Disruption of a Gene Essential for Sulfoquinovosyldiacylglycerol Biosynthesis in *Sinorhizobium meliloti* Has No Detectable Effect on Root Nodule Symbiosis

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The sulfolipid sulfoquinovosyldiacylglycerol is commonly found in the thylakoid membranes of photosynthetic bacteria and plants. While there is a good correlation between the occurrence of sulfolipid and photosynthesis, a number of exceptions are known. Most recently, sulfoquinovosyldiacylglycerol was discovered in the non-photosynthetic, root nodule-forming bacterium *Sinorhizobium meliloti*. This discovery raised the questions of the phylogenetic origin of genes essential for the biosynthesis of this lipid in *S. meliloti* and of a function of sulfolipid in root nodule symbiosis. To begin to answer these questions, we isolated and inactivated the *sqdB* gene of *S. meliloti*. This gene and two other genes located directly 3' of *sqdB* are highly similar to the *sqdB*, *sqdC*, and *sqdD* genes known to be essential for sulfolipid biosynthesis in the photosynthetic, purple bacterium *Rhodobacter sphaeroides*. This observation confirms the close phylogenetic kinship between these two species. Furthermore, the reduced similarity of *sqdB* to the plant ortholog *SQD1* of *Arabidopsis thaliana* does not support a previous *sqd* gene transfer from the plant as a consequence of close symbiosis. A sulfolipid-deficient mutant of *S. meliloti* disrupted in *sqdB* is capable of inducing functional nodules and does not show an obvious disadvantage under different laboratory culture conditions. Thus far, no specific function can be assigned to bacterial sulfolipid, in either nodule-associated or free-living cells. *S. meliloti* contains a rich set of polar membrane lipids some of which, including sulfolipid, may become critical only under growth conditions that still need to be discovered.

Additional keyword: Rhizobium.

The sulfolipid sulfoquinovosyldiacylglycerol widely occurs in photosynthetic organisms ranging from bacteria to seed plants (Benning 1998; Haines 1973; Harwood 1980; Heinz 1993), where it is associated with photosynthetic membranes. However, the analysis of sulfolipid-deficient null mutants of the purple bacterium *Rhodobacter sphaeroides* and the cyanobacterium *Synechococcus* strain PCC7942 suggested that sulfolipid is not as generally essential for photosynthesis as one might be tempted to think based on its prevalence in photosynthetic organisms. However, it is required for growth under phosphate-limiting growth conditions in *R. sphaeroides* and *Synechococcus* strain PCC7942 (Benning et al. 1993; Güler et al. 1996). There are now several confirmed reports of the presence of sulfoquinovosyldiacylglycerol in nonphotosynthetic bacteria: a gram-positive, extremely thermoacidophilic bacterium from the genus *Alicyclobacillus* formerly called *Bacillus acidocaldarius* (Langworthy et al. 1976), several *Caulobacter* and *Brevundimonas* spp. (Abraham et al. 1997), and *Sinorhizobium meliloti* (formerly called *Rhizobium meliloti*; Cedergreen and Hollingsworth 1994). The interaction of *S. meliloti* or similar bacteria, collectively called rhizobia, with the roots of leguminous host plants leads to the formation of nodules in which atmospheric nitrogen is fixed. Lipo-chitin oligosaccharides produced by the bacteria act as nodulation signals (Dénarié et al. 1996; Schultze et al. 1994). Furthermore, certain cyclic glucans (Breedveld and Miller 1994), exopolysaccharides (Leigh and Walker 1994), lipopolysaccharides (Noel 1992), and K-like capsular polysaccharides (Reuhs et al. 1995) are required for the infection process and for the colonization of the root nodule. The importance of these compounds for successful symbiosis has been determined by the analysis of the respective biochemical mutants. In many cases, such mutants lead to the formation of nodules incompetent for nitrogen fixation, but free-living cells are not impaired in their growth rates. Because bacteroids are in close contact with plant membranes during the infection process in the nodules, it seems conceivable that certain membrane lipids in the plant or bacteroids may be important for successful nodule formation. *S. meliloti* shows a rich complement of membrane lipids (Goldfine 1987) and changes its membrane lipid composition

