Sinorhizobium Meliloti, A Bacterium Lacking The Autoinducer-2 (AI-2) Synthase, Responds To AI-2 Supplied By Other Bacteria

C. S. Pereira

J. R. McAuley

M. E. Taga

See next page for additional authors

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Authors

C. S. Pereira, J. R. McAuley, M. E. Taga, K. B. Xavier, and Stephen T. Miller

Sinorhizobium meliloti, a bacterium lacking the autoinducer-2 synthase, responds to AI-2 supplied by other bacteria

Catarina S. Pereira[1], **J. Randall McAuley**[2], **Michiko E. Taga**[3], **Karina B. Xavier**[1,4], and **Stephen T. Miller**[2,*]

[1]Bacterial Signaling Laboratory, Instituto Gulbenkian de Ciência, Oeiras, Portugal

[2]Department of Chemistry and Biochemistry, Swarthmore College, Swarthmore, PA, USA

[3]Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA

[4]Bacterial Signaling Laboratory, Instituto de Tecnologia Química e Biológica, Oeiras, Portugal

SUMMARY

Many bacterial species respond to the quorum sensing signal autoinducer-2 (AI-2) by regulating different niche specific genes. Here, we show that *Sinorhizobium meliloti*, a plant symbiont lacking the gene for the AI-2 synthase, while not capable of producing AI-2 can nonetheless respond to AI-2 produced by other species. We demonstrate that *S. meliloti* has a periplasmic binding protein that binds AI-2. The crystal structure of this protein (here named SmLsrB) with its ligand reveals that it binds (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF), the identical AI-2 isomer recognized by LsrB of *Salmonella typhimurium*. The gene encoding SmLsrB is in an operon with orthologs of the *lsr* genes required for AI-2 internalization in enteric bacteria. Accordingly, *S. meliloti* internalizes exogenous AI-2, and mutants in this operon are defective in AI-2 internalization. *S. meliloti* does not gain a metabolic benefit from internalizing AI-2, suggesting that AI-2 functions as a signal in *S. meliloti*. Furthermore, *S. meliloti* can completely eliminate the AI-2 secreted by *Erwinia carotovora*, a plant pathogen shown to use AI-2 to regulate virulence. Our findings suggest that *S. meliloti* is capable of 'eavesdropping' on the AI-2 signaling of other species and interfering with AI-2-regulated behaviors such as virulence.

Keywords

Sinorhizobium meliloti; *Erwinia carotovora*; AI-2; luxS; quorum sensing

INTRODUCTION

Quorum sensing is a cell-cell signaling process that enables bacteria to regulate gene expression as a function of population density. It is becoming increasingly apparent that quorum sensing signals, called autoinducers, can provide bacteria more information than simply the number of cells in the vicinity. By sensing combinations of various autoinducer signals in the environment, bacteria can determine, for instance, the species composition of the population or if they are inside or outside their host. Bacteria translate the information provided by the different autoinducers into specific gene expression responses leading to the promotion or inhibition of group behaviors such as bioluminescence, biofilm formation, and production of virulence factors. Additionally, some bacteria have mechanisms that enable

*To whom correspondence should be sent: Stephen Miller, Department of Chemistry and Biochemistry, 500 College Ave, Swarthmore, PA 19081, USA, (610) 957-6063 (tel) (610) 328-7355 (fax), smiller1@swarthmore.edu.

them to interfere with other species' ability to correctly sense and respond to autoinducer signals. It is likely that this interference with quorum sensing provides a benefit during competition for colonization of a common niche.

Most autoinducers are species specific; however, one autoinducer, autoinducer-2 (AI-2), and its synthase, LuxS, have been identified in many bacteria including both gram-negative and gram-positive species. Likewise, bacterial species have been shown to respond to AI-2 with behaviors such as bioluminescence in *Vibrio harveyi* (Mok *et al.*, 2003; Schauder *et al.*, 2001; Waters and Bassler, 2006), motility in *Helicobacter pylori* (Rader *et al.*, 2007), interference with AI-2 regulated quorum sensing in *Escherichia coli* (Xavier and Bassler, 2005a), cell division and stress response in *Streptococcus mutans* (Sztajer *et al.*, 2008), virulence and formation of biofilms in *Vibrio cholerae* (Hammer and Bassler, 2003, 2007; Miller *et al.*, 2002; Xavier and Bassler, 2005a) and *Staphylococcus epidermis* (Li *et al.*, 2008), and mutualistic biofilm growth in co-cultures of *Actinomyces naslundii* and *Streptococcus oralis* (Rickard *et al.*, 2006). AI-2 is hypothesized to play an important role in enabling cross-species communication by allowing bacteria to regulate gene expression in response to the density and species composition of the bacterial populations they encounter. In some species (such as the examples given above), AI-2 has been shown to be the chemical signal responsible for inducing regulation of those specific phenotypes; in other species, the role played by AI-2 might be more complex and further studies are needed to distinguish between the metabolic effect of disrupting the AI-2 synthase and the responses caused by the signal itself (Turovskiy *et al.*, 2007; Vendeville *et al.*, 2005).

Despite the large number of studies identifying AI-2 regulated phenotypes (reviewed in in (Hardie and Heurlier, 2008; Xavier and Bassler, 2003)), the mechanisms of AI-2 detection and signal transduction have only been determined in two *Vibrio* species (*V. harveyi* and *V. cholerae*) (Chen *et al.*, 2002; Lenz *et al.*, 2004; Miller *et al.*, 2002; Mok *et al.*, 2003; Waters and Bassler, 2006) and the enteric bacteria *Salmonella typhimurium* and *E. coli* (Taga *et al.*, 2001; Taga *et al.*, 2003; Xavier and Bassler, 2005b; Xavier *et al.*, 2007). LuxS catalyzes the production of 4,5-dihydroxy-2,3-pentanedione (DPD) from *S*-ribosylhomocystine, however, DPD is not directly recognized by these species as AI-2 (Chen *et al.*, 2002; Miller *et al.*, 2004; Schauder *et al.*, 2001). Rather, crystal structures of the AI-2 receptor/ligand complexes revealed that these bacterial species recognize different adducts of DPD as AI-2 signals. In *V. harveyi*, the AI-2 signal is formed by cyclization of DPD, followed by hydration and addition of borate. In enteric bacteria, the LsrB protein recognizes an AI-2 moiety that lacks boron and is a different stereoisomer than the signal recognized by *V. harveyi* (Fig. 1A). Importantly, although different bacterial species recognize chemically distinct molecules as AI-2, these molecules interconvert spontaneously in solution, allowing different bacterial species to respond to one another (Miller *et al.*, 2004; Xavier and Bassler, 2005a).

