Rhizobial homologs of the fatty acid transporter FadL facilitate perception of long-chain acyl-homoserine lactone signals

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Quorum sensing (QS) using N-acyl homoserine lactones (AHLs) as signal molecules is a common strategy used by diverse Gram-negative bacteria. A widespread mechanism of AHL sensing involves binding of these molecules by cytosolic LuxR-type transcriptional regulators, which requires uptake of external AHLs. The outer membrane is supposed to be an efficient barrier for diffusion of long-chain AHLs. Here we report evidence that in *Sinorhizobium meliloti*, sensing of AHLs with acyl chains composed of 14 or more carbons is facilitated by the outer membrane protein $FadL_{Sm}$, a homolog of the *Escherichia coli* $FadL_{Ec}$ long-chain fatty acid transporter. The effect of $fadL_{Sm}$ on AHL sensing was more prominent for longer and more hydrophobic signal molecules. Using reporter gene fusions to QS target genes, we found that $fadL_{Sm}$ increased AHL sensitivity and accelerated the course of QS. In contrast to $FadL_{Ec}$, $FadL_{Sm}$ did not support uptake of oleic acid, but did contribute to growth on palmitoleic acid. $FadL_{Sm}$ homologs from related symbiotic α-rhizobia and the plant pathogen *Agrobacterium tumefaciens* differed in their ability to facilitate long-chain AHL sensing or to support growth on oleic acid. $FadL_{At}$ was found to be ineffective toward long-chain AHLs. We obtained evidence that the predicted extracellular loop 5 of $FadL_{Sm}$ and further α-rhizobial FadL proteins contains determinants of specificity to long-chain AHLs. Replacement of a part of loop 5 by the corresponding region from α-rhizobial FadL proteins transferred sensitivity for long-chain AHLs to $FadL_{At}$.

autosignaling | facilitated diffusion | Rhizobiaceae

Quorum sensing (QS) is a widespread mechanism of cell–cell signaling that allows bacteria to coordinate their gene expression in response to local population density. Communication occurs via small hormone-like molecules called QS signals. As an integrative part of the global regulatory network, QS molecules have a crucial role in controlling many bacterial traits, including virulence, symbiosis, and development and maintenance of population and community structures (reviewed in ref. 1).

QS molecules must cross the bacterial cell envelope to act as diffusible signals in cell–cell communication. Bacteria use a wide variety of signaling molecules and various signal detection and transduction mechanisms in QS. The most common QS signals in Gram-negative bacteria are *N*-acyl-homoserine lactones (AHLs). AHLs are composed of a fatty acid chain of 4–18 carbons attached to an invariant homoserine lactone ring. Following the fatty acid nomenclature, AHL molecules with acyl chains composed of 12 or more carbons are defined as long-chain AHLs. *Vibrio harveyi* and *Vibrio cholerae* use membrane sensor kinase phosphorelay mechanisms to transmit the extracellular signal to the intracellular regulatory protein (2). In many other systems, AHLs are supposed to enter the cell and directly bind to their cognate LuxR-type transcription regulators in the cytoplasm.

It is assumed that short-chain AHLs can diffuse freely across the bacterial cell envelope, whereas long-chain AHLs likely require a transport mechanism. In *Pseudomonas aeruginosa*, the multidrug efflux pump MexAB has been shown to actively export 3-oxo-C12-HSL (3). The uptake mechanism of long-chain AHLs remains enigmatic, however. The amphipathic properties of these molecules resemble those of fatty acids. It has been shown that the lipopolysaccharide layer of the outer membrane is an efficient barrier for long-chain fatty acids (LCFAs) (4). In *Escherichia coli*, their import across the outer membrane requires the β-barrel FadL protein, which facilitates diffusion of LCFAs C12–C18 (5, 6).

Considering the similar nature of LCFAs and long-chain AHLs, we asked whether FadL-like proteins are the missing players promoting uptake of long-chain AHLs by Gram-negative bacteria. To address this question, we investigated the role of FadL in QS of *Sinorhizobium meliloti*, an α-proteobacterium that has been well studied for its nitrogen-fixing symbiotic interaction with legume plants (7). *S. meliloti* produces a variety of long-chain AHLs, including C18-, C16:1-, 3-oxo-C16:1-, 3-oxo-C14-, and C12-HSLs (8, 9). AHLs with an acyl chain containing 14 or more carbons may be characteristic of α-proteobacteria, being identified in *Rhodobacter capsulatus*, *Agrobacterium vitis*, and several distant marine species as well (10–12). *S. meliloti* strains 1021 and 2011 have a single AHL-based QS system (Fig. 1).

The long-chain AHLs are produced by the synthase SinI (13). *sinI* expression requires the transcription activator SinR and is strongly enhanced by the major LuxR-type regulator ExpR in the presence of AHLs, resulting in positive feedback (14). ExpR also represses transcription of *sinR* at high AHL concentrations, which provokes negative feedback regulation of *sinI* (15). Furthermore, ExpR has a broad regulatory influence on the transcriptome, including activation of exopolysaccharide (EPS) biosynthesis and repression of motility genes (16–18). Long-chain AHL-dependent

Significance

Bacterial intercellular communication is crucial for developing population and community structures and for pathogenic and symbiotic interactions with eukaryotic hosts. Many Gram-negative bacteria use N-acyl-homoserine lactones (AHLs) in quorum sensing-related signaling. Although it is likely that specific transport mechanisms are required for long-chain AHLs to overcome the outer membrane, mechanisms promoting uptake have not been reported so far. Here we present evidence that homologs of the outer membrane long-chain fatty acid transporter FadL facilitate uptake of long-chain AHLs, which closes an important gap in our understanding of quorum sensing signaling. Our findings suggest that bacteria responding to long-chain AHLs have evolved specificity of FadL toward these signal molecules and have partly lost the ability to transport long-chain fatty acid by this protein.