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in response to environmental stresses such as phosphate deprivation (Geiger et al. 1999). The discovery of sulfolipid in *S. meliloti* raised two questions (Cedergreen and Hollingsworth 1994): (i) Are *S. meliloti* *sqd* genes evolutionary derived from close bacterial relatives or from the host plant during the intimate symbiotic relationship? and (ii) What, if any, role does sulfolipid play in nodule formation and function? With the availability of four *sqd* genes from *R. sphaeroides* (Benning and Somerville 1992a, 1992b; Rossak et al. 1995) as well as cyanobacterial and plant *sqdB* orthologs (Essigmann et al. 1998; Güler et al. 1996), these questions can be experimentally addressed. The most highly conserved sulfolipid gene in bacteria, *sqdB*, and its ortholog in plants, *SQD1*, encode for an enzyme involved in the biosynthesis of UDP-sulfoquinovose from UDP-glucose and an unknown sulfur donor (Essigmann et al. 1999; Mulichak et al. 1999). While the function of the bacterial *sqdA* gene in sulfolipid biosynthesis remains unclear, *sqdC* and *sqdD* are thought to catalyze the transfer of sulfoquinovose from UDP-sulfoquinovose to diacylglycerol (Rossak et al. 1995, 1997). Here, we describe the isolation and characterization of *sqd* genes from *S. meliloti*, as well as the inactivation of *sqdB* and subsequent analysis of the resulting sulfolipid-deficient mutant with regard to bacterial growth, nodule formation, and nodule function.

RESULTS

Isolation of a putative *sqdB* ortholog and surrounding DNA from *S. meliloti*.

Relying on the strong conservation of *sqdB* DNA and amino acid sequences from different bacteria, degenerate primers against the *sqdB* gene from *R. sphaeroides* were designed. These primers were used to polymerase chain reaction (PCR) amplify and clone the corresponding fragment from *S. meliloti* genomic DNA, giving rise to plasmid pBW1 as shown in Figure 1. The insert of pBW1 served as homologous probe for screening of a genomic phage library of *S. meliloti* by DNA/DNA hybridization. Several overlapping clones hybridizing to the probe were isolated. The clone containing the longest insert, pBW9, was sequenced. Sequence analysis and preliminary comparison to the *sqd* genes from *R. sphaeroides* revealed two complete and one partial open reading frame (Fig. 1). The predicted amino acid sequence of the first open reading frame showed high sequence similarity to the *sqdB*, the

second to the *sqdD*, and the third to the *sqdC* gene products of *R. sphaeroides*. Because only the 5' end of *sqdC* was present on pBW9, and because no other 3' extending clones were available, a cosmid library containing genomic DNA fragments of *S. meliloti* was probed with the pBW1 insert. The cosmid cosSL1 completely containing all three genes was isolated. A 10-kb *Pst*I fragment derived from this cosmid was subcloned (pBW41) and used as the template to complete sequencing of *sqdC*. The assembled sequence of 3,989 bp was submitted to GenBank (accession no. AF194444). Detailed analysis revealed an open reading frame (base pairs 612 to 1829) encoding a putative protein of 406 amino acids with high amino acid sequence similarity to the *sqdB* gene product of *R. sphaeroides* (79% identity). The second open reading frame (base pairs 1829 to 2701) encoded a putative protein of 291 amino acids with a 62% identity to the *sqdD* gene product of *R. sphaeroides*, and the third open reading frame (base pairs 2701 to 3624) encoded a putative protein of 308 amino acids with 47% identity to the *sqdC* gene product of *R. sphaeroides*.

The putative *sqd* genes of *S. meliloti* are most likely of bacterial and not plant origin.

