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Striking Complexity of Lipopolysaccharide Defects in a Collection of *Sinorhizobium meliloti* Mutants

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Although the role that lipopolysaccharide (LPS) plays in the symbiosis between *Sinorhizobium meliloti* and alfalfa has been studied for over a decade, its function in this process remains controversial and poorly understood. This is largely due to a lack of mutants affected by its synthesis. In one of the definitive studies concerning this issue, Clover et al. (R. H. Clover, J. Kieber, and E. R. Signer, J. Bacteriol. 171:3961-3967, 1989) identified a series of mutants with putative LPS defects, judged them to be symbiotically proficient on *Medicago sativa*, and concluded that LPS might not have a symbiotic function in *S. meliloti*. The mutations in these strains were never characterized at the molecular level nor was the LPS from most of them analyzed. We have transduced these mutations from the Rm2011 background from which they were originally isolated into the sequenced strain Rm1021 and have characterized the resulting strains in greater detail. We found the LPS from these mutants to display a striking complexity of phenotypes on polyacrylamide electrophoresis gels, including additional rough LPS bands and alterations in the molecular weight distribution of the smooth LPS. We found that some of the mutants contain insertions in genes that are predicted to be involved in the synthesis of carbohydrate components of LPS, including *ddhB*, *lpsB*, *lpsC*, and *lpsE*. The majority, however, code for proteins predicted to be involved in a wide variety of functions not previously recognized to play a role in LPS synthesis, including a possible transcription elongation factor (GreA), a possible queuine synthesis protein, and a possible chemotaxis protein. Furthermore, using more extensive assays, we have found that most of these strains have symbiotic deficiencies. These results support more recent findings that alterations in LPS structure can affect the ability of *S. meliloti* to form an effective symbiosis.

The bacterium *Sinorhizobium meliloti* is capable of forming a symbiosis with alfalfa, in which it reduces nitrogen from the atmosphere into ammonia for the plant in exchange for other nutrients. Establishing a symbiosis requires close association between the plant and bacterium. The bacteria live intracellularly within the plant in an organ called the root nodule. From the time at which the bacteria colonize the roots of the plant to the development of nitrogen-fixing nodules, the two organisms are in intimate contact. It is therefore not surprising that this developmental process involves many components of the bacterial cell envelope. Various cell surface polysaccharides have proven to be involved, including lipopolysaccharide (LPS), K antigen, exopolysaccharides, and cyclic β-(1,2)-glucans (36).

LPS comprises a large percentage of the bacterial outer envelope and consists of three components: lipid A, containing both sugar and fatty acid molecules, which serves to anchor the LPS to the cell envelope; the core, a nonrepeating oligosaccharide; and the O antigen, consisting of repeating oligosaccharide subunits. In the case of *S. meliloti*, a heterogeneous population of LPS molecules, with and without the O antigen, is present on the surface of the bacteria. In addition, LPS core composition analysis of an *S. meliloti lpsB* mutant suggests that there is more than one species of core (5). Alterations of the LPS structure in *S. meliloti* (5, 31, 37), as well as in other *Rhizobium* species (38), have been found to affect the ability of these strains to form an effective symbiosis with their host plants.

Mutants with LPS alterations typically exhibit characteristic phenotypes resulting from alterations of their outer membranes. These phenotypes include rough colony morphologies, alterations in sensitivity to certain classes of compounds such as detergents and cationic antimicrobial peptides, and alterations in phage sensitivity. Clover et al. (11) took advantage of some of these signatures of LPS alterations to isolate a large collection of putative *S. meliloti* LPS mutants. Some of these mutants were selected based on resistance to the phages ϕM5 or ϕM10, which kill the wild-type strain. Others were isolated on the basis of increased sensitivity to the detergent deoxycholate. These mutants seemed to be symbiotically proficient on alfalfa plants, and it was concluded that there is no specific symbiotic function for *S. meliloti* LPS. For the past decade, this work has been considered a definitive study of LPS mutants in *S. meliloti*. However, most of these mutants were never demonstrated to actually have alterations to their LPS structure and none of their mutations were characterized at the molecular level. Moreover, the ability of most of these mutants to establish a symbiosis or to fix nitrogen was not assessed in a quantitative fashion.