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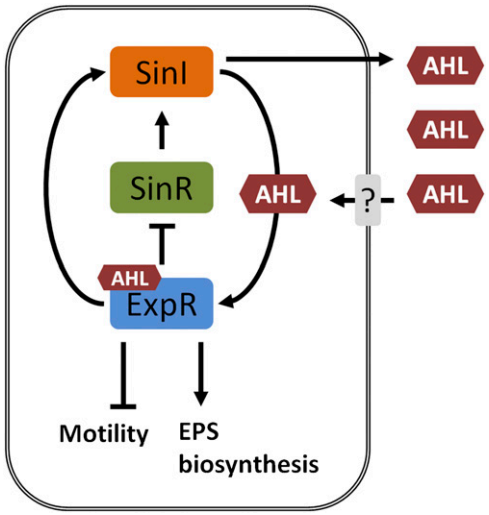

Fig. 1. Simplified scheme of the *S. meliloti* QS regulatory system (13). Expression of *sinI* encoding a long chain-AHL synthase is dependent on SinR. Transcription of *sinR* is repressed by ExpR at high AHL levels. ExpR binds AHLs and activates expression of *sinI* and EPS biosynthesis genes, and represses expression of motility genes. The long-chain AHL uptake mechanism is the subject of this study.

gene regulation by ExpR has been explored in great detail (19, 20), and in a genomic search we identified a single *fadL* homolog in *S. meliloti*. Thus, we chose this rhizobial species to test our hypothesis that FadL is able to facilitate long-chain AHL uptake.

Here we present evidence for the *S. meliloti* FadL homolog promoting uptake of long-chain AHLs and describe its impact on the AHL concentration-dependent course of QS. We report data strongly suggesting that specificity of FadL proteins for long-chain AHLs resides in the extracellular loop 5 (L5) of this outer membrane protein.

Results

***S. meliloti* FadL Facilitates Sensing of C16:1-HSL and Supports Growth on Palmitoleic Acid.** To test whether FadL has a role in the uptake of long-chain AHLs in *S. meliloti*, we analyzed the response of *sinI* mutant Sm2B4001 (15) (referred to as *sinI* strain hereinafter) to externally supplied C16:1-HSL in the presence and absence of the $fadL_{Sm}$ gene. Because the *sinI* strain is unable to synthesize AHLs, and these signal molecules are sensed by the cytosolic LuxR-type regulator ExpR (16, 17), transcriptional activation of its target genes requires AHL uptake. In this setup, we determined activation of galactoglucan biosynthesis gene expression by judging culture morphology on tryptone-yeast (TY) agar and monitoring activity of the *wgeA* promoter. *wgeA* is the first gene of the *wge* operon of the galactoglucan biosynthesis gene cluster (21).

Activation of the *wgeA* promoter by externally added C16:1-HSL was observed in the *sinI* strain, but not in the *sinI fadL* mutant. Ectopic complementation by P_{lac}-driven $fadL_{Sm}$ restored the response of this mutant to C16:1-HSL in the presence of 100 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Fig. 2*A*). Consistent with this finding, addition of 200 nM or more C16:1-HSL to TY agar resulted in a mucoid phenotype of the *sinI* strain, whereas the *sinI fadL* mutant stayed dry with up to 5,000 nM C16:1-HSL (*SI Appendix*, Fig. S1). Thus, the *sinI fadL* strain lost its ability to respond to C16:1-HSL with activation of galactoglucan biosynthesis gene expression, strongly suggesting a role for $FadL_{Sm}$ in C16:1-HSL sensing.

In the *sinI* strain, the promoter activity of *sinI* was found to be highly sensitive to externally supplied C16:1-HSL (15). Rising AHL concentrations in the growth medium first increased (positive feedback state) and then decreased (negative feedback state) *sinI* promoter activity (15). Whereas this characteristic bell-shaped response curve was obtained for the *fadL*-sufficient *sinI* strain, activation of the *sinI* promoter in the *sinI fadL* mutant was observed at an ~100-fold greater external C16:1-HSL concentration (Fig. 2*B*). This finding suggests that also in the absence of *fadL*, C16:1-HSL is perceived by *S. meliloti*, but with much lower sensitivity. Complementation of the *sinI fadL* mutant with P_{lac}-driven $fadL_{Sm}$ fully restored the WT response in presence of 100 μM IPTG. Lowering the level of ectopic $fadL_{Sm}$ expression by omitting IPTG shifted the *sinI* promoter activation curve toward higher AHL concentrations (Fig. 2*B*). This suggests a positive correlation between $fadL_{Sm}$ expression level and the magnitude of response to added C16:1-HSL.