In *S. typhimurium* and *E. coli*, AI-2 induces the production of a transport apparatus responsible for internalizing, phosphorylating, and processing of the AI-2 signal (Fig. 1B). The genes encoding this transport system are in the operon *lsr* (for LuxS Regulated), along with other genes involved in AI-2 processing and response (Taga *et al.*, 2001; Taga *et al.*, 2003; Xavier and Bassler, 2005b). The Lsr transport system internalizes endogenously produced AI-2 as well as AI-2 produced by other bacterial species, eliminating the signal from the environment. Thus, in cultures composed of different species, these enteric bacteria are capable of interfering with the AI-2-mediated signaling of other species by disrupting their ability to regulate group behaviours (Xavier and Bassler, 2005a). Recently, Demuth and co-workers have studied the function of the Lsr homologue in the oral pathogen *Aggregatibacter (Actinobacillus) actinomycetemcomitans* and showed that this organism is also capable of internalizing AI-2 from the environment via the Lsr system. Further, they

demonstrated that the LsrB homologue is required to mediate the complete, AI-2-dependent activation of biofilm formation in this organism (Shao *et al.*, 2007a;Shao *et al.*, 2007b).

Of the bacteria that have a complete genome sequence in the KEGG database as of this writing, there are 16 different species with protein sequences that have greater than 60% sequence identity to the LsrB protein from *S. typhimurium*. Interestingly, two of these bacterial species, *Sinorhizobium meliloti* and *Rhodobacter sphaeroides*, do not have orthologs to the LuxS protein from *S. typhimurium*. This leads us to hypothesize that although these bacteria do not make their own AI-2 they could use their LsrB homologues to recognize AI-2 produced by other species. Additionally, sequence analysis revealed that *S. meliloti* also has orthologs to all the proteins of the Lsr systems from the enteric bacteria (Fig. 1B and 1C), except LsrE (which is present in *S. typhimurium* but not in *E. coli* and has no known function), suggesting that this operon could be involved in AI-2 internalization in *S. meliloti*. It should be noted that the LsrF orthologue (*SMb20498*) has the lowest sequence homology to its *S. typhimurium* counterpart and is not located in the same operon, raising the doubt as to whether it plays the same role as *E. coli* and *S. typhimurium* LsrF.

S. meliloti is a soil bacterium well known for its capacity to establish a symbiotic relationship with legume plants from the genera *Medicago, Melilotus* and *Trigonella*. *S. meliloti* symbiosis is initiated under nitrogen-limiting conditions by the exchange and recognition of specific signals between the plant and the bacteria. *S. meliloti* has at least two, and in some strains three, quorum sensing systems dependent on homoserine lactone-type autoinducers (Gonzalez and Marketon, 2003). While it has been shown that these species-specific quorum sensing systems regulate functions crucial for symbiosis between this bacterium and its host, they are not expected to facilitate bacterial inter-species quorum sensing. Here, we show that *S. meliloti* does not produce the inter-species signal AI-2 but does contain a functional AI-2 receptor protein, and furthermore that *S. meliloti* recognizes the same form of AI-2 previously described for *S. typhimurium*: (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF). *S. meliloti* is able to internalize exogenously supplied AI-2 from its environment, and it responds to the AI-2 signal by up-regulating transcription of its Lsr-like operon. By importing and responding to a signal it does not produce, *S. meliloti* is apparently employing a different strategy for AI-2-based signaling than that employed by previously characterized species possessing the Lsr system, in effect eavesdropping on other bacteria rather than participating in the conversation.

RESULTS

S. meliloti contains an AI-2 Binding Protein

The SMb21016 hypothetical protein from *S. meliloti* is 72% identical to the LsrB protein from *S. typhimurium* and, significantly, the gene *SMb21016* is located in an operon that includes orthologs to all the genes necessary for AI-2 internalization in *S. typhimurium* (Fig. 1B and 1C). To demonstrate that the protein encoded by *SMb21016*, the putative *S. meliloti* AI-2 receptor gene, is capable of binding AI-2, we cloned and overexpressed the protein in an *E. coli* strain producing AI-2. The candidate protein was purified and tested for AI-2 binding. Ligand was released from the receptor by thermal denaturation and the denatured protein removed from solution by pelleting. The resulting supernatant containing released ligand was subsequently tested for its ability to induce bioluminescence in a reporter strain of *V. harveyi*, MM32, which produces light only in the presence of exogenous AI-2.

Ligand released from the *S. meliloti* AI-2 receptor induced a light response similar to that of ligand released from *S. typhimurium* LsrB (Fig. 2, black bars). To confirm that the light response was specifically induced by AI-2, receptor proteins from *S. meliloti* and *S. typhimurium* were overexpressed in FED101, an *E. coli* strain lacking LuxS and thus unable

to produce AI-2. As expected, denaturation of these proteins released no ligand capable of inducing light production in MM32 (Fig. 2, white bars). As a further control, ribose binding protein from *E. coli* was overexpressed as a GST-fusion and tested for AI-2 binding ability; the protein showed no ability to bind AI-2 in this bioassay (Fig. 2). Thus, the periplasmic binding protein (PBP) encoded by the *S. meliloti SMb21016* has the capacity to bind AI-2, and we named it SmLsrB (for *S. meliloti* LsrB).

Structure of the *S. meliloti* AI-2-receptor complex

To identify the form of AI-2 recognized by *S. meliloti*, we determined the crystal structure of the SmLsrB/AI-2 complex. SmLsrB crystallized with ligand bound, and the structure was solved at 1.8Å resolution by molecular replacement (bound ligand was omitted from the molecular replacement model). The *S. meliloti* AI-2 receptor has a classic PBP fold, with two α/β domains connected via a three-stranded hinge. The structure is very similar to that of LsrB from *S. typhimurium*, with an RMSD of 0.6Å. As with other PBPs, the receptor binds the ligand in a cleft between the two domains (Fig. 3A). The structure has been deposited in the PDB with ID code 3EJW.

After the structure of the protein was modeled and refined, electron density corresponding to the ligand was easily interpretable. This density was modeled as *R*-THMF (Fig. 3B), the same form of AI-2 recognized by *S. typhimurium* (Fig. 1A). The electron density does not make it possible to unequivocally rule out the enantiomer *S*-THMF as the ligand, but the assignment of *R*-THMF is consistent with the chemical environment of the binding site. If the *R*- form of AI-2 is bound, the methyl group on the cyclized ligand is positioned in a hydrophobic pocket, surrounded by residues Phe43, Ala224, Leu268, and Trp269 (Fig. 3C). In contrast, modeling the *S*- form of the ligand into the binding site leads to the less energetically favorable positioning of a hydrophilic hydroxyl group in this hydrophobic pocket. Further, in the *S*- form, the nearest potential hydrogen bonding partner for this hydroxyl group is the backbone nitrogen from Ala224, but the interatomic distance of 4.12Å and poor geometry rule out a significant hydrogen bond interaction. When the *R*- form is modeled into the binding site electron density, this hydroxyl group is 3.35Å from the backbone oxygen of Pro222 (within hydrogen bonding distance) and 3.85Å from the side chain of Gln169 and thus reasonably positioned for a beneficial electrostatic interaction. Thus, we expect that these interactions selectively stabilize the receptor-ligand complex with bound *R*-THMF. Furthermore, these interactions are consistent with those observed previously in the *S. typhimurium* LsrB/AI-2 complex; in that case, higher resolution electron density further supported the assignment of *R*-THMF as the bound form of AI-2. Thus, all the evidence supports the conclusion that *R*-THMF is the predominant species recognized by *S. meliloti* as AI-2.