The analysis of the organization of the putative *S. meliloti* *sqd* genes and their similarity to the presumed orthologs from *R. sphaeroides* suggests that they have a common bacterial ancestor. Because the bacterial *sqdB* genes and the plant ortholog *SQD1* from *A. thaliana* are known to be highly conserved (Benning 1998), they provide an ideal basis for an analysis of relatedness and evolution of sulfolipid head group biosynthesis in different organisms. Figure 2 shows a tree predicting the evolutionary relatedness of the *sqdB/SQD1* genes based on amino acid sequence identity. It is apparent that the two *sqdB* orthologs from *R. sphaeroides* and *S. meliloti* are closest to each other and that *SQD1* is most distant from *sqdB* of *S. meliloti*. This result provides a satisfying answer to the question of the origin of *sqd* genes in *S. meliloti* raised by Cedergreen and Hollingsworth (1994). It seems unlikely that *S. meliloti* acquired *sqd* genes from the host plant during the symbiotic interaction. Rather, this analysis confirms the close relationship between *R. sphaeroides* and *S. meliloti*, which are both classified in the α division of gram-negative bacteria (Woese 1987).

The *sqdB* gene of *S. meliloti* is essential for sulfolipid biosynthesis.

To obtain functional proof for the *sqdB* ortholog of *S. meliloti*, the respective gene was inactivated by targeted gene

Fig. 1. Restriction map of *Sinorhizobium meliloti* genomic DNA containing (A) *sqd* genes and (B) clone and gene location. Insertion point and orientation of 0.9-kb DNA fragment (gray arrow) containing neomycin phosphotransferase (*npt*) cassette in mutant SL11 are indicated. Restriction sites: B, *Bgl*II; E, *Eco*RI; H, *Hind*III; N, *Nco*I; P, *Pst*I; S, *Sal*I).

Fig. 2. Phylogenetic tree based on similarity of bacterial SQDB and plant SQD1 proteins. Length of the branches is drawn to scale (10 substitutions per 100 residues are as indicated).

disruption. A neomycin phosphotransferase cassette was inserted into the *sqdB* open reading frame, leading to the isolation of mutant strain SLD11. Figure 1B shows the arrangement of genes in the mutant. Comparison of genomic DNA from the wild type and SLD11 by DNA/DNA hybridization confirmed the complete loss of wild-type genome copies in SLD11 and their replacement with mutant genome copies (Fig. 3). Most informative was a restriction digest with *Eco*RI probed with the insert of pBW1, giving rise to a diagnostic 3.2-kb band in the wild type and a 4.2-kb band in SLD11. To test the primary consequences of the *sqdB* gene disruption in

Fig. 3. Southern blot of genomic DNA from wild type (WT) and SLD11 digested with enzymes as indicated and probed with the insert of pBW1. Approximate fragment sizes (kb) are indicated.

Fig. 4. Thin-layer chromatography of [35 S]sulfate labeled extracts from wild type (WT) and SLD11. Dilutions of extracts (1:3 or 1:10) are indicated. Lipids were visualized by exposure to X-ray film.

S. meliloti, lipids of the wild type and SLD11 were compared by thin-layer chromatography (TLC) following labeling of the cells with [35 S]sulfate (Fig. 4). A single band co-chromatographing with bacterial and plant sulfolipid was detected in the wild-type samples that was completely absent in the extracts of SLD11, suggesting that this strain is a sulfolipid-deficient null mutant. When cosmid cosSL1 carrying *sqdB* and other adjacent genes was reintroduced into SLD11, sulfolipid biosynthesis was restored, consistent with genetic complementation (data not shown). Thus, *sqdB* of *S. meliloti* is a functional ortholog of the bacterial and plant *sqdB/SQD1* genes that is essential for sulfolipid biosynthesis in *S. meliloti*.

Sulfolipid deficiency has only subtle effects on growth of *S. meliloti*.