Based on the foregoing observations, we asked whether the native expression of $fadL_{Sm}$ is regulated by QS. Previous transcriptome studies detected negative regulation of $fadL_{Sm}$ by *expR* independent of *sinI* (17, 22). We applied a *fadL* promoter-EGFP fusion to monitor *fadL* expression levels. Neither knockout of *expR* in the WT background (*SI Appendix*, Fig. S2*A*) nor addition of C16:1-, 3-oxo-C16:1-, or 3-oxo-C14-HSL to the cultures of the *sinI* strain strongly influenced *fadL* promoter activity (*SI Appendix*, Fig. S2*B*). Moreover, purified His_6-tagged ExpR did not bind to the promoter region of *fadL* (*SI Appendix*, Fig. S2*C*). Our data imply that *fadL* is expressed under standard growth conditions, and that its expression level is not influenced by QS.

Further evidence that $FadL_{Sm}$ contributes to sensing of AHLs was obtained using *Agrobacterium tumefaciens* AHL indicator strain NTL4 (pZLR4) (23). This strain responds to AHLs by β-galactosidase production and thus can be used to detect AHLs in a semiquantitative manner. When the complete $fadL_{Sm}$ gene including its native promoter was introduced into this indicator strain on the low-copy vector pPHU231, its sensitivity to C16:1-HSL increased (Fig. 2*C*), indicating that $fadL_{Sm}$ also promotes sensing of long-chain AHLs in *A. tumefaciens*.

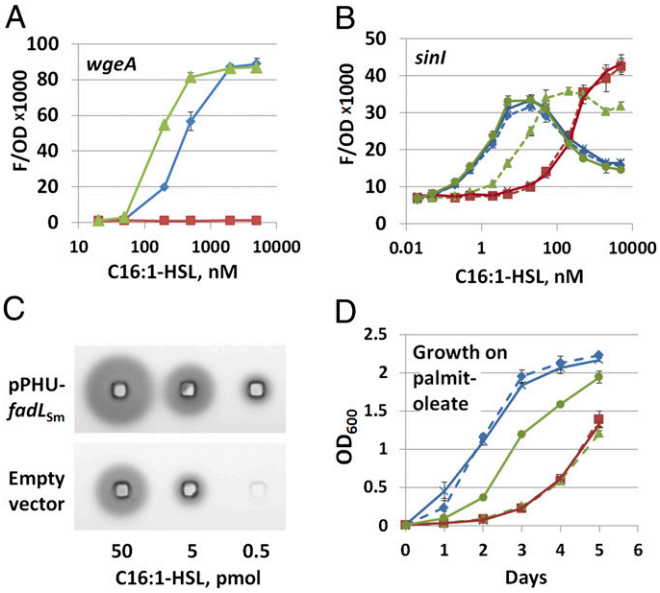

Fig. 2. *S. meliloti* FadL facilitates the response to externally added C16:1-HSL. (*A* and *B*) *wgeA* (*A*) and *sinI* (*B*) promoter activities estimated as fluorescence mediated by promoter-EGFP fusions on the addition of C16:1-HSL at various concentrations. Fluorescence measurements were performed at 24 h after inoculation. (*C*) C16:1-HSL detection by *A. tumefaciens* indicator strain NTL4 (pZLR4) expressing or not expressing $fadL_{Sm}$. Images were taken at 24 h after the addition of AHLs. (*D*) Growth on minimal medium with palmitoleic acid as the sole carbon source. Blue, *sinI* pSRKKm; red, *sinI fadL* pSRKKm; green; *sinI fadL* pFadLSm. Solid lines indicate cultures grown with IPTG, broken lines indicate cultures grown without IPTG. Error bars show SDs of three biological replicates.

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Given that the *E. coli fadL* homolog is essential for growth on oleic (C18:1) and palmitic (C16:0) acids (5), we tested the growth of *S. meliloti sinI* and *sinI fadL* strains on minimal medium with these fatty acids as the sole carbon source. Because *S. meliloti* did not grow irrespective of *fadL* status (*SI Appendix*, Fig. S1), $FadL_{Sm}$ is unlikely to facilitate uptake of these fatty acids. Similar assays with *sinI*-sufficient strains produced the same result. The possibility that the nongrowth phenotype resulted from metabolic incompetence was ruled out by the experiments described below, which strongly suggest that oleic and palmitic acid support growth of *S. meliloti* if a heterologous *fadL* facilitating uptake of these fatty acids is expressed. The *sinI* strain was able to grow with palmitoleic acid (C16:1) as the sole carbon source, whereas the *sinI fadL* strain showed poor growth (Fig. 2*D*). The WT-like growth phenotype was essentially restored by ectopic expression of P_{lac}-driven $fadL_{Sm}$ in the presence of 250 μM IPTG, implying that $FadL_{Sm}$ contributes to uptake of palmitoleic acid.

***S. meliloti* FadL Facilitates Sensing of 3-Oxo-C16:1- and 3-Oxo-C14-HSLs, but Not of AHLs with Shorter Acyl Chains.** Because *S. meliloti* is known to produce and detect a range of long-chain AHLs, we asked whether sensing of AHLs other than C16:1-HSL is also facilitated by *fadL*. Similar to the promoting effect of *fadL* on sensing of externally added C16:1-HSL, this gene also had a positive effect on the response to 3-oxo-C16:1-HSL, in terms of stimulating *wgeA* and *sinI* promoter activities and mucoidity of agar culture. However, compared with the *sinI fadL* mutant, the presence of *fadL* lowered the activating concentration of 3-oxo-C16:1-HSL by only ∼40-fold (*SI Appendix*, Fig. S3 *A* and *B*) whereas an ∼100-fold lower threshold was determined for C16:1-HSL (Fig. 2*B*).