S. meliloti removes exogenously supplied AI-2 from extracellular medium

S. meliloti does not have an ortholog to the *luxS* gene from *S. typhimurium*, and thus we expected that this bacterium does not produce AI-2. Accordingly, cell-free culture fluids collected from *S. meliloti* cultures grown on LBMC do not possess AI-2 activity, as measured by induction of bioluminescence in a *V. harveyi* BB170 AI-2-reporter assay (Fig. 4A, squares).

We cultured *S. meliloti* in medium supplemented with *in vitro* synthesized AI-2 and followed the AI-2 activity in the cell-free culture fluids over time. As shown in Fig. 4A, the initial AI-2 activity due to the addition of synthetic AI-2 results in 100,000-fold induction of bioluminescence in the *V. harveyi* assay (Fig. 4A, black triangles). However, after 12 hours of growth, the AI-2 activity measured in the cell-free culture fluids decreases drastically to only 20-fold induction. This decrease in extracellular AI-2 activity is consistent with *S.*

meliloti having a functional Lsr operon and, thus, the ability to remove exogenously supplied AI-2 from its environment.

The decrease in apparent AI-2 levels in *S. meliloti* cultures supplemented with AI-2 could also be due to accumulation of an inhibitor of the *V. harveyi* AI-2 response or to the degradation of AI-2 by some extracellular factor. To eliminate the first possibility, we added AI-2 to *S. meliloti* cell-free culture fluids collected after 24 hours of growth (OD=6.0). These samples of cell-free culture fluids were subsequently tested in the *V. harveyi* bioluminescence assay and found to give high-level light induction (Fig. S1). Thus, no inhibitor was present. To control for the possibility of AI-2 degradation, we collected cell-free culture fluids from *S. meliloti* grown in the presence of AI-2 for 18 hours (OD=3.6). As shown in Fig. S2A, no AI-2 activity can be detected in these cell-free culture fluids. We then supplemented this cell-free culture fluid with synthetic AI-2 and incubated this sample for 24 hours at 30°C to determine the stability of AI-2 in this cell-free sample. Results from a BB170 bioluminescence assay show that AI-2 activity did not decrease during this incubation period (Fig. S2B). Therefore, we conclude that the decrease of extracellular AI-2 activity observed during growth of *S. meliloti* results from AI-2 internalization by the cells and not from inhibition of AI-2 detection or degradation of AI-2 by an extracellular factor.

Increased transcription of the *lsr*-like operon *ait* occurs in response to AI-2

In *S. typhimurium* and *E. coli*, the *lsr*-operon is induced by the presence of AI-2. Induction of *lsr* in these bacteria leads to increased production of the AI-2 transport proteins and, thus, a positive feedback loop resulting in increased removal of AI-2 from the extracellular medium. As shown in Fig. 1B and 1C, the *S. meliloti* orthologs to the proteins of the *S. typhimurium* Lsr transport system (LsrA, LsrC, LsrD, and LsrB) have a sequence identity above 49% to their orthologs. Thus, we predicted that the *S. meliloti lsr*-like operon was involved in the removal of AI-2 from culture fluids reported above and we named the *S. meliloti lsr*-like operon *ait* (for <u>a</u>uto<u>i</u>nducer <u>t</u>ransporter). This name was chosen because the name *lsr* has already been given to another gene in *S. meliloti* with an unrelated function (Luo *et al.*, 2005).

We anticipated that *S. meliloti* would respond to exogenously supplied AI-2 with a similar up-regulation of its *ait* operon. To test this premise, we used RT-PCR to determine whether the addition of AI-2 to *S. meliloti* cultures would induce transcription of this operon. As predicted, levels of *aitB* (*SMb21016*) mRNA were higher in the culture that was supplemented with synthetic AI-2 than in AI-2-free cultures (Fig. 4B, black and white bars respectively). Moreover, induction of *aitB* transcription decreases overtime, an observation in accordance with the decrease of extracellular AI-2 during growth shown in Fig. 4A (triangles).

S. meliloti aitK and *aitA* mutants are impaired in the ability to remove AI-2 from the medium

To verify if the *S. meliloti* Lsr-like system (Ait) was capable of internalizing AI-2, we constructed a mutant in *aitA* (previously *SMb21019*), the *S. meliloti* gene homologous to *lsrA* from *S. typhimurium* (Fig. 1B). In *S. typhimurium* and *E. coli*, this gene is predicted to encode the ATP-binding subunit of the Lsr transporter. Comparison of AI-2 activity in cell-free culture fluids of *S. meliloti* WT cultures with cultures of the *aitA* mutant shows that the mutant is defective in AI-2 internalization (Fig. 5, diamonds and squares respectively), supporting our prediction that this operon encodes a functional AI-2 transporter that accounts, at least partially, for the observed AI-2 internalization.

In *S. typhimurium* and *E. coli*, the phenotype of the *lsrK* mutant with respect to AI-2 internalization is even more pronounced than a transport mutant because phosphorylation of AI-2 is required for trapping the signal inside the cell (Taga *et al.*, 2001; Taga *et al.*, 2003; Xavier and Bassler, 2005b). As shown in Fig. 5, this is also true for the *S. meliloti* mutant in *aitK* (previously *SMb21022*) the *lsrK* orthologue (Fig. 5, triangles). In cultures of the *aitK* mutant, AI-2 activity persists in the extracellular medium for much longer than in wild type *S. meliloti*. The fact that *aitA* transport mutant is less defective than the *aitK* mutant in AI-2 removal suggests that at least one more transport mechanism for AI-2 exists, a finding consistent with previous results in enteric bacteria (Taga *et al.*, 2001; Taga *et al.*, 2003; Xavier and Bassler, 2005b). Nonetheless, our data indicate that in *S. typhimurium, E. coli*, and *S. meliloti* the alternate system(s) for AI-2 uptake is less efficient in AI-2 transport than the Lsr and Ait systems.

We have also tested the ability of the *S. meliloti aitA* mutant to colonize its host, the plant *Medicago sativa*, and found that plants inoculated with the *aitA* mutant or the *aitK* mutant were indistinguishable from WT-inoculated plants. Additionally, the *aitA* mutant did not have a competitive defect when co-inoculated with the WT (data not shown). We interpret this result to mean that the *S. meliloti* Ait system is not essential in this single-species symbiosis process. This result is not surprising since, as we demonstrate in Fig. 4B, the Ait system in *S. meliloti* is only induced in the presence of exogenously supplied AI-2.