To begin to understand the role of sulfolipid in membranes of non-photosynthetic bacteria, in this case *S. meliloti*, growth of mutant and wild-type cells was compared in minimal medium containing sufficient or growth-limiting amounts of phosphate. In photosynthetic bacteria such as the purple bacterium *R. sphaeroides* or the cyanobacterium *Synechococcus* strain PCC7942, phosphate limitation specifically affects growth of sulfolipid-deficient null mutants and causes changes in membrane lipid composition (Benning et al. 1993; Güler 1996). Based on this observation, it has been proposed that sulfolipid substitutes for anionic phospholipids under phosphate-limiting growth conditions, thereby permitting maintenance of a functional photosynthetic membrane (Benning 1998). Comparing growth of the wild type and SLD11 in media with sufficient and 65-fold lower initial amounts of phosphate revealed a slight delay in growth of SLD11 under both conditions (Fig. 5). Growth rates for both strains were reduced in low-phosphate medium, but under the chosen conditions

Fig. 5. Growth of wild type (WT) (●) and SLD11 (▲) in (A) phosphate sufficient and (B) phosphate depleted minimal medium. Three independent cultures each were analyzed and averaged. Standard error bars are smaller than symbol size.

there was no specific effect on growth of the sulfolipid-deficient null mutant, contrary to what was observed for photosynthetic bacteria (Benning et al. 1993; Güler et al. 1996). Cultures of both strains reached the same final optical density, but this could have been due to the accumulation of secreted material (e.g., polysaccharides) during later stages of the culture, and not due to actual cell divisions and growth. Despite the lack of dramatic reduction in growth of the mutant even under phosphate-limiting growth conditions, the lipid composition qualitatively changed, as had been observed for photosynthetic bacteria grown in low-phosphate medium (Table 1). However, the increase in the relative pool size of phosphatidylglycerol in the mutant compared with the wild type, a decrease in the pool size for this lipid in the wild type under phosphate limitation but not in the mutant, and an increase in the pool size of sulfolipid under phosphate limitation in the wild type, were more subtle in *S. meliloti* than observed in *R. sphaeroides* or *Synechococcus* strain PCC7942. Either the employed conditions did not lead to the extent of phosphate starvation needed to see differences in growth and lipid composition of *S. meliloti* wild type and mutant, or sulfolipid is not as critical in non-photosynthetic bacteria under phosphate-limiting growth conditions as it is in photosynthetic bacteria.

The *sqdB* gene of *S. meliloti* is not essential for nodule formation or function.

The discovery of sulfoquinovosyldiacylglycerol in *S. meliloti* tempted speculation that this lipid, typically found only in photosynthetic organisms, may play a role in the symbiotic association of *S. meliloti* and its host alfalfa (Cedergreen and Hollingsworth 1994). To test whether SLD11 can form functional nodules, aseptically grown alfalfa seedlings were inoculated with the wild type or the sulfolipid-deficient strain SLD11, or treated with water as a control, and further incubated on minimal medium lacking a nitrogen source. Plants inoculated with either wild type or SLD11 formed nodules and flourished, while the control plants lagged behind in growth and showed with time increasing signs of nitrogen deprivation. Closer inspections of the plant roots revealed nodules on plants inoculated with bacteria and no nodules on water-treated plants. Cross sections of nodules from plants

inoculated with either bacterium were reddish pink. Furthermore, there was no difference in the number of nodules developing over time on the roots of plants treated with wild-type or mutant bacteria (Table 2). Lipid analysis of bacteria reisolated from the nodules to confirm the bacterial phenotypes showed the same membrane lipid composition as found in cells used for inoculation. These observations suggest that there are no adverse effects on the development and function of nodules derived from *S. meliloti* lacking the sulfolipid sulfoquinovosyldiacylglycerol.