Analysis of *sinI* promoter activation dynamics in response to externally supplied 3-oxo-C14-, 3-oxo-C12-, and 3-oxo-C10-HSLs revealed a moderate effect of *fadL* on 3-oxo-C14-HSL sensing (∼10-fold difference compared with a *fadL*-sufficient strain) and no influence on the response to AHLs with shorter acyl chains (*SI Appendix*, Fig. S3*C*). Moreover, the *A. tumefaciens* NTL4 (pZLR4) indicator strain also showed increased sensitivity to 3-oxo-C16:1-HSL in the presence of $fadL_{Sm}$ (*SI Appendix*, Fig. S3*D*). Thus, $fadL_{Sm}$ contributes to sensing of C16:1-, 3-oxo-C16:1-, and 3-oxo-C14-HSLs, but not to perception of molecules with shorter acyl chains.

***fadL* QS Phenotype.** We have presented genetic evidence indicating that FadL promotes sensing of extracellularly supplied AHLs. In a *sinI* mutant, *fadL* had a significant impact on AHL-mediated gene regulation. We asked to what extent *fadL*-facilitated AHL sensing contributes to QS-mediated gene regulation in a *sinI*-positive strain that is able to produce AHLs. For this purpose, we determined promoter activities of several ExpR-regulated genes in the WT and *sinI* strains and their *fadL* mutant derivatives grown without addition of AHLs to the cultures. Promoter activities of *wgeA, wggR,* SMc04171, and SMa2111, known to be activated by ExpR and AHLs (17, 19), were decreased in the *fadL* strain (Fig. 3*A* and *SI Appendix*, Fig. S4). Because QS negatively regulates motility gene expression in an AHL-dependent manner (19, 24), we also analyzed the expression of *flaA* encoding a flagellin, and found that *flaA* promoter activity was strongly increased in the *fadL* strain (Fig. 3*B*). In contrast, the *fadL* mutation did not cause any change in the activities of the tested promoters in the *sinI* mutant that is unable to synthesize AHLs. The differences in activities of *expR*-regulated promoters between *fadL*-positive and *fadL*-negative strains were strongest at the beginning of batch culture growth and declined toward the stationary growth phase; thus, the $fadL_{Sm}$-dependent response to long-chain AHLs likely is most important in the initial phase of AHL-dependent regulation, when external levels of AHLs are supposed to be low.

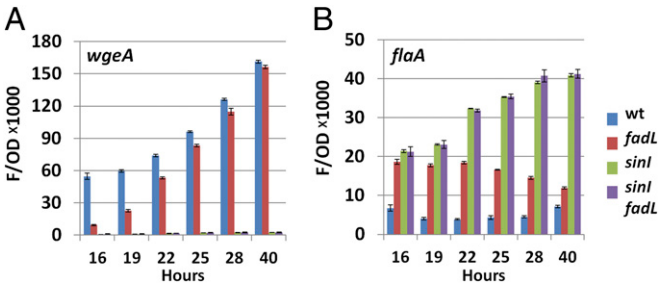

Fig. 3. In WT background, $fadL_{Sm}$ contributes to ExpR-dependent QS gene regulation. *wgeA* (*A*) and *flaA* (*B*) promoter activities estimated as fluorescence mediated by promoter–EGFP fusions. Time is denoted in hours after inoculation. Error bars show SDs of three biological replicates.

We also investigated the impact of $fadL_{Sm}$ on accumulation of QS signals in the culture medium. Samples of WT, *fadL*, *sinI*, and *sinI fadL* TY culture supernatants were obtained in the late exponential growth phase and applied to the *A. tumefaciens* NTL4 (pZLR4) indicator strain. The supernatant of the *fadL* strain induced a stronger response than that of the WT, whereas the *sinI* and *sinI fadL* supernatants induced no response (*SI Appendix*, Fig. S5). Thus, the *fadL* mutation resulted in increased accumulation of AHLs in the medium.

***fadL* Homologs from Related Bacteria Differ in Their Ability to Promote Growth on Fatty Acids and to Facilitate a Response to Long-Chain AHLs.** FadL homologs are present in other symbiotic rhizobia and in the plant pathogen *A. tumefaciens*, but are not found in Bradyrhizobiaceae. Phylogenetic analysis revealed strong similarities in FadL amino acid sequences among *Sinorhizobium* species and weaker similarities with representative FadL homologs from *Rhizobium*, *Mesorhizobium,* and *Agrobacterium* (Fig. 4*A* and *SI Appendix*, Fig. S6). Because these bacteria use AHL-based QS (8, 25–27), we asked whether promotion of long-chain AHL sensing is a common trait of their FadL proteins. For this, *fadL* homologs from these species were ectopically expressed in the *S. meliloti sinI fadL* mutant by introduction of plasmid-localized heterologous *fadL* genes driven by an IPTG-inducible *lac* promoter. Resulting strains were assayed for EPS production on TY agar supplemented with C16:1-HSL (*SI Appendix*, Table S1 and Fig. S1) and for *sinI* promoter activity at increasing concentrations of C16:1-HSL or 3-oxo-C16:1-HSL (Fig. 4*B* and *SI Appendix*, Fig. S7). Whereas $fadL_{Smed}$, $fadL_{Sf}$, and $fadL_{Ml}$ performed similarly to $fadL_{Sm}$, the $fadL_{At}$-complemented strain differed only slightly from the control strain carrying the empty vector. $FadL_{Re}$ was less efficient than $FadL_{Sm}$ in mediating *sinI* promoter activation in response to C16:1-HSL (Fig. 4*B*) and hardly contributed to 3-oxo-C16:1-HSL sensing (*SI Appendix*, Fig. S7).