S. meliloti does not grow with AI-2 as a sole carbon source

Some bacteria, such as *Variovorax paradoxus*, are capable of degrading specific acyl-homoserine lactone autoinducers and using these molecules as a source of nitrogen and energy when grown in minimal medium (Leadbetter and Greenberg, 2000). This led us to test whether, in conditions of low nutrient availability, *S. meliloti* could similarly use AI-2 as a sole carbon source. We grew cultures of *S. meliloti* in minimal medium (M9) using AI-2 as a carbon source and compared growth to cultures grown in M9 supplemented with a variety of sugars (glucose, sucrose, and ribose). *S. meliloti* was able to grow on glucose, sucrose, and ribose as a sole carbon source (at a concentration of 2 mM) but not when the same concentration of AI-2 was provided (Fig. S3). After 72 hours of incubation the OD_{600} of a culture of *S. meliloti* in M9 supplemented with 2 mM AI-2 remained as low as the control where no carbon source was added. Additionally, our results (Fig. 4A and Fig. 5) show that in rich medium (LBMC) addition of AI-2 to cultures of *S. meliloti* WT or *ait* mutants does not affect the growth rate of these strains. Thus, we have no evidence indicating that *S. meliloti* can gain any metabolic benefit from internalizing AI-2.

S. meliloti clears AI-2 produced by *Erwinia carotovora* in co-cultures of these two species

We have shown that *S. meliloti* is capable of removing exogenously supplied synthetic AI-2 from culture fluids and that the *S. meliloti* Ait system is involved in this process. Importantly, we also showed that the Ait system is induced only when exogenous AI-2 is supplied to the culture. To test if *S. meliloti* grown in the presence of an AI-2 producing bacterial species could use the Ait system to remove AI-2 produced by that species, we cultured *S. meliloti* in the presence of *Erwinia carotovora* (wild type strain SCC3193). *E. carotovora* is a Gram-negative plant pathogen that can co-exist with *S. meliloti* in the rhizosphere of several plants. *E. carotovora* has a *luxS* homolog and has been shown to produce AI-2 (Laasik *et al.*, 2006). Accordingly, AI-2 activity was detected in cell-free culture fluids of cultures of *E. carotovora* and in co-cultures with wild type strains of both *S. meliloti* and *E. carotovora* (Fig. 6, triangles and circles respectively) but not when *S. meliloti* was co-cultured with an *E. carotovora luxS* mutant (strain SCC6023) incapable of producing AI-2 (Fig. 6, crosses). In assays of mixed cultures of the wild type strains of *S. meliloti* and *E. carotovora*, we observed that AI-2 activity in culture cell-free fluids increased for 4 hours

and then began to decrease (Fig. 6, circles). After 6 hours, AI-2 activity in this culture was almost undetectable. In contrast, AI-2 activity in cell-free culture fluids remained high when *E. carotovora* is grown as a pure culture (Fig. 6, triangles) or in mixed cultures of *E. carotovora* and the *S. meliloti aitK* mutant (Fig. 6, squares), showing that the disappearance of AI-2 from the extracellular medium in these mixed cultures requires *S. meliloti* with a functional Ait system.

DISCUSSION

As of this writing, approximately 40% of the nearly 800 sequenced bacterial genomes contain the *luxS* gene (based on genomes with homologues to the *luxS* gene of *S. typhimurium* with an e-value smaller than 10^{-12} (http://www.genome.jp/kegg/)), suggesting that there are a large number of bacterial species capable of producing DPD, the AI-2 precursor. Additionally, *luxS*/AI-2 has been implicated in the regulation of a variety of niche-specific functions. For these reasons, AI-2 has been proposed to function as a universal bacterial signal. Here we provide the molecular mechanism for AI-2 detection and response in *S. meliloti*, an organism that lacks the AI-2 synthase and thus is incapable of producing its own AI-2. We have determined the crystal structure of the AI-2/receptor complex in *S. meliloti* and have shown that transcription of this receptor is dependent on exogenously supplied AI-2, produced either synthetically or by organisms capable of synthesizing AI-2.

This work demonstrates that AI-2 signaling can influence levels of gene expression in non-AI-2 producers, a fact that increases the range of species with the potential to be involved in exchange of, or response to, the AI-2 molecule beyond those that carry the *luxS* gene. The *S. meliloti* response to AI-2 emphasizes the role of AI-2 as an inter-species signal; because *S. meliloti* is incapable of producing AI-2, other organisms are the only source of AI-2 in the environment. Significantly, the *S. meliloti* case is not an isolated example; in fact, Surette and colleagues have shown (Duan and Surette, 2007) that expression of several genes in *Pseudomonas aeruginosa*, another organism lacking *luxS*, is influenced by the presence of AI-2, although the molecular mechanism involved in this process has yet to be defined. We predict that this phenomenon is not limited to these two species, and other species lacking LuxS will be shown to respond to AI-2.

Understanding the role played by AI-2 across species requires understanding of the molecular mechanisms by which different species recognize and respond to AI-2. To date, the AI-2 receptors of the marine bacterium *V. harveyi* and the human pathogen *S. typhimurium* have been characterized. Here, by studying AI-2 response in a plant symbiont, we expand our understanding of the molecular mechanism of AI-2 detection into a new environmental niche, the soil. Previous work has shown that *S. typhimurium* and *V. harveyi* recognize chemically distinct forms of the AI-2 molecule and that levels of the various forms of AI-2 present in a particular environment are dictated by the chemistry of that environment (Chen *et al.*, 2002; Miller *et al.*, 2004). In this work, we demonstrate that *S. meliloti* recognizes the same form of AI-2 as the enteric bacterium *S. typhimurium* despite the fact that these bacteria are usually isolated from chemically different niches (the soil and the human gut).

Previous work has shown that *S. typhimurium* and *E. coli* have the ability to internalize AI-2 via their Lsr system, thus removing the molecule from the environment. These species can use this ability to interfere with AI-2 based signaling of other species (Xavier and Bassler, 2005a). Here we show that *S. meliloti* also has a functional AI-2-inducible Lsr-like system (Ait) capable of removing AI-2 from the environment. *S. meliloti* colonizes the rhizosphere of several legume plants and therefore it shares its habitat with many AI-2-producing

bacterial species. Our results show that *S. meliloti* can use the Ait system to clear the AI-2 signal produced by *E. carotovora*, a plant pathogen that can co-exist with *S. meliloti* in the rhizosphere and that has been reported to regulate virulence by AI-2 quorum sensing (Laasik *et al.*, 2006). Thus, it is reasonable to presume that, like the enteric bacterium, *S. meliloti* can use the AI-2 internalization system for interference. However, this strategy of interference likely functions somewhat differently for *S. meliloti* than for the enteric species, since in *S. meliloti* the *ait* operon can only be induced in the presence of AI-2 produced by other bacterial species. Thus, unlike other previously characterized bacterial species, a population of *S. meliloti* cannot up-regulate AI-2 internalization in response to fluctuations in its own population density. Instead, a population of *S. meliloti* could sense the AI-2 produced by its neighbors, leading to induction of its *ait* operon and thus interference with the AI-2 mediated behaviors of other species in the vicinity. Moreover, *S. meliloti* presumably does so without allowing the other species to detect its presence via AI-2 mediated quorum sensing, effectively eavesdropping on its neighbors. It is tempting to speculate that the ability of *S. meliloti to* interfere with the quorum sensing of plant pathogens that use AI-2 to regulate virulence could be beneficial to the plant, decreasing the virulence of pathogens like *E. carotovora*. The identification and characterization of the *S. meliloti* AI-2 dependent Ait system has provided us an excellent tool to begin studying the influence of inter-species bacterial signaling on bacteria-plant interactions, both symbiotic and pathogenic.