DISCUSSION

Since the discovery and structural elucidation of the “plant sulfolipid” sulfoquinovosyldiacylglycerol by Benson and co-workers 40 years ago (Benson 1963; Benson et al. 1959), researchers have speculated about a specific role for this lipid in photosynthetic organisms. However, based on mutant analysis it now seems clear that sulfolipid is not generally essential for photosynthesis, but is of conditional importance under phosphate-limiting growth conditions, at least in purple bacteria with non-oxygenic photosynthesis and in cyanobacteria with oxygenic, plantlike photosynthesis (Benning et al. 1993; Güler et al. 1996). The nodule-forming bacterium *S. meliloti* is closely related to the purple bacterium *R. sphaeroides* (Woese 1987), but cannot carry out photosynthesis. The analysis of the sulfolipid-deficient mutant of *S. meliloti* revealed qualitative changes in lipid composition in response to phosphate deprivation (Table 1) similar to those observed for *R. sphaeroides* or the cyanobacterium *Synechococcus* strain PCC7942, but, unlike those photosynthetic bacteria, growth was not specifically reduced under phosphate-limiting conditions (Fig. 5B). This observation suggests that sulfolipid may have a distinct function in photosynthetic bacteria different from that in non-photosynthetic bacteria, but only under certain conditions, when anionic lipids such as phosphatidylglycerol become scarce and need to be replaced by a non-phosphorous lipid. If the *sqd* genes are not simply “ancestral baggage” passed on from the progenitor of *R. sphaeroides* and *S. meliloti*, what is the function of sulfolipid in *S. meliloti*? Because sulfolipid does not seem to be critical for the growth of free-living cells of *S. meliloti*, at least under the conditions tested, the question arises whether this lipid may be important for bacteroids. However, inoculation of roots with *S. meliloti* inactivated in *sqdB* leads to the formation of functional nod-

Table 1. Membrane lipid composition of *Sinorhizobium meliloti* wild type and SLD11 following incubation on high phosphate (1.3 mM) or low phosphate (0.02 mM) minimal medium

Lipid ^a	Composition (% of total ¹⁴ C) ^b			
	High phosphate		Low phosphate	
	Wild type	SLD11	Wild type	SLD11
PG	11.7 ± 0.3	12.8 ± 0.8	4.2 ± 0.3	10.4 ± 0.6
CL	4.2 ± 1.1	4.1 ± 0.7	5.3 ± 0.7	6.0 ± 0.8
PE/MMPE/DMPE	22.8 ± 3.3	20.6 ± 1.0	5.4 ± 0.3	5.0 ± 1.8
PC	46.5 ± 3.3	49.7 ± 0.3	6.2 ± 6.2	10.1 ± 1.1
SQDG	2.2 ± 0.2	ND ^c	3.2 ± 0.1	ND ^c
OL	10.1 ± 0.4	10.0 ± 0.7	8.6 ± 3.3	8.9 ± 0.4
DGTS	2.5 ± 0.7	2.7 ± 0.3	67.1 ± 3.0	59.5 ± 3.5

^a CL, cardiolipin; DGTS, diacylglycerol-*N,N,N*-trimethylhomoserine; DMPE, *N,N*-dimethyl-phosphatidylethanolamine; MMPE, *N*-monomethyl-phosphatidylethanolamine; OL, ornithine lipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SQDG, sulfoquinovosyldiacylglycerol.

^b Mean ± standard deviation; *n* = 3.

^c Not detectable.

Table 2. Nodule formation on roots of alfalfa seedlings

Days ^a	Nodules per plant ^b			
	Experiment 1		Experiment 2	
	WT	SLD11	WT	SLD11
5	0	0	0	0
10	0.7	0.3	1.2	0.3
15	1.0	1.0	1.5	1.3
20	1.3	1.3	3.0	2.3
25	1.5	2.0	3.0	2.7
30	1.8	2.0	3.2	3.5
35	2.3	2.0	3.2	3.5
40	2.3	2.0	3.2	3.5
45	2.5	2.0	3.2	3.5

^a Days after inoculation with bacteria.

^b Mean value derived from six plants.

ules indistinguishable from those induced by wild-type cells. Unless there is a second pathway for sulfolipid biosynthesis independent of the here-described *sqd* genes and specific for bacteroids, sulfolipid clearly has no specific function in nodule formation and nitrogen fixation. Thus, one can only speculate that the capability to synthesize sulfolipid increases the metabolic plasticity of *S. meliloti*, a predisposition for swift adaptations to changes in the environment.