Involvement of FadL homologs in C16:1-HSL sensing was further confirmed in a motility assay. C16:1-HSL (2,000 nM) inhibited swimming motility of the *sinI fadL* strain ectopically expressing $fadL_{Sm}$, but not of the control strain carrying the empty vector. All homologs but $fadL_{At}$ enabled inhibition of the *sinI fadL* swimming phenotype, with $fadL_{Re}$ conferring only partial inhibition (Fig. 4*C*).

Expression of $fadL_{Re}$, $fadL_{Ml}$, and $fadL_{At}$ in the *sinI fadL* strain allowed for significant growth on oleic acid (Fig. 4*D* and *SI Appendix*, Table S1 and Fig. S1), whereas $fadL_{Ml}$ and $fadL_{At}$ also supported poor growth on palmitic acid (*SI Appendix*, Fig. S1). In contrast, complementation with $fadL_{Smed}$ and $fadL_{Sf}$ did not change the nongrowth phenotype of the *sinI fadL* strain on these fatty acids. Thus, the analyzed FadL homologs likely differ in their ability to promote LCFA uptake.

Extracellular L5 Contains Determinants of Specificity to C16:1-HSL. To identify the regions of $FadL_{Sm}$ that determine its ability to promote

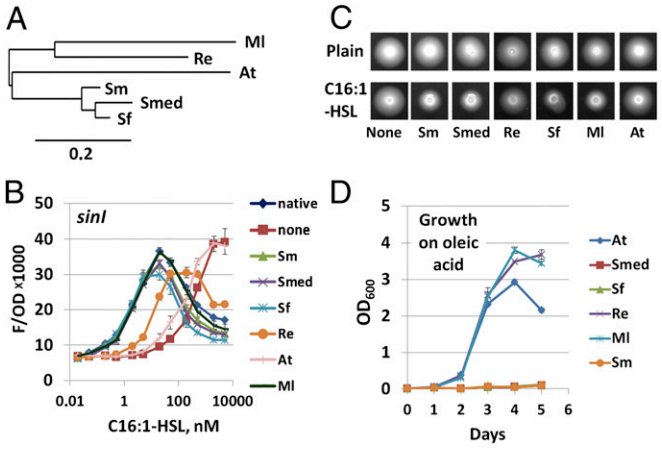

Fig. 4. FadL from related species differs in the ability to facilitate C16:1-HSL sensing and to support growth on oleic acid. (*A*) Phylogenetic tree of FadL protein sequences. (*B*) *sinI* promoter–EGFP fusion activity on addition of different concentrations of C16:1-HSL to the *sinI fadL* strain complemented with *fadL* from different species. Fluorescence measurements were performed at ca. 24 h after inoculation. (*C*) Inhibition of swimming motility on 0.3% agar by 2,000 nM C16:1-HSL promoted by expression of *fadL* from different species in the *S. meliloti sinI fadL* mutant. (*D*) Growth of the *sinI fadL* strain complemented with *fadL* of different origins in minimal medium with oleic acid as sole carbon source. Heterologous *fadL* genes were ectopically expressed from the P_{lac} promoter in the presence of 100 μM (*B* and *C*) or 250 μM (*D*) IPTG. Sm, *S. meliloti* 1021; Smed, *S. medicae* WSM419; Sf, *S. fredii* HH103; Re, *R. etli* CFN42; Ml, *M. loti* MAFF 303099; At, *A. tumefaciens* C58. Error bars show SDs of three biological replicates.

sensing of long-chain AHLs, we first applied a loss-of-function approach. Several $FadL_{Sm}$ regions were replaced with the corresponding $FadL_{At}$ sequences at positions that were conserved in most of the FadL proteins facilitating long-chain AHL sensing but not in $FadL_{At}$ (*SI Appendix*, Fig. S5). The $FadL_{Sm}$ protein tertiary structure, predicted based on its homology (21% identical amino acids) to $FadL_{Ec}$ (6), constitutes a putative β-barrel with 14 transmembrane β-strands and seven partially disordered extracellular loops. The extracellular loops L3 and L5 of $FadL_{Ec}$ contain the amino acids R157, K317, and E319, key residues of the high-affinity substrate binding site (6).

The nonconserved regions of $FadL_{Sm}$ L3 and L5 were replaced by the corresponding $FadL_{At}$ sequences (*SI Appendix*, Table S1 and Fig. S6). In the *sinI fadL* mutant, the resulting $fadL_{Sm}$ variants were assayed for their ability to support growth on oleic acid as the sole carbon source and their response to C16:1-HSL in terms of EPS production (*SI Appendix*, Table S1 and Fig. S1). Loss of the ability to promote C16:1-HSL sensing was observed on replacement SmAt308-318 in L5 (*SI Appendix*, Table S1 and Figs. S1 and S6). Further amino acid exchanges narrowed the region essential for induction of EPS production by C16:1-HSL to residues 315–318 (Fig. 5*A* and *SI Appendix*, Table S1 and Fig. S1). Moreover, these replacements conferred growth on oleic acid, a property of *A. tumefaciens*, but not of the *S. meliloti* WT. Mutation T318IN was sufficient to abolish the response to C16:1-HSL and replacement T318N was sufficient to decrease this response, whereas growth on oleic acid was enabled. Amino acid exchanges in the majority of the other selected regions had no effect; however, replacements in L2 and L4 permitted growth on oleic acid without affecting the C16:1-HSL–mediated response (*SI Appendix*, Table S1).