We present here a more thorough characterization of some of these mutants. We have identified the open reading frames (ORFs) containing the Tn*5* insertions and have found them to be located in a remarkably diverse set of genes. Some code for proteins that contribute directly to LPS structure, while others

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probably affect LPS in an indirect manner. We have also confirmed that the majority of these mutants physically alter the structure of LPS and exhibit a striking complexity of phenotypes. Moreover, we have characterized these mutants with respect to a variety of free-living phenotypes, including resistance to an expanded set of phages and sensitivity to deoxycholate and low pH. When we used quantitative methods to measure symbiotic efficiency to test these mutants for symbiotic phenotypes, we found that most of them are symbiotically defective.

MATERIALS AND METHODS

Strains, growth conditions, and phage assays. *S. meliloti* strains were grown at 30°C in Luria-Bertani (LB) medium supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ unless otherwise stated. *Escherichia coli* strains were grown at 37°C in LB medium. Antibiotics were used at the following concentrations: neomycin, 200 μg/ml; kanamycin, 50 μg/ml. For phage resistance assays, 5-μl drops of phage lysate (10^7 to 10^8 PFU) were spotted onto lawns of *S. meliloti* cells on LB agar plates. For purifying bacterial LPS, strains were grown to an optical density at 600 nm (OD_{600}) of ~0.8. All strains were transduced as previously described (18) to ensure that the phenotypes observed for each strain correlated with their transposon insertions.

Cloning transposon insertions. Transposon insertions were cloned using a whole-genome shotgun approach. Genomic DNA from each mutant was purified, digested with *Eco*RI and *Bam*HI (New England Biolabs), and ligated into pUC19 with T4 ligase (New England Biolabs) by using standard methods (2). The ligation mixture was transformed into DH5α cells made competent by $RbCl_2$ (30). Cells were plated on LB with kanamycin in the case of Tn*5* insertions and gentamicin and spectinomycin in the case of Tn*5-233* (12) insertions to select for the transposon drug markers, and plasmids from resistant colonies were purified using a Qiagen plasmid minikit (catalog no. 12123). Sequences adjacent to the transposons were determined using primers specific for the Tn*5* repeat element. Reactions were performed with dye terminator chemistry on the MegaBASE 1000 DNA sequencer (Amersham Biosciences).

Zone-of-inhibition and gradient assays. Zone-of-inhibition and gradient assays were performed as described by Ferguson et al. (17). Strains were grown overnight in LB medium to stationary phase and then diluted to an OD_{600} of 0.2. For the deoxycholate (DOC) zone-of-inhibition assay, cells (100 μl) were added to 3 ml of LB soft agar and spread evenly on plates containing 25 ml of LB medium. Zones of inhibition were measured after 2 days of growth at 30°C. For sodium DOC sensitivity assays on gradient plates, 200 ml of LB agar was poured on 22.5-cm-square plates containing a gradient of DOC from 0 to 25 mM. A total of 60 μl of each strain was then spread in an even line across the plate, the plates were incubated for 3 days at 30°C, and the zone of inhibition was measured. Strains were scored as very sensitive (−−−) when they were sensitive to 0.025 M DOC, moderately sensitive (−−) when they were sensitive to 0.25 M DOC, and slightly sensitive (−) when the length of the growth streak on gradient plates was 60% or less of the length of the wild-type growth streak. Tests of acid sensitivity were similarly performed on gradient plates of pH 7 to 5.5 buffered with 35 mM 2-(*N*-morpholino)ethanesulfonic acid (MES). Strains were scored as slightly sensitive when the length of their growth streaks on pH gradient plates was 85% or less of the wild-type streak length and moderately sensitive when their growth streaks were 80% or less of the wild-type streak length. All tests were performed at least three times for each strain.

Cationic peptide assays. For all cationic peptide assays, *S. meliloti* strains were grown to an OD of ~1 in LB. Next, cells (100 μl) were aliquoted to a 96-well microtiter dish, 1 μl of cationic peptide was added, and the dishes were incubated at 30°C with shaking for 1 h. The titers of the cultures were then determined by spotting a series of dilutions on LB plates. The final concentration of melittin was 20 μg/ml, and that of polymyxin B was 5 μg/ml. Cationic peptide sensitivity assays of each strain were performed at least three times. For strains assayed in triplicate, all three values were included in the average. For those assayed at least four times, the highest and lowest values were excluded from the average.

Plant assays. Seedlings of alfalfa were nodulated on petri dishes of Jensen agar as previously described (33). Three-day-old seedlings were inoculated with approximately 10^7 bacteria added to each plate in 1 ml of water. Plant height, leaf color, and nodule phenotypes were scored after 3 to 4 weeks.