The discovery of sulfoquinovosyldiacylglycerol in *S. meliloti* by Cedergreen and Hollingsworth (1994) also poses the interesting question of the origin of *sqd* genes in this bacterium. It seemed plausible that the *sqd* genes of *S. meliloti* were acquired by lateral gene transfer from plants as a result of symbiosis. However, comparison of *sqdB* orthologs from plants and bacteria clearly excludes this possibility, because *S. meliloti sqdB* is most distant from the plant ortholog (Fig. 2). On the other hand, the *sqdB* gene products of *R. sphaeroides* and *S. meliloti* are very similar in sequence and confirm the close phylogenetic kinship of the two bacteria (Woese 1987). It should be pointed out that there are actually photosynthetic, nodule-forming rhizobia (So et al. 1994). Thus, it seems plausible that *R. sphaeroides* and *S. meliloti* evolved from a common photosynthetic progenitor. While *S. meliloti* lost its photosynthetic capability, it maintained functional *sqd* genes. In conclusion, rhizobia are interesting not only because of their symbiotic interaction with plants but also because they are placed in the evolutionary tree at a branch point of photosynthetic and non-photosynthetic bacteria. The sulfolipid biosynthetic pathway is an interesting example of the evolution of a pathway primarily, but not exclusively, associated with photosynthetic organisms and a more extensive comparison of *sqd* gene structures throughout the phylogenetic tree may provide novel insights into the evolution of photosynthetic organisms.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions.

Strains and plasmids used in this study are listed in Table 3. *Sinorhizobium (Rhizobium) meliloti* strains were grown at 29°C in tryptone/yeast extract (TY) medium (Beringer 1974) or in minimal medium (Sherwood 1970) with succinate (8.3 mM) replacing mannitol as carbon source (de Rudder et al. 1997). *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) medium. Antibiotics were added when required to obtain the following final concentrations (in mg of medium per liter): streptomycin, 500; gentamicin, 60; neomycin, 50; piperacillin, 40; and tetracycline, 2 for *S. meliloti*; and carbenicillin, 100; gentamicin, 10; kanamycin, 50; and tetracycline, 20, for *E. coli*.

Construction of an *sqdB* probe and screening of genomic libraries.

An approximately 700-bp genomic DNA fragment of the sinorhizobial *sqdB* ortholog was PCR amplified with degenerated primers TTGGATCCCCSCCTATTTCRATGAAG and TTGGTACCTGCGKGGRTTGGGCAGG, annealing temperature 58°C. These primers were designed based on highly conserved regions of the *sqdB* genes from *R. sphaeroides* 2.4.1 (Benning and Somerville 1992b) and *Synechococcus* strain PCC7942 (Güler et al. 1996). The DNA fragment was blunt end-ligated into an *EcoRV*-digested pBSISK⁺ vector to obtain pBW1. The insert of plasmid pBW1 was subsequently used to screen for larger genomic clones containing the full-length *S. meliloti sqdB* and adjacent genes. Two different genomic libraries of *S. meliloti* 1021 were used: a cosmid library and a newly prepared lambda-ZAP-Express (Stratagene, La Jolla, CA) library. For this purpose, genomic DNA was