It has been argued that some species gain mainly a metabolic benefit from internalization of AI-2 (Winzer *et al.*, 2002b); if this were the case, a non-AI-2 producing species could be acting as a "free-rider" in a mixed-species environment where other species are producing AI-2. Although this remains a possibility, our results indicate that *S. meliloti* gains no metabolic benefit from metabolizing AI-2, at least under our growth conditions. We did not observe an increase in the growth rate of *S. meliloti* cultured in the presence of AI-2 in either complex medium or in minimal medium with AI-2 as sole carbon source, nor did the *S. meliloti ait* mutants show a growth defect in the presence or absence of AI-2.

Some bacteria are capable of degrading acyl-homoserine lactone signals produced by other species (Dong *et al.*, 2000; Leadbetter and Greenberg, 2000; Wang *et al.*, 2007; Zhang *et al.*, 2004). While the producing species use these molecules for species-specific quorum sensing, at least one bacterium, *Variovorax paradoxus*, is able to use these signal molecules as an energy source (Leadbetter and Greenberg, 2000). Although the benefit derived by *V. paradoxus* from removing autoinducer signals from the environment might be only metabolic, *S. meliloti* does not gain a metabolic benefit from internalizing AI-2 and therefore would be expected to gain another advantage. This supports the possibility that *S. meliloti* is using AI-2 internalization as a means to interfere with the quorum sensing of competitive species.

An alternative hypothesis for the function of the AI-2-response in *S. meliloti* is that AI-2 is used to distinguish between being in the soil, a mixed-species environment where, presumably, it encounters AI-2 produced by bacteria such as *Erwinia* or any of several bacillus, and being in its plant host where it exists inside nodules colonized exclusively by a single-species culture of *S. meliloti* and, thus, in a niche where it will encounter no AI-2. Given that *S. meliloti* lacks the ability to produce its own AI-2, it is clear that any benefits derived from AI-2 recognition and transport must arise from inter-species interactions.

EXPERIMENTAL PROCEDURES

Protein Production

AI-2 receptor proteins from *S. typhimurium* and *S. meliloti*, and RbsB from *E. coli* were cloned from each species' genomic DNA (*S. typhimurium* 14028, *S. meliloti* Rm1021, *E.*

coli MG1655, respectively) into plasmid pGEX-4T1 for expression as glutathione-S-transferase (GST) fusion proteins. In all cases, the amino-terminal signal peptides, as determined by the program SignalP (Bendtsen *et al.*, 2004), were omitted from the construct. The primers used to PCR amplify the genes (Stfgex1 and Stfgex2 for the *S. meliloti* AI-2 receptor, Styl1 and Styl2 for *S. typhimurium* LsrB, and Strbs1 and Strbs2 for *E. coli* RbsB) are shown in Table S1.

Plasmids were transformed into *E. coli* strains BL21 and FED101 (BL21, *luxS* mutant), and protein expression was induced by the addition of 0.1 mM isopropyl β-D-thiogalactopyranoside. After induction, the bacteria were grown for 6 hours at 22°C before harvesting.

Cells were resuspended in 25 mM Tris, pH 8.0, 150 mM NaCl, 1 mM DTT and lysed using a M-110Y Microfluidizer (Microfluidics). The lysates were then clarified by centrifugation and fusion proteins purified by affinity chromatography using glutathione agarose (Sigma-Aldrich). Proteins for luminescence assays were eluted from the affinity resin using 25 mM Tris, pH 8.0, 150 mM NaCl, 1 mM DTT, 10 mM reduced glutathone. Eluted protein was concentrated to approximately 10 mg/ml for the luminescence assay.

To prepare the *S. meliloti* receptor protein for crystallization, the GST-fusion protein was digested with thrombin for 18 hours at 4°C while still bound to the glutathione agarose. The receptor protein was then eluted from the agarose in resuspension buffer and subsequently swapped into 25 mM HEPES pH 7.0, 1mM DTT using G25 resin (GE Healthcare). The receptor protein was further purified by ion exchange chromatography using a SouceQ column (GE Healthcare) with a gradient from 0 to 350 mM NaCl. As a final purification step, the protein was subjected to size exclusion chromatography on an Superdex 75 column (GE Healthcare), eluting in 25 mM HEPES pH 7.0, 150 mM NaCl, 1mM DTT. The protein was then concentrated to approximately 10 mg/ml.

AI-2 Binding Assay

Ligands were released from purified receptor proteins by heating the protein samples (10 min, 70°C). The denatured protein was then pelleted and the supernatants used in the luminescence assay. For this assay, the *V. harveyi* strain MM32 (*luxN*::Cm, *luxS*::Tn*5*Kan) was used as a reporter. Because this strain has an insertion in the AI-1 receptor (LuxN) it does not respond to autoinducer-1, and since it is a *luxS* mutant, it does not produce AI-2; thus the strain will only produce light in response to exogenous AI-2 and is effective for discriminating between the presence and absence of AI-2. *V. harveyi* MM32 was grown for 16 hr in AB medium at 30°C and subsequently diluted 1:5000 into fresh AB medium containing 10% released autoinducer sample or buffer. The bacteria were then grown at 30°C and luminescence measured using a Wallac Victor2 1420 multilabel counter. Bioluminescence produced by MM32 is reported as counts per second (c. p. s.) as measured by the instrument.

Crystallization and Structure Determination

Crystals of the *S. meliloti* AI-2 receptor protein expressed in *E. coli* BL21 (LuxS[+]) were grown via the sitting drop method with a well solution of 0.1 M Tris-HCl pH 8.5, 0.2 M MgCl$_2$, 30% w/v PEG 4000 and developed in approximately two weeks at room temperature. Crystals were frozen in mother liquor, and data were collected at 100K using an R-AXIS-IV image plate detector mounted on a Rigaku 200HB generator. The crystals (P2$_1$, a = 57.85, b = 71.49, c = 68.04, β = 98.69) diffracted to 1.8Å resolution. Data were processed using MOSFLM (Leslie, 1992) and CCP4 (CCP4, 1994).