Replacements of $FadL_{Sm}$ L5 segments with the corresponding $FadL_{Re}$ or $FadL_{Ml}$ sequences resulted in constructs that enabled growth on oleic acid, but did not abolish the ability to enhance C16:1-HSL sensing (Fig. 5*A* and *SI Appendix*, Table S1 and Fig. S1). Again, the C-terminal portion of the nonconserved region of L5 was sufficient to confer the substrate specificity properties of the source protein to the recipient. Whereas replacement SmRe316-317 enabled growth on oleic acid, exchange SmRe309-312 in the N-terminal part of this region had no effect (*SI Appendix*, Table S1). These data suggest that in α-rhizobial FadL proteins, L5, especially region 315–318, carries specificity determinants for LCFA and/or AHL uptake.

We further tested this hypothesis in a gain-of-function approach designed to confer specificity for C16:1-HSL to $FadL_{At}$ via changes in the nonconserved part of L5. We found that indeed the *sinI fadL* strain, expressing a $fadL_{At}$ variant carrying the L5 encoding sequence derived from $fadL_{Sm}$, responded to C16:1-HSL with EPS production, but observed no growth on oleic acid (Fig. 5 and *SI Appendix*, Fig. S1). Analysis of further replacements in this region confirmed the importance of the C-terminal portion of L5 for substrate specificity. Moreover, loop length also affected the response to C16:1-HSL. Replacement AtSm300-310, which shortened the loop by six amino acids, had the same effect as the exchange of the whole region; in contrast, replacement AtSm305-310, which reduced the loop by only one amino acid, had little effect (Fig. 5*A* and *SI Appendix*, Fig. S1). Furthermore, $FadL_{At}$ also promoted the ability to sense C16:1-HSL when the putative L5 specificity region was replaced with the $FadL_{Re}$ sequence. In that case, growth on oleic acid was not abolished, corresponding to the properties of the L5 source protein (Fig. 5).

For a comparison of expression levels and stability of the recombinant proteins, selected $FadL_{Sm}$ and $FadL_{At}$ variants were C-terminally extended with 3× FLAG tag, and Western blot analysis was performed. Although FLAG-tagged $FadL_{At}$ appeared slightly more abundant than FLAG-tagged $FadL_{Sm}$, the amino acid replacements in L5 did not significantly affect the concentrations of the variants of both proteins detected in the *sinI fadL* strain (*SI Appendix*, Fig. S8). The minor differences in the abundance of FLAG-tagged FadL proteins are not likely responsible for the different oleic acid utilization and C16:1-HSL sensing phenotypes conferred by the limited exchanges of amino acids in L5.

Discussion

AHLs are generally assumed to enter bacterial cells by diffusion. This concept probably is satisfactory for the majority of bacterial species studied so far that produce and perceive AHLs with acyl chains of up to 12 carbons; however, longer acyl chains are presumed to impede diffusion of AHLs (reviewed in ref. 28), suggesting an additional route of uptake. This is relevant to several α-proteobacteria, including *S. meliloti*, which communicate via long-chain AHLs with acyl chains of 14 or more carbons (8, 10–12).

In *S. meliloti*, AHLs exert their action by direct binding to the cytosolic transcriptional regulator ExpR (14, 19); thus, external AHLs must cross the cell envelope to be sensed by the cells. We found that long-chain AHL sensing by *S. meliloti* was greatly facilitated by the FadL protein, as well as by its homologs from four other symbiotic α-rhizobia. These homologs of the *E. coli* LCFA transporter $FadL_{Ec}$ were predicted to adopt the β-barrel structure characteristic of FadL channels, suggesting that they act as long-chain AHL transporters.

In *S. meliloti*, the *sinI* promoter is the most sensitive promoter known to be induced by AHLs, with a minimal activating concentration of 5 nM (13, 18). The observed residual activation of the *sinI* promoter in the *fadL* mutant implies that AHLs can enter the cell via *fadL*-dependent and *fadL*-independent mechanisms. The contribution of $fadL_{Sm}$ to the sensing of extracellularly supplied AHLs was found to be inversely correlated with the length and hydrophobicity of the AHL molecules. The most prominent effect of $fadL_{Sm}$ was observed for C16:1-HSL, the longest and most hydrophobic AHL tested in this study. Shorter acyl chains of the AHL reduced *fadL* dependency. Thus, we assume that shorter and less hydrophobic AHL molecules are more efficient in diffusion through the outer membrane compared