Table 3. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics or genotype	Reference or source
<i>Sinorhizobium (Rhizobium) meliloti</i>		
Sm1021	Str ^r of SU47	Meade et al. 1982
SLD11	Sm1021 with <i>npt</i> (Kan ^r) in <i>sqdB</i>	This study
<i>Escherichia coli</i>		
DH5αF ⁺	F' <i>endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 thi-1 recA1 gyrA96 relA1</i> Δ(lacIZYA-argF)U169 <i>deoR</i> (ΦF80 <i>dlacZ(lacZ)M15</i>)	Woodcock et al 1989
XL-1 Blue	<i>endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 thi-1 recA1 gyrA96 relA1</i> Δ <i>lac</i> [F' <i>proAB lacI^qΔM15</i> Tn10 (Tet ^r)]	Stratagene, La Jolla, CA
XL0LR	D(<i>mcrA</i>)183 Δ(<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1 thi-1 recA1 gyrA96 relA1</i> Δ <i>lac</i> [F' <i>proAB 1 lacI^qΔM15</i> Tn10 (Tet ^r)] Su ⁻ λ ^r	Stratagene
Plasmids		
pLAFR3	Broad-host-range cosmid vector, Tet ^r	Staskawicz et al. 1987
pUC19	Cloning and expression vector, Amp ^r	Yanisch-Perron et al. 1985
pBK-CMV	Cloning and expression vector, Amp ^r	Stratagene
pBSISK ⁺	Cloning and expression vector, Amp ^r	Stratagene
pRK2013	Helper plasmid for triparental mating, Kan ^r	Figurski and Helinski 1979
cosSL1 pLAFR3	Containing 24-b genomic DNA from <i>S. meliloti</i> surrounding <i>sqd</i> genes	This study
pMP3510	Broad-host-range plasmid, IncP, Tet ^r	Spaink et al. 1995
pTB3131	<i>npt</i> of Tn5 in pUC19	M. Schobert
pBW1	0.7 kb polymerase chain reaction fragment of sinorhizobial <i>sqdB</i> , blunt-end cloned in pBSISK ⁺	This study
pBW9	3.5-kb fragment carrying the sinorhizobial <i>sqdB</i> , <i>sqdD</i> genes and a part of <i>sqdC</i> in pBK-CMV	This study
pBW20	3.3-kb <i>AgeI/XbaI</i> fragment of pBW9 in pUC19	This study
pBW22	pBW20 with <i>npt</i> of pTB3131 inserted in <i>NcoI</i> of <i>sqdB</i>	This study
pBW24	4.2-kb <i>KpnI/XbaI</i> fragment of pBW22 in pMP3510	This study
pBW41	10-kb fragment of cosSL1 inserted into <i>PstI</i> of pUC19	This study

isolated from *S. meliloti* 1021 wild type, partially digested with *Sau3A*, and 2- to 4-kbp fragments were ligated into prepared phage arms according to the manufacturer's instructions. The phage library was screened by DNA/DNA-hybridization with Hybond N⁺ filters (Amersham, Piscataway, NJ) and a [³²P]phosphate-labeled probe. Hybridization was performed at 66°C overnight in hybridization buffer (0.255 M Na_xPO₄, 6.65% sodium dodecyl sulfate (SDS), 1 mM EDTA, 10 g of bovine serum albumin per liter). The filters were washed twice at 66°C for 20 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.5% (wt/vol) SDS prior to exposure. In vivo excision of a positive clone according to the manufacturer's instructions gave rise to plasmid pBW9 with an insert size of 3.5 kb. The cosmid library was screened with a digoxigenin (DIG)-labeled probe at 68°C overnight according to the manufacturer's instructions (Boehringer, Mannheim, Germany). The same conditions as for the cosmid library were used for the Southern blot. Routine nucleic acid manipulations were done as described (Sambrook et al. 1989).

Insertional inactivation of *sqdB*.

