). This fragment was PCR amplified using forward primer 5′-gcgtaagcttatgatcttcatccaccttg and reverse primer 5′-attcggatccttgtgccgtcaggagatcagt.

Consecutive subculturing of S. meliloti

For the consecutive subculturing of the three *S. meliloti* WT strains, Rm41, Rm8530 and Sm3B3001 on TY agar, the bacterial strains were firstly streaked on a TY agar plate to obtain single colonies. From each strain, two single colonies were selected for glycerol stocks and served as biological replicates. Three consecutive subculturing experiments were performed. In the first experiment, bacterial strains from the glycerol stock were grown on TY agar containing 10 μg ml^{-1} of nalidixic acid and incubated at 30°C. After incubation, bacteria were recovered from the agar surface and re-streaked on fresh TY agar using a sterile glass pipette. This second agar culture was counted as the second consecutive culture. Throughout the subculturing experiments, bacteria grew as a lawn on the agar. From each agar culture, cells were also re-suspended in TY broth, diluted and plated on TY agar to observe 50–100 colonies. This process of consecutive subculturing was repeated at 3.5–7 day intervals until at least 50% of the colonies exhibited a dry phenotype. For the third experiment, in which pWBPwgeAmCherry was introduced into all strains as an colorimetric indicator of galactoglucan production, the TY agar contained 20 μg ml^{-1} gentamicin and cultures were firstly incubated at 30°C for 2 days and then at room temperature for 5 days. Dry/white mutants were arbitrarily selected for analysis of the *expR* sequence.

Competitive solid medium culture assay

Strain G was the mucoid/WT strain Sm2B3001, while strain R carried either plasmid pWBPwgeAmCh if carrying a functional *expR* or pWBP20911mCh if carrying a non-functional *expR*. Plasmid pWBP20911mCh carried a fusion of *mCherry* to the promoter of the gene SMb20911, previously shown to be highly active only the in the absence of a functional *expR* (Charoenpanich *et al*., 2013). Starter cultures were grown overnight in TY supplemented with gentamicin. The strains were diluted to OD_{600} = 0.001, mixed at a ratio of 9 (G) : 1 (R), and 1 μl of the mixture was spotted onto the growth surface. For growth, we used TY agarose at a concentration of 1.5%, since this is the standard concentration for agar cultivations. The advantage with agarose is lower background fluorescence. The agarose culture was then incubated with the growth surface on top at 30°C. Fluorescence and bright-field photographs of the colonies were made at various time intervals (up to 24 days) using a stereo fluorescence microscope (Nikon SMZ1000). Care was taken to capture both the bright-field image and its corresponding fluorescence image at the same depth of focus.

Competitive liquid culture assay

Strain G was either the mucoid/WT strain SM2B3001 or one of the *expR*$^{+}$ mutants, *exoB* (Sm2B3001ΔexoB) and *wgeB* (Sm2B3001wgeB) and carried a plasmid with the *wge* promoter fused to *egfp* (pWBPwgeAegfp). Strain R was labelled with mCherry by either plasmid pWBPwgeAmCh if carrying a functional *expR* or pWBP20911mCh if carrying a non-functional *expR*. Strains were grown separately in a TY broth starter culture at 30°C overnight. The bacteria were then diluted in fresh TY to OD_{600} = 0.001 and mixed at a ratio of 9 (G) : 1 (R) in a final volume of 5 ml. After 7 days incubation at 30°C, the cultures were serially dilution and plated on multiple TY agar plates to obtain at least 200 green or red colonies per culture.

Competition assays on alfalfa plants

Surface-sterilized alfalfa *(Medicago sativa* L. cv. Aragón) seedlings were grown in hydroponic cultures under axenic conditions in glass tubes containing nitrogen-free nutrient solution as described by Olivares and colleagues (1980). To determine competitive ability, 12-day-old plants (a total of 12 replicates, one in each tube) were inoculated with 1 ml of a bacterial suspension. Prior to this inoculation, bacteria were grown to exponential phase (OD_{600} = 0.5 to 0.6) in TY medium supplemented with antibiotics where appropriate for plasmid maintenance. To obtain the 1:1 ratio required in the co-inoculation mixture, strains were diluted (≈ 100-fold) from their respective cultures to matching ODs and mixed together in sterile distilled water. This suspension was tested and contained approximately 10^{6} cfu. To determine nodule occupancy by competing strains, one of the strains was marked with the pGUS3 plasmid which contains an *nfeD-gusA* fusion (stains nodule blue after a β-glucuronidase assay) while the second strain did not carry pGUS3 (García-Rodríguez and Toro, 2000). To determine nodule occupancy, roots were collected 14–15 days after inoculation, briefly washed with water, and incubated overnight in the dark at 37°C in 1 mM X-Gluc (5-bromo-chloro-3-indolyl-β-D-glucuronide, Apollo Scientific, UK) in 50 mM