For competition assays, mutant and wild-type strains were mixed together in a 1:1 ratio and incubated on alfalfa plants as described above. At 4 weeks, three nodules were cut from each plant, sterilized in 50% Clorox bleach for 1 min, rinsed, and crushed in a solution of LB medium with 0.3 M glucose. Serial dilutions were then spread on LB plates. To determine the relative proportions of mutant bacteria, they were replica plated onto plates containing the drug to which their Tn*5* insertions confer resistance.

Acetylene reduction assays were performed by placing two plants in a vial with 1 ml of acetylene and incubating for 2 and 4 h for *Medicago sativa* and *M. truncatula*, respectively. The relative amounts of ethylene produced were measured by gas chromatography. Three to four measurements were taken for each strain. Mutants were scored as $Fix^{+/-}$ when their acetylene reduction levels were 25% or less of that of the wild-type strain and Fix^- when acetylene reduction rates were 3% of wild-type levels or less.

LPS analysis. LPS was prepared by a variation of the method described by Niehaus et al. (37). Cells were grown overnight in mannitol glutamate salts minimal medium to an OD_{600} of 0.8. Aliquots (1 ml) were centrifuged for 5 min at 2,500 × *g* rpm, resuspended in 30 μl of lysis buffer (1 M Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 4% β-mercaptoethanol, 10% glycerol, 0.005% bromophenol blue) and incubated at 100°C for 10 min. Proteinase K (10 μl) was then added to each sample (2.5 mg/ml in lysis buffer), the mixtures were incubated for 1 h at 60°C and centrifuged for 15 min at 15,000 × *g*, and the supernatant was mixed with sample buffer (120 mM Tris HCl [pH 6.8], 3% SDS, 9% β-mercaptoethanol, 30% glycerol, and 0.03% bromophenol blue) in a 1:2 ratio.

A volume of 5 μl of the final preparation was then applied to gels for SDS–16.5% polyacrylamide gel electrophoresis (PAGE), and electrophoresis was carried out with a Tricine buffer system (34). Finally, gels were silver stained (50).

RESULTS AND DISCUSSION

Transduction of mutations into Rm1021. Since the mutants isolated by Clover et al. (11) were obtained by screening populations of *S. meliloti* mutagenized by Tn*5*, it seemed likely that their phenotype was derived from the Tn*5* insertion. Nevertheless, before carrying out further characterization we transduced the Tn*5* insertion in each strain from the less-widely-used SU47 RCR-Rm2011 background to the sequenced strain Rm1021 (3, 8, 19, 21). Rm1021 and Rm2011 are both derived from strain SU47 and are extremely closely related. The new and the old strain designations are shown in Table 1.

Mutant characterization and sequence analysis. To determine whether these strains had alterations in their LPS, we ran samples of their LPS on SDS-PAGE silver-stained gels (Fig. 1; lanes 1 and 11 contain LPS from the wild-type strain). In the case of the parental Rm1021 strain, smooth LPS and rough LPS migrate as distinct species. The smooth LPS produced by the wild-type strain runs as a series of nondistinct bands. The rough LPS migrates faster on the gel and consists of one prominent upper band and a very faint lower band. The mutant strains exhibited a striking complexity of phenotypes, with most of the mutants having their own unique LPS banding patterns that differed from that of the wild-type strain.

We then further characterized the mutants by determining the sites of their transposon insertions. To do this, we cloned a portion of the transposon containing the drug resistance marker, along with flanking chromosomal DNA, and sequenced several hundred base pairs outwards from the insertion site. By comparing these sequences to those of the newly sequenced *S. meliloti* genome (3, 8, 19, 21), we were able to determine the ORFs in which the transposon insertions were located (Table 1). A subset of the mutants from this analysis had defects in a wide variety of genes involved in carbohydrate synthesis and sugar modification. Surprisingly, however, we found that the majority of these mutants contained insertions in genes involved in unexpected processes whose role in LPS synthesis is probably less direct.