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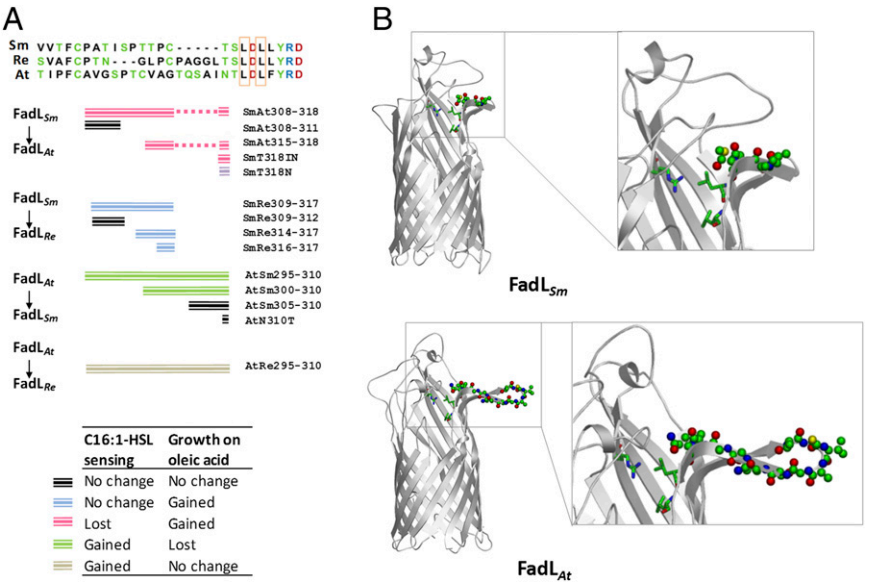

Fig. 5. Extracellular L5 contains determinants of specificity to C16:1-HSL. (*A*) Protein sequence alignment of the part of L5 from *S. meliloti*, *R. etli*, and *A. tumefaciens* containing the specificity region, representation of regions that were replaced in either FadL$_{Sm}$ or FadL$_{At}$, and phenotypes of the *fadL sinI* strain complemented with the different constructs. Residues L320 and L322 corresponding to HAS amino acids K317 and E319 in FadL$_{Ec}$ are boxed. (*B*) Three-dimensional structure model of FadL$_{Sm}$ and FadL$_{At}$ predicted by SWISS MODEL in the automated mode. Amino acids R178, L320, and L322 of FadL$_{Sm}$ (R169, L312, and L314 of FadL$_{At}$) corresponding to the key residues R157, K317, and E319 of the FadL$_{Ec}$ HAS are represented by rods; amino acids 315–318 of FadL$_{Sm}$ and 300–310 of FadL$_{At}$ of the AHL/fatty acid specificity region, by spheres. Color code: green, carbon; blue, nitrogen; red, oxygen; yellow, sulfur.

with the longer molecules, the uptake of which is likely facilitated by FadL.

In the *S. meliloti* WT, FadL$_{Sm}$ had a significant impact on the sensing of long-chain AHLs at early growth stages when external AHL concentrations are supposed to be low. At culture saturation, when the signal molecules are assumed to accumulate, its effect was diminished. This finding is consistent with the *fadL*-independent AHL sensing observed in the *sinI* mutant, which became significant at higher external AHL concentrations. Recently, Charoenpanich et al. (19) described the temporal expression program of ExpR-mediated regulation, in which different target promoters have different external C16:1-HSL concentration thresholds for their activation or repression. The impact of *fadL* on QS-regulated promoters determined in the present study is in good agreement with these different thresholds. ExpR-mediated repression of motility, requiring high external AHL concentrations, was found to be more dependent on FadL-facilitated sensing than activation of EPS biosynthesis genes and two further targets that required intermediate AHL levels. Thus, FadL-mediated long-chain AHL uptake increases sensitivity and accelerates progression of the QS response.

The *E. coli* homolog FadL$_{Ec}$ is required for uptake of exogenous oleic and palmitic acids, but not for reutilization of membrane lipids (5, 29). Thus, LCFA uptake through FadL likely is crucial only for utilization of exogenous fatty acids. We found that FadL$_{Sm}$ and its close homologs FadL$_{Smed}$ and FadL$_{Sf}$ did not facilitate oleic acid uptake, whereas the more distantly related FadL$_{Re}$, FadL$_{Ml}$, and FadL$_{At}$ did. For soil-dwelling symbiotic rhizobia, free fatty acids are not likely important growth factors, whereas enhanced sensing of long-chain AHLs may be advantageous; thus, it is tempting to speculate that the capacity for uptake of oleic acid was lost from sinorhizobial FadLs during evolution.

The ability of FadL to promote long-chain AHL sensing was found to be conserved among three main genera of nitrogen-fixing rhizobia, *Sinorhizobium*, *Mesorhizobium* and *Rhizobium*. In contrast, the homolog from the plant pathogen *A. tumefaciens* was not able to facilitate long-chain AHL sensing. To date, the AHL pattern of *S. meliloti* is the sole symbiotic rhizobia that has been studied in detail; however, it is conceivable that closely related species produce long-chain AHLs as well. Interestingly, a *fadL* homolog was not found in Bradyrhizobiaceae. *Bradyrhizobium japonicum* was reported to produce unusual isovaleryl-HSL QS signals (30) whereas *A. tumefaciens* QS involves AHLs with C_6 or C_8 acyl chains (27). Thus, we speculate that species synthesizing AHLs with acyl chains of 14 or more carbons, or coming into contact with species producing them, might have evolved FadL to facilitate their sensing.

Facilitated diffusion, the mechanism of LCFA uptake by the *E. coli* FadL protein, involves accommodation of the fatty acid in an extracellular high-affinity binding pocket, which triggers a conformational change (6, 31). We found that the nonconserved region of the extracellular L5 determined specificity to C16:1-HSL and/or oleic acid. Replacement of this region in FadL$_{Sm}$ or FadL$_{At}$ with the region from FadL$_{Sm}$, FadL$_{Re}$, FadL$_{Ml}$, or FadL$_{At}$ conferred functionality of the source FadL. Nonetheless, no particular signature of C16:1-HSL or oleic acid specificity could be identified because of the high heterogeneity of the amino acid sequences. In FadL$_{Sm}$, the exchange of residues 315–318 comprising the C-terminal portion of L5 with the corresponding FadL$_{At}$ or FadL$_{Re}$ sequence was sufficient for conversion to the source protein functionality. The single T318N exchange significantly decreased the response to C16:1-HSL.