sodium-phosphate buffer (pH 7.5) with sodium dodecyl sulfate 1% (wt/vol). Nodule occupancy was determined by counting blue and white nodules. If the strain without the plasmid is the sole occupant of a nodule, that nodule will remain white. If a nodule contains the strain with pGUS3 or a mixture of both strains, it will turn blue. In this way, white nodules were indicative of sole occupancy by the strain lacking pGUS3.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Loss-of-function mutation in *expR* is the cause of the non-mucoid phenotype.
A. Mucoid strains eventually turn dry upon serial subculturing.
B. Mutant phenotypes were complemented via a plasmid-borne copy of *expR*.
C. Amino acid sequence of ExpR and mapped mutations. N-terminal AHL-binding domain is in blue and C-terminal DNA binding domain in coral red. Locations of each mutation are indicated (underlined) and numbered. (See Table S1 for detailed description of the mutations.)
D. Mutations in *expR* correlates with loss of ExpR activity.
Fig. S2. All ExpR variants are defect in activity.
A. Western blot analysis of ExpR and mutant derivatives in *S. meliloti* strains as indicated (3001, Sm2B3001; 2011, Rm2011). His-tagged proteins purified from *E. coli* (far right) were included as controls.
B. His-tagged proteins from each step of purification from *E. coli* were collected and analysed using SDS-PAGE. Lane are as follows: (i) uninduced whole cells, (ii) induced whole cells, (iii) crude extract, (iv) 1st wash with 20 mM imidazole, (v) 3rd wash with 100 mM imidazole and (vi) eluate with 1 M imidazole. A239P represents one of the mutant variants which could be overexpressed but not purified. M107V and W218C represent mutant variants which could be purified.
C, D. EMSA with purified $(His)_6$-ExpR was used to test DNA binding activity. Labelled DNA included the promoter region of *pstS* as a negative control (upper panel, left), while the promoter region of *wgeA* (upper panel, right) and *sinI* (lower panel) were used to show binding activity.

E, F. Promoters of *wgeA* and *sinI* fused to *egfp* were used to test transcription activation by the ExpR variants. Promoter activity was induced by IPTG-induced ectopic expression of *expR* and variants in an *expR/sinI* double mutant strain (Sm2B4011) which lacks both a functional *expR* and the ability to produce AHLs. Only the WT ExpR and the W218C variant were able to activate promoters of *wgeA* and *sinI* in the presence of $C_{16:1}$-HL. However, W218C showed a significantly lower activation. Error bars were calculated from four biological replicates.

Fig. S3. Growth curves of WT (Sm2B3001), *expR* mutant (Rm2011) and the *exoB* mutant (Sm2B3001ΔexoB) grown in TY broth with shaking at 30°C. OD_{600} was measured at the time points indicated. The results are the average of three biological replicates. Error bars indicate standard deviation.

Table S1. List of *expR* sequence variants from dry/white phenotype mutants which invaded WT strains during the consecutive subculturing experiments described in Fig. S1. Each of the mutant variants is assigned a number from 1 to 36. This number is correlated with the location of the mutation in the *expR* sequence relative to the translation start. Each variation is described at the DNA and the amino acid level. Lastly, the parental strain is indicated. Of the 36 mutants listed, 30 spontaneously arose in our experiments. The other six (mutants 8, 12, 13, 17, 18 and 21) arose elsewhere.

Table S2. Bacterial strains and plasmids used in this work.

Appendix S1. ••.

Chapter 10: Acknowledgements

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Chapter 11: Statutory Declaration

I herewith formally declare that I have written the submitted dissertation independently. I did not use any outside support except for the quoted literature and other sources mentioned in the paper.

I clearly marked and separately listed all of the literature and all of the other sources which I employed when producing this academic work, either literally or in content.

This dissertation has not been submitted in its present or similar form to any other domestic or foreign university in connection with a doctoral application or for other examination purposes.

I am aware that the violation of this regulation will lead to failure of the thesis.

_____

(Pornsri Charoenpanich)

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