For inactivation of the *sqdB* open reading frame, the plasmid pBW9 (Fig. 1B) was digested with *AgeI* and *XbaI*. The ends of the resulting 3.3-kb DNA fragment were filled in and ligated into pUC19 digested with *SmaI*, giving rise to pBW20. The *sqdB* open reading frame was cut with *NcoI* and filled in, and the neomycin phosphotransferase cassette of pTB3131 inserted in opposite orientation relative to *sqdB*, giving rise to pBW22. The 4.2-kb insert was released with *KpnI/XbaI* and inserted into the broad-host-range vector pMP3510 to obtain pBW24. Plasmid pBW24 was transferred into *E. coli*. The resulting strain was used for triparental mating. For this purpose, the *S. meliloti* wild type, helper strain, and *E. coli* strain carrying the plasmid pBW24 were grown to an OD₆₂₀ of 0.3 and mixed in a ratio of 5:2:1 (vol/vol). Following an overnight incubation at 29°C on TY agar-solidified medium, the exconjugants were selected in the presence of streptomycin, tetracycline, and piperacillin at concentrations indicated above. In a second conjugation step, the IncP-incompatible plasmid pGM2 was transferred into the exconjugants to select for loss of pBW24 and concomitant integration of the kanamycin cassette into the genome as previously described (Østeras et al. 1998). The selection of the knock-out mutants was performed on TY medium with piperacillin, gentamicin, and neomycin added as indicated above. To screen for double recombinant mutants, the positive clones were also streaked onto TY medium containing tetracycline. Putative candidates were further analyzed by Southern hybridization to ensure complete segregation of wild-type genome copies. The finally isolated, *sqdB*-deficient *S. meliloti* strain was designated SLD11.

In vivo labeling with [¹⁴C]acetate or [³⁵S]sulfate and lipid analysis.

The lipid compositions of *S. meliloti* 1021 wild type and mutant strains were determined following labeling with [¹⁴C]acetate. Conditions were chosen to ensure homogeneous labeling of the different pools. Cultures (1 ml) of wild type and mutant were grown in minimal medium with two different concentrations of phosphate (1.3 and 0.02 mM) following in-

oculation with precultures containing the same media. After addition of 0.4 µCi [¹⁴C]acetate (60 nCi/nmol), the cultures were incubated for 24 h. To specifically visualize the sulfolipid, labeling experiments with [³⁵S]sulfate were performed in minimal medium (1 ml) with a reduced sulfate concentration (0.1 mM) containing 1 µCi carrier-free [³⁵S]sulfate. The cells were harvested by centrifugation, washed with 500 ml of water and resuspended in 100 ml of water. The lipids were extracted according to Bligh and Dyer (1959). Prior to quantification, the radioactive lipid extracts were analyzed on high-performance TLC silica gel 60 F254 plates (Merck, Darmstadt, Germany). In the case of one-dimensional TLC the solvent consisted of chloroform/methanol/water (14:6:1, vol/vol); in the case of two-dimensional TLC analysis the same solvent was used in the first dimension and chloroform/methanol/glacial acetic acid (13:5:2, vol/vol) in the second dimension. After exposure to Biomax MR-1 film (Kodak, Rochester, NY), the lipids were visualized by iodine staining. Quantification of ¹⁴C-labeled lipids was done by transferring lipid containing silica to vials and subsequent scintillation counter analysis.

Nodulation test on alfalfa.

Seeds of *Medicago sativa* (alfalfa) were surface sterilized and germinated as previously described (van Brussel et al. 1986). The seedlings were transferred to Jensen-Agar (van Brussel et al. 1982) and inoculated with mutant and wild-type strain cultures grown in minimal medium (OD₆₂₀ = 0.01). Controls were inoculated with sterilized water. The plants were incubated under natural day light (approximately 10 h per day) at room temperature for 10 or 12 weeks. During the first weeks, the seedlings were examined for root nodules every 3 to 4 days. Bacteria were reisolated from the nodules according to Somasegaran and Hoben (1994) and tested for the presence of sulfolipid as described above.

Computer analysis.

Multiple sequence alignments were performed (PileUp) and relationships were calculated (Distances) with GCG software version 10 (Genetics Computer Group, Madison, WI). A dendrogram based on the SQDB or SQD1 amino acid sequences from different organisms was constructed with GrowTree of the GCG package according to Sneath and Sokal (1973).

Nucleotide sequence.

A 3,989-bp long nucleotide sequence containing the *sqdB*, *sqdC*, and *sqdD* genes of *S. meliloti* has been deposited at GenBank (accession no. AF194444).

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