The structure of *S. meliloti* LsrB (SmLsrB) was solved via molecular replacement with PHENIX (Adams *et al.*, 2002), using LsrB from *S. typhimurium* (PDB ID: 1TJY) as a search model. The automatically generated partial structure was used as a starting point for building in Coot (Emsley and Cowtan, 2004). The structure was refined using data to 1.8Å with PHENIX. To avoid bias, the ligand was omitted from the search model and not built until the protein structure had been completed and refined. The binding site electron density was clearly interpretable and modeled as *R*-THMF. A prime-and-switch map was also calculated via PHENIX to confirm the identity of the bound ligand. Refinement parameters for the ligand were generated with the eLBOW module of PHENIX. The final structure contains two copies of SmLsrB, including all residues in the expressed construct (29 – 343), two copies of the bound ligand, and 1113 water molecules. The structure has good geometry (Table 1), with only two residues per chain (Asp118 and Leu268) outside the allowed regions of the Ramachandran plot. Clear density exists for both of these residues. The final R_{cryst} and R_{free} were 0.168 and 0.214 respectively. All molecular images were generated using PyMOL (DeLano, 2002).

AI-2 synthesis

DPD protected with cyclohexylidene was synthesized as reported previously (Semmelhack *et al.*, 2005). The protective group was removed with H_2SO_4 followed by neutralization with potassium phosphate buffer, pH 7 as described in Xavier, 2007 (Xavier *et al.*, 2007).

Bacterial strains and growth conditions

The *S. meliloti* strains used in this study are derived from the wild-type strain Rm1021 (Meade *et al.*, 1982). To construct the *aitA*::pJH104 mutant (MET2000), a 300 bp internal fragment of *aitA* was amplified by PCR from an *S. meliloti* Rm1021 colony with the primers P113 and P114 (Table S1), ligated into the suicide plasmid pJH104 at the SpeI and XhoI restriction sites, and transformed into *E. coli* DH5α. The resulting plasmid was introduced into Rm1021 by triparental mating, and integration of the plasmid was selected by growth on neomycin (0.2 mg/ml). To construct the in-frame deletion of *aitK* (MET2002), a 750 bp region upstream of the *aitK* open reading frame was amplified by PCR with primers P117 and P118 (Table S1), and a 500 bp region downstream of *aitK* was amplified with primers P120 and P121 (Table S1). The two PCR products were ligated in tandem into the plasmid pK18 mob sacB (Schafer *et al.*, 1994) at the BamHI, PstI, and HindIII restriction sites, transformed into *E. coli*, and introduced into Rm1021 by triparental mating. Neomycin resistant exconjugants were plated on TY with 10% sucrose to select for a second recombination event. Neomycin sensitive colonies were screened by PCR for deletion of *aitK*. The *E. carotovora* ssp. *carotovora* strains used are the wild type SCC3193 (Pirhonen, 1991) and its isogenic *luxS*::Cm mutant SCC6063 (Laasik *et al.*, 2006). *S. meliloti* and *E. carotovora* strains were grown at 30°C with aeration in Luria–Bertani broth (LB) supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ (LBMC).

For the studies of *S. meliloti* growth on different carbon sources, *S. meliloti* was grown overnight in LBMC at 30°C. The culture was washed three times in M9 minimal medium (Glazebrook and Walker, 1991) with no carbon source and used to inoculate M9 media with biotin and sucrose, glucose, ribose or AI-2 to a final concentration of 2 mM. As a control, a culture was grown with M9 media and biotin but no carbon source. Cultures were grown at 30°C with agitation for 72 hours and growth was monitored by optical density (OD_{600}). Each carbon source was tested in duplicate.

AI-2 activity in *S. meliloti* and *E. carotovora* cultures

To monitor extracellular AI-2 activity in *S. meliloti* cultures during growth, overnight cultures were diluted to OD = 0.08 into LBMC medium with and without 80 µM chemically

synthesized AI-2. Aliquots were collected at the times indicated and used for measurement of optical density at 600 nm (OD_{600}) and preparation of cell-free culture fluids. The AI-2 activity in cell-free culture fluids was measured using the *V. harveyi* BB170 (*luxN*::Tn5Kan) bioluminescence reporter assay, as described previously (Bassler *et al.*, 1993; Bassler *et al.*, 1994). Cell-free culture fluids were prepared by filtration of liquid cultures (Surette and Bassler, 1998, 1999). The filtered samples were analyzed in duplicate. A similar procedure was used to measure AI-2 activity in *E. carotovora* cultures, either in single cultures or in co-culture with *S. meliloti*. When *S. meliloti* was grown in co-culture with *E. carotovora*, or when single cultures of *E. carotovora* were tested, no synthetic AI-2 was supplied.

AI-2 activity in cell-free culture fluids is reported as fold induction of light produced by BB170 and is calculated as the ratio of light produced by BB170 supplemented with sample to light produced by BB170 supplemented with *S. meliloti* growth medium (LBMC). When required, serial dilutions of the cell-free fluids in LBMC were tested and the values were calculated from the dilution of each sample that resulted in half-maximal induction (approximately 500 fold). (In these experiments, BB170 was used because it allows determination of the fold induction of each sample in relation to the background luminescence when the response is not saturated (Bassler *et al.*, 1993) and is thus appropriate for quantifying the AI-2 in the cell-free fluids. The MM32 assay, used in the AI-2 receptor binding assay, above, is more effective in discriminating between the presence and absence of AI-2.)

Quantitative real-time PCR analysis

To measure the induction of *aitB* transcription by AI-2 in *S. meliloti* culture, aliquots were collected throughout growth in the presence or absence of 80 µM AI-2. Cells were collected by centrifugation at 16,000g for 10 min. 50 µL of 5 mg/ml lysozyme was added to each sample, which were then incubated on ice for 5 minutes. Samples were frozen in liquid nitrogen and stored at −80°C until the RNA was extracted.

S. meliloti RNA was extracted with Trizol reagent (Invitrogen) and chloroform according to the manufacturer's protocol. RNA was precipitated using isopropanol, washed with 75% ethanol, and diluted in DEPC water. RNA samples were diluted to a concentration of 200 ng/ul and treated with DNase I (Roche). The RNeasy Mini kit from QIAGEN was used to clean the RNA. cDNA was generated in 100-µL reactions, each containing 20 µg of RNA, 5x First Strand Buffer (Invitrogen), 100 mM DTT (Invitrogen), 10 mM dNTPs (ABI), random hexamers (Roche), and SuperScript II reverse transcriptase (Invitrogen). The reverse transcriptase reactions were undertaken in a thermocycler with steps of 10 min at 25°C, 50 min at 42°C, and 15 min at 72°C. Identical reactions were performed without reverse transcriptase enzyme to ensure the absence of genomic DNA contamination. Quantitative RT-PCR reaction mixtures contained 5 µl of cDNA template, 2 µl gene-specific primers, 12.5 µl of SYBR Green Mix (Applied Biosystems), and 5.5 µl H_2O. For each reaction, 10 µl of reaction mixture were loaded into 384-well optical reaction plates (Applied Biosystems) using a platemate 2×2 automated liquid pipettor (Matrix), with six replicates of each sample. Real-time PCR reactions were carried out on an ABI Prism 7900HT Sequence Detector (Applied Biosystems). Real-time PCR primers were designed using Primer Express 2.0 (ABI Software) and are listed in Table S1. *hfq* or *rpsL* transcripts were used as endogenous controls for the reactions, and RNA levels were quantified using absolute quantification (standard curve analysis).