TABLE 1. Mutant genotypic information

Strain	Transduced from	Insertion type/location	AA Position[a]	Location	Possible function or similarity of gene product
SmGC5	Rm7662	Tn*5-233*/*lpsB*	213/351	Chrom[b]	Glycosyl transferase involved in LPS core biosynthesis
SmGC6	Rm7609	Tn*5-233*/*lpsC*	93/270	Chrom	Glycosyl transferase
SmGC7	Rm7539	Tn*5*/*lpsE*	251/340	Chrom	Glycosyl transferase
SmGC8	Rm7665	Tn*5*/*lpsE*	261/340	Chrom	Glycosyl transferase
SmGC9	Rm7597	Tn*5*/*ddhB*	78/356	Chrom	CDP-glucose 4,6-dehydratase
SmGC10	Rm7659	Tn*5-233*/*ddhB*	216/356	Chrom	CDP-glucose 4,6-dehydratase
SmGC11	Rm7598	Tn*5*/*rkpK*	365/437	Chrom	UDP-glucose 6-dehydrogenase
SmGC12	Rm7663	Tn5-233/SMb21425	44/258	pSymB	Conserved hypothetical protein (1 transmembrane helix predicted)
SmGC13	Rm7661	Tn5-233/SMb21425	200/258	pSymB	Conserved hypothetical protein (1 transmembrane helix predicted)
SmGC14	Rm7666	Tn*5*/*greA*	91/158	Chrom	Transcription elongation factor
SmGC15	Rm7543	Tn*5*/*ropB1*	91/212	Chrom	Conserved outer-membrane protein
SmGC16	Rm7602	Tn*5*/SMb20515	619/1161	pSymB	Putative chemotaxis methyltransferase protein
SmGC17	Rm7536	Tn*5*/SMc00984	251/324	Chrom	Conserved hypothetical protein containing a Sua5/YciO/YrdC family signature domain, a possible nucleic acid binding motif
SmGC18	Rm7535	Tn*5*/SMc00644	438/753	Chrom	Transmembrane protein with ATP/GTP binding site (P-loop)
SmGC19	Rm7550	Tn*5*/*queA*	71/360	Chrom	*S*-adenosylmethionine:tRNA-ribosyltransferase-isomerase (queuine synthetase)
SmGC20	Rm7551	Tn*5*/*ppiB*	20/169	Chrom	Peptidyl-prolyl *cis-trans* isomerase from cyclophilin family
SmGC21	Rm7600	Tn*5*/SMc04209	65/301	Chrom	No sequence similarity
SmGC22	Rm7660	Tn5-233/ND[c]			

[a] Amino acid position of insertion from beginning of protein/total length of protein.
[b] Chrom, chromosome.
[c] ND, not determined.

Only one of these mutants contained an insertion in *lpsB*, the best-characterized *S. meliloti* gene involved in LPS synthesis. *lpsB* mutants have a dramatically altered core structure and display a characteristic downward shift in both their rough and smooth LPS on gels in SDS-PAGE (5). While the *lpsB* mutant displayed a characteristic shift in rough and smooth LPS, the remaining mutants all exhibited banding patterns unique from those of the *lpsB* phenotype. For this reason we constructed double mutants between *lpsB* and all of the unlinked mutants. Surprisingly, the double mutants all displayed phenotypes on gels that were indistinguishable from that of *lpsB* alone (data not shown). This suggests that these unlinked insertions are in genes whose products alter the LPS structure either in steps subsequent to that involving LpsB or in a fashion requiring LpsB.

We also tested the symbiotic proficiency of these strains on the host plants *M. sativa* (alfalfa) and *M. truncatula* (Table 2). While our results were consistent with the previous finding that these strains were all capable of forming an effective symbiosis on alfalfa (11), our more detailed analysis indicated that most of these strains are less proficient at forming a symbiosis than the wild-type strain. Most strains are out-competed when coinoculated on alfalfa plants with the wild-type strain, and the majority had reduced nitrogenase activity on *M. truncatula* as measured by acetylene reduction assays.

Moreover, we examined several additional free-living phenotypes of these strains, including their resistance to a large collection of phages and their sensitivities to deoxycholate (DOC), low pH (Table 2), and the cationic peptides melittin and polymyxin B (Table 3). DOC sensitivity may indicate the relative integrity of the outer envelopes of the mutant bacteria. Sensitivity to low pH might be informative, because the plant membrane compartment can be between pH 5.5 and 6 (39). Alterations in LPS structure often result in changes in cationic peptide sensitivity (1, 16). Since cationic peptides are part of the innate defense response of many organisms, including plants, alterations in cationic peptide sensitivities might reflect a susceptibility to the plant's defenses against bacteria. We have found that some mutants have differential sensitivity to melittin and polymyxin B. This may have implications for the relative ability of these peptides to bind to altered LPS or for differences in the ways these peptides cross or destabilize the outer membrane (14).