FadL$_{Sm}$ T318 is situated in close proximity to L320, corresponding to FadL$_{Ec}$ K317, an essential component of the high-affinity substrate binding pocket (6). The exchange T318N introduced an additional amino group, which could increase local polarity, and thus favors fatty acid versus AHL accommodation. Thus, the sequence of the specificity region might affect the properties of the binding pocket, resulting in different affinities to free fatty acid or AHL. A leucine residue at the position corresponding to K317 has been identified as characteristic of FadL homologs transporting aromatic compounds, but not fatty acids (32). Each FadL homolog analyzed in the present study had a leucine residue at this position irrespective of its ability to support growth on oleic acid, suggesting that the architecture of FadL-binding pockets likely is more heterogeneous than previously assumed.

Materials and Methods

Strains, Plasmids, and Growth Conditions. The strains and plasmids used in this study are listed in *SI Appendix*, Table S2. *S. meliloti* was cultured at 30 °C on TY or MOPS-buffered minimal medium. *A. tumefaciens* was grown at 30 °C on LB or mannitol-glutamate minimal (MGM) medium. *E. coli* was grown at 37 °C on LB. Media compositions and antibiotic concentrations are provided in *SI Appendix*.

For growth assays on fatty acids as the sole carbon source, MOPS-buffered minimal medium was supplemented with 250 μM IPTG and 5 mM palmitoleic acid, oleic acid, or palmitic acid as the sole carbon source. To improve the solubility of the free fatty acids, 0.5% Brij58 was added to the medium. Liquid cultures were inoculated to set a starting OD_{600} of 0.01, and OD_{600} was determined at 24-h intervals. For assays of growth on solid medium, 2 μL of cell suspension in sterile 0.9% NaCl at $OD_{600} = 0.01$ prepared from fresh TY agar cultures was spotted onto the agar medium surface and photographed after 5 d (oleic acid) or 10 d (palmitic acid) of growth. Negative control plates without carbon source and positive controls with mannitol instead of the fatty acids were analyzed in parallel.

For EPS production assays, 3 μL of cell suspensions in sterile 0.9% NaCl at $OD_{600} = 1$ were prepared from fresh TY agar cultures and spotted onto TY agar plates with 100 μM IPTG and 200 mg/L kanamycin. C16:1-HSL or oxoC16:1-HSL was added to the agar as 1,000× DMSO stocks at the indicated concentrations. The phenotype was documented after ∼24 h.

Motility assays were performed on soft TY agar (standard TY agar diluted with water 1:5, final agar concentration 0.3%) containing 100 mg/L kanamycin, 100 μM IPTG, and 2000 μM C16:1-HSL. Then 2 μL of stationary TY culture was spotted onto the soft agar plate, and the phenotype was documented after ∼48 h of growth.

Genetic Manipulations. Constructs used in this work were generated using standard techniques. The primers used are listed in *SI Appendix*, Table S3. The *fadL* KO mutant was generated by integration of plasmid pG19mob2ΩHMB-fadL into the *S. meliloti* genome. Promoter-EGFP fusions were generated by fusing the promoter region including up to 30 bp downstream of the native start codon to the EGFP coding sequence in plasmid pPHU231-EGFP. Plasmid pPHU-fadLSm was generated by insertion of the *fadL* promoter region and coding sequence into plasmid pPHU231. *fadL* complementation constructs were obtained by insertion of the *fadL* coding sequence downstream of an IPTG-inducible *lac* promoter in the broad host range vector pSRKKm. Amino acid exchanges in FadL were generated by site-directed mutagenesis applying splicing by overlap extension PCR.

Detection of AHLs by the *A. tumefaciens* Indicator Strain. A 5-μL AHL sample containing DMSO solution or 20 μL of culture supernatant was added to wells in MGM agar containing 80 mg/L X-Gal mixed with *A. tumefaciens* NTL4 (pZLR4) indicator strain cultures at a 1:1 ratio. AHL diffusion to the medium resulted in X-Gal staining of areas of different size depending on AHL concentration and sensitivity of the indicator strain to a particular AHL. The response zone was measured as the distance from the border of the well to the border of the halo.

EGFP Fluorescence Measurements. For promoter-EGFP assays, cultures were grown in 96-well plates in a volume of 100 μL with shaking at 1,200 rpm. AHLs were added as 1,000 DMSO stocks at appropriate concentrations. Ectopic *lac* promoter-driven expression of *fadL* was induced with 100 μM IPTG. EGFP fluorescence was calculated as relative fluorescence units (F/OD), which represent fluorescence values divided by optical density. Background F/OD that typically ranged between 500 and 1000 F/OD units was included in the values shown in Figs. 2*B* and 4*B* and *SI Appendix*, Figs. S2*B*, S3*C*, and S7. Otherwise, background fluorescence of control strains carrying pPHU231-EGFP was subtracted. Fluorescence of three independent transconjugants of each strain was measured.

Further details of the procedures outlined above and additional methods are described in *SI Appendix*.

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