Acknowledgments

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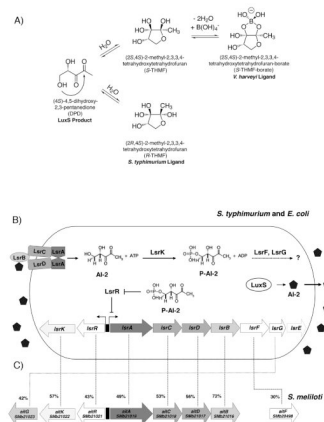
Fig. 1. The interconversion of DPD into the known AI-2 ligands and the AI-2-dependent internalization system

A. Proposed equilibrium between the currently known forms of AI-2 and their common precursor, DPD. The *V. harveyi* and *S. typhimurium* ligands have previously been shown to interconvert in solution.

B. *S. typhimurium* and *E. coli* Lsr-mediated transport and processing of AI-2. In *S. typhimurium* and *E. coli*, AI-2 is produced within the cell by LuxS and is secreted to the medium. As the concentration of extracellular AI-2 increases, AI-2 binds to the periplasmic binding protein LsrB and is internalized by the Lsr system, an ABC-type transport system. Once in the cytoplasm, AI-2 is phosphorylated (P-AI-2) by LsrK. P-AI-2 binds to the repressor of the *lsr* operon, LsrR, inactivating LsrR, relieving repression, and inducing transcription of *lsr*. This causes a rapid increase in the production of the Lsr transporter and, consequently removal of AI-2 from the environment. P-AI-2 is further processed by a mechanism not fully understood involving LsrG and LsrF. The *lsrE* gene is also present in the operon of *S. typhimurium* but not in *E. coli* and its function is not known.

C. The *lsr* orthologues in *S. meliloti* operon. *S. meliloti* has orthologs to all the genes of the *lsr* operon except *lsrE*. We named the *S. meliloti lsr*-like operon *ait* (for autoinducer transporter). The percent identity to the Lsr proteins from *S. typhimurium* is shown.
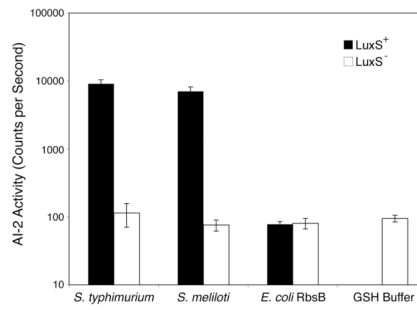
Fig. 2. Binding of AI-2 to potential receptor proteins
Light produced by *V. harveyi* strain MM32 (LuxN⁻, LuxS⁻) was assayed following the
addition of ligand released from purified protein expressed in either LuxS⁺ (black bars) or
LuxS⁻ (white bars) *E. coli* (strains BL21 and FED101, respectively). The *E. coli* ribose
binding protein RbsB and protein-free GSH-buffer were included as negative controls. AI-2
activity is reported as c.p.s. of MM32 bioluminescence. Error bars represent the standard
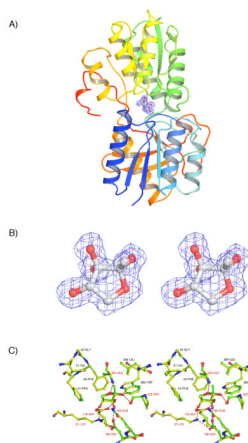deviations for three independent cultures.

Fig. 3. Structure of *S. meliloti* LsrB and its ligand
A. Ribbon diagram of SmLsrB. The ribbon diagram is colored in rainbow order from N- to C- terminus. The bound ligand and the corresponding electron density are shown.
B. Stereoview of 2Fo-Fc ligand electron density. The DPD isomer *R*-THMF is shown modeled into non-protein electron density in the receptor binding site.
C. Comparison of the of the *S. meliloti* and *S. typhimurium* LsrB AI-2 binding sites. Overlay of the SmLsrB (green) and *S. typhimurium* LsrB (yellow) binding sites based on overall alignment of the protein structures as calculated by PyMOL. Residue numbers are from the *S. meliloti* sequence. Dashed red lines indicate potential hydrogen bonds and the interacting residues are labeled in red.
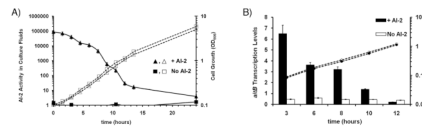
Fig. 4. *S. meliloti* internalization of exogenously supplied AI-2

A. Extracellular AI-2 activity in *S. meliloti* cultures. Wild-type *S. meliloti* Rm1021 was cultured in LBMC in the presence (triangles) and absence (squares) of *in vitro* synthesized AI-2 and aliquots were taken at the specified times. AI-2 activity in cell-free culture fluids is reported as fold induction of light production by *V. harveyi* BB170 (solid lines). Samples with fold inductions above 1000 were diluted in LBMC and values shown were calculated taking account the dilution factor. Cell growth was monitored by optical density (dashed lines).