The defects in these LPS mutants are similar to those of mutant with mutations in the *lpsB* and *bacA* genes, which also lead to alterations in LPS, sensitivity to low pH, alterations in cationic peptide sensitivities, and symbiotic defects (5, 17, 23), suggesting that in some instances these phenotypes are correlated.

Mutations in genes predicted to encode enzymes directly involved in carbohydrate or LPS synthesis. (i) *lpsBEDC*. Four of the mutants had insertions in the *lpsBEDC* cluster adjacent to *greA*. All of these *lps* genes have previously been shown to be involved in LPS synthesis (32), consistent with our findings that this collection of mutants contains insertions in genes that affect LPS structure. Mutant SmGC5 had an insertion in *lpsB*, mutant SmGC6 had an insertion in *lpsC*, and mutants SmGC7 and SmGC8 had insertions in *lpsE*. None of the mutants contained insertions in *lpsD*. *lpsB* mutants have already been characterized in detail. The *lpsB* gene encodes a type 1 glycosyl transferase required for LPS core synthesis (5, 31, 32, 37). The *lpsB* mutant identified in this study, SmGC5, had phenotypes similar to those of other *lpsB* mutants, including a dramatic shift in both the smooth and the rough LPS on gels in SDS-PAGE indicative of the LPS core alteration. It was also similar to other *lpsB* mutants in having resistance to a large number of phages and exhibiting deoxycholate sensitivity. The fact that *lpsB* mutants are resistant to certain phages suggests that these phages require specific LPS epitopes that are no longer present in the *lpsB* mutant for binding to the bacterial surface during the infection process. While we found that some of the mutants in this collection were resistant to the same subset of phage