B. Expression of the *S. meliloti aitB* transcript in the presence and absence of AI-2. RNA levels in cultures of wild-type *S. Meliloti* RM1021 grown in the presence (black bars) and absence (white bars) of *in vitro* synthesized AI-2 were measured using quantitative real-time PCR. *aitB* transcript levels are reported as fold increase of *aitB* transcript in relation to the *rpsL* transcript. Cell growth in the presence (black circles) and in the absence (white circles) of AI-2 was measured by optical density.
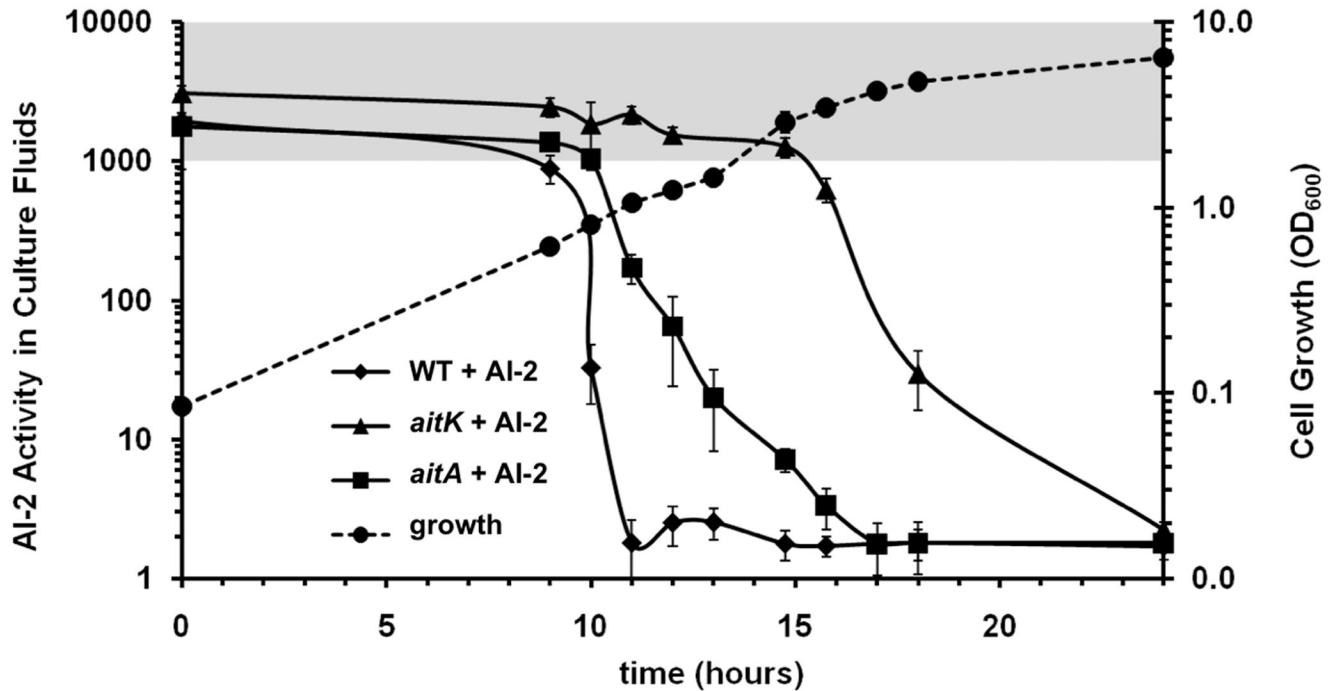
Fig. 5. Extracellular AI-2 activity in *S. meliloti ait* mutants
Cultures of the following *S. meliloti* strains, RM1021 (WT, diamonds), MET2000 (*aitA*, squares) and MET2002 (*aitK*, triangles), were grown in LBMC with *in vitro* synthesized AI-2. AI-2 activity in the cell-free culture fluids is reported as fold induction of light production by *V. harveyi* BB170 (solid lines) and cell growth was monitored by optical density (dashed lines). The average optical density of the three cultures is shown (circles) with the corresponding standard deviation. Fold inductions above 1000 correspond to AI-2 concentrations that saturate the *V. harveyi* bioassay and are highlighted by the grey shadow
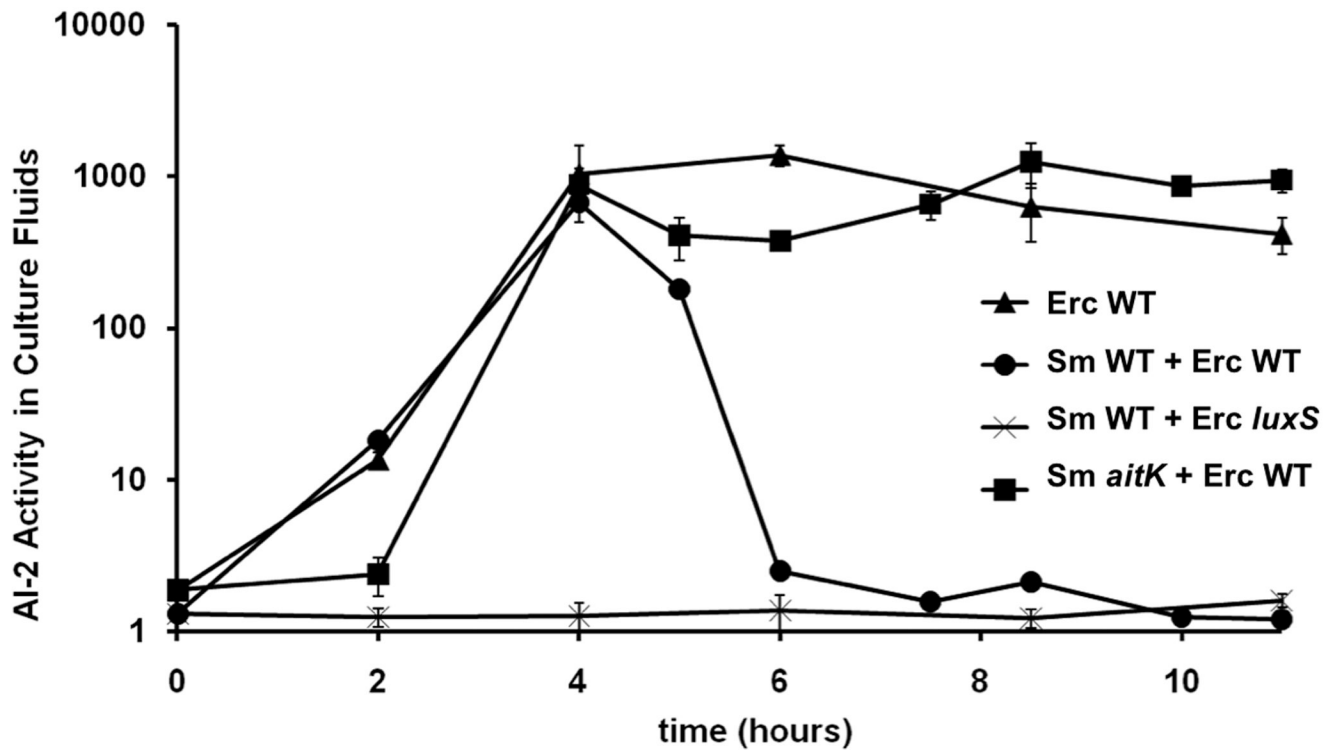
Fig. 6. Extracellular AI-2 activity in co-cultures of *S. meliloti* with *E. carotovora*
Extracellular AI-2 activity was measured in a pure culture of *E. carotovora* WT strain
SCC3193 (triangles) or co-cultures of the following combinations: *S. meliloti* (WT) with *E. carotovora* WT strain SCC3193 (circles),*S. meliloti* (WT) with *E. carotovora luxS* strain
SCC6023 (crosses), and *S. meliloti aitK* strain MET2002 with *E. carotovora* WT strain
SCC3193 (squares). All strains were grown in LBMC medium. AI-2 activity in the cell-free
culture fluids is reported as fold induction of light production by *V. harveyi* BB170.

Table 1

Crystallographic data and refinement statistics.

Data	
Resolution (Å)	1.8 (1.9–1.8)
Unique Reflections	50186 (7394)
R_{merge} (outer shell)	0.059 (0.348)
Mean $I/\sigma I$ (outer shell)	15.3 (2.6)
Completeness (%)	98.8 (100)
Multiplicity	2.6 (2.5)

Refinement	
R_{cryst}/R_{free}	0.168/0.214
RMS deviation	
Bond Length (Å)	0.007
Bond Angle (°)	1.007
Dihedrals (°)	19.074
Average B factor	
Non-solvent	17.20
Waters	28.74
All atoms	19.26