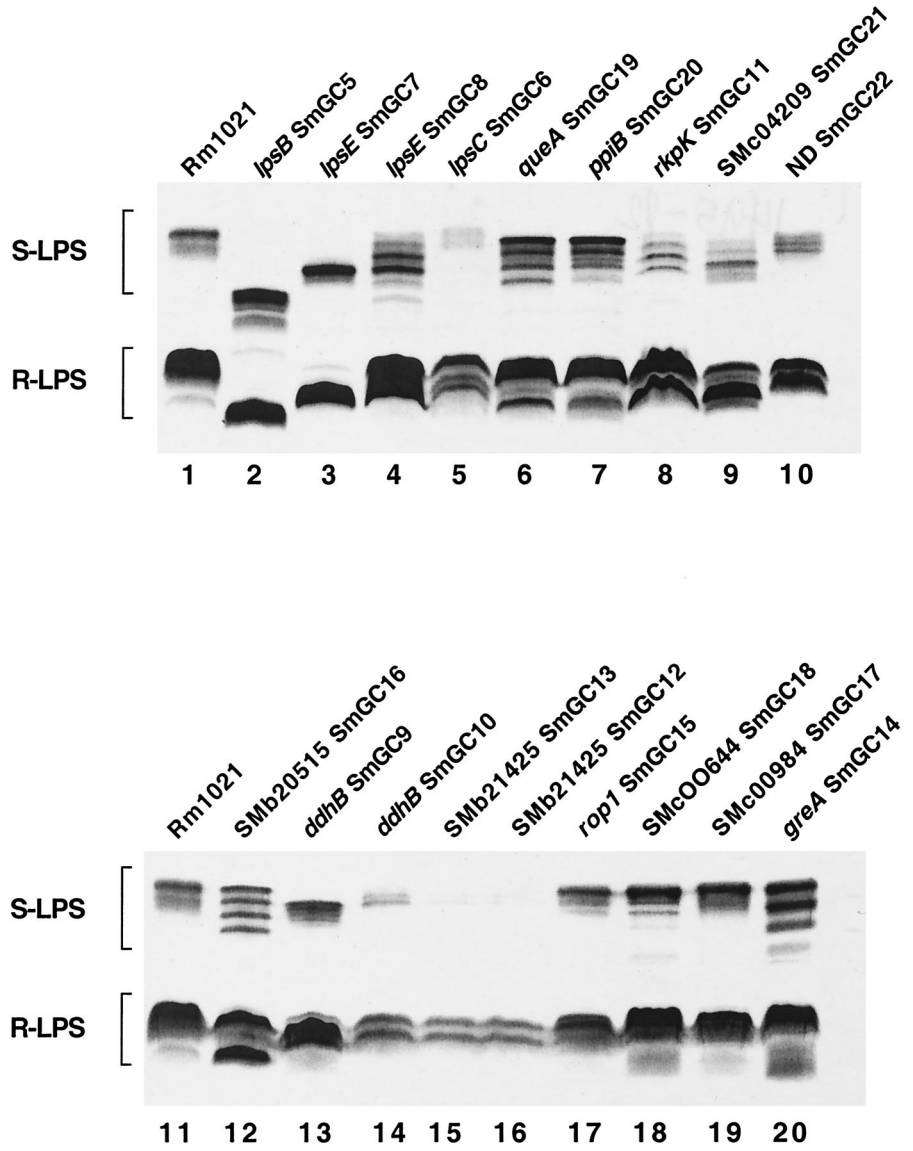

FIG. 1. DOC PAGE analysis of mutant strains showing rough LPS and smooth LPS. Lanes: 1, Rm1021; 2, SmGC5, *lpsB*; 3, SmGC7, *lpsE*; 4, SmGC8, *lpsE*; 5, SmGC6, *lpsC*; 6, SmGC19, *queA*; 7, SmGC20, *ppiB*; 8, SmGC11, *rkpK*; 9, SmGC21, Smc04209; 10, SmGC22, sequence not determined (ND); 11, Rm1021; 12, SmGC16, Smb20515; 13, SmGC9, *ddhB*; 14, SmGC10, *ddhB*; 15, SmGC13, SmB21425; 16, SmGC12, SmB21425; 17, SmGC15, *ropB1*; 18, SmGC18, Smc00644; 19, SmGC17, Smc00984; 20, SmGC14, *greA*.

strains as *lpsB*, none of the mutants were resistant to any phage to which *lpsB* remained sensitive. Perhaps these other phages do not depend on binding to the LPS for infection. SmGC5 also resembled previously isolated *lpsB* mutants in being very sensitive to melittin and polymyxin B.

The mutant SmGC6 contains an insertion in *lpsC*. The *lpsC* gene product has similarities to β-1 to 4-glycosyl transferases from *Neisseria meningitidis* and *Aquifex aeolicus*. The *lpsC* mutant produces reduced amounts of smooth LPS on gels in PAGE. This mutant has reduced nitrogenase activity on alfalfa and is Fix$^-$ on *M. truncatula*. Despite having severe alterations to its LPS, it is resistant to only one phage and has wild-type sensitivity to DOC, low pH, and cationic peptides.

The mutants SmGC7 and SmGC8 both contain insertions in the *lpsE* gene. The start codon of *lpsE* and the termination codon of *lpsD* overlap by 2 bp, suggesting that their translations can be coupled. LpsD and LpsE exhibit stronger sequence similarity to each other (53%) than to any other sequences in GenBank. Like *lpsB*, these genes are members of the type 1 glycosyltransferase family, a large family of proteins that transfer activated sugars to a variety of substrates, including glycogen and lipopolysaccharides. They are also members of the COG0438 RfaG glycosyltransferase family. The *E. coli* RfaG (WaaG) protein is involved in LPS core biosynthesis, and *rfaG* mutants exhibit a deep rough phenotype that includes resistance to certain bacteriophages and lower levels of expression of some outer-membrane proteins (7, 22, 43). SmGC8, the strain containing an insertion located further downstream in *lpsE*, displays a phenotype on gels in SDS-PAGE that is similar to a previously published phenotype (32). The upstream inser-

TABLE 2. Mutant phenotypes

Strain	Insertion[a]	DOC[b]	pH[b]	Phage sensitivity[c]							Plant assays result		
				M1	M5	M6	M9	M10	M14	Other phages[d]	*M. sativa* phenotype[e]	Alfalfa mutant/ total ratio[f]	*M. truncatula* phenotype[g]
Rm1021	Wild type	+	+	S	S	S	S	S	S	S	Fix^+	NA[h]	Fix^+
SmGC1	*lpsB*	−−−	−−	R	R	R	R	R	R	S	$Fix^{+/-}$	ND[i]	$Fix^{+/-}$
SmGC5	*lpsB*	−−−	−−	R	R	R	R	R	R	S	$Fix^{+/-}$	0.02	$Fix^{+/-}$
SmGC7	*lpsE*	−	+	R	R	R	R	R	R	S	Fix^+	0.021	Fix^-
SmGC15	*ropB1*	−−−	−−	R	R	R	R	S	S	S	Fix^+	0.003	Fix^+
SmGC14	*greA*	−−−	−−	S	r	S	r	S	S	S	$Fix^{+/-}$	0.056	Fix^-
SmGC18	c00644	−−−	−	S	S	S	S	S	S	S	Fix^+	0.06	$Fix^{+/-}$
SmGC11	*rkpK*	−−	−	S	S	S	S	S	S	S	$Fix^{+/-}$	0.030	Fix^-
SmGC9	*ddhB*	+	+	R	R	R	R	R	R	S	Fix^+	0.041	$Fix^{+/-}$
SmGC21	c04209	+	+	r	r	r	r	r	r	S	Fix^+	0.043	Fix^-
SmGC16	c20515	+	+	R	R	R	r	S	S	S	Fix^+	0.30	Fix^-
SmGC19	*queA*	+	+	r	R	r	S	S	S	S	Fix^+	0.023	$Fix^{+/-}$
SmGC20	*ppiB*	+	+	r	R	r	S	S	S	S	$Fix^{+/-}$	0.093	$Fix^{+/-}$
SmGC8	*lpsE*	+	+	S	r	S	S	r	r	S	Fix^+	ND	ND
SmGC6	*lpsC*	+	+	S	S	S	S	R	S	S	$Fix^{+/-}$	0.30	Fix^-
SmGC10	*ddhB*	+	+	S	S	S	S	R	S	S	Fix^+	0.42	Fix^-
SmGC22	ND	+	+	S	S	S	S	R	S	S	Fix^+	0.11	Fix^-
SmGC12	b21425	+	+	S	S	S	S	R	S	S	Fix^+	0.41	$Fix^{+/-}$
SmGC13	b21425	+	+	S	S	S	S	R	S	S	Fix^+	0.28	Fix^-
SmGC17	c00984	+	−−	S	S	S	S	S	S	S	Fix^-	ND	Fix^-

[a] Numbers refer to numbers of ORFs from *S. meliloti* genome project. c, chromosomal ORFs; b, ORFs on *psymB*.
[b] −, slightly sensitive to DOC and/or acid; −−, moderately sensitive to DOC and/or acid; −−−, very sensitive to DOC and/or acid; +, wild-type sensitivity to DOC and/or acid.
[c] M1, phage ϕM1; M5, phage ϕM5; M6, phage ϕM6; M9, phage ϕM9; M10, phage ϕM10; M14, phage ϕM14. S, phage sensitive; R, phage resistant; r, slightly phage resistant.
[d] ϕM7, ϕM11, ϕM12, ϕM12H1, and ϕM19 (all strains were sensitive to these phages).
[e] Fix^+, nitrogen fixation occurs at wild-type levels (as measured by acetylene reduction assays). $Fix^{+/-}$, nitrogen fixation occurs at reduced levels. Fix^-, inoculated bacteria do not fix nitrogen. Phenotypes are those of *M. sativa* plants inoculated with LPS mutants.
[f] Data are ratios of numbers of mutant bacteria/total numbers of bacteria recovered in coinoculation assays on alfalfa.
[g] Phenotypes are those of *M. truncatula* plants inoculated with LPS mutants.
[h] NA, not available.
[i] ND, not determined.

tion SmGC7, however, has a more pronounced LPS phenotype. This mutant resembles *lpsB* mutants in that both the rough and smooth LPS run faster on gels in PAGE, it has the same full phage resistance pattern as *lpsB* mutants, and it is slightly sensitive to DOC. This may indicate that the Tn*5* insertion in the strain SmGC7 insertion eliminates activity of *lpsE* whereas the insertion in SmGC8 results in a partially active protein. Both SmGC7 and SmGC8 are very sensitive to

TABLE 3. Cationic peptide sensitivity

	Average % survival after 1 h in 20 μg of melittin/ml	Melittin survival ratio of Rm1021/mutant[a]	Average % survival after 1 h in 5 μg of polymyxin B/ml	Polymyxin B survival ratio of Rm1021/ mutant[a]
Rm1021	1.20	1.0	54	1.0
lpsB SmGC1	3.0×10^{-3}	400.0	9.8×10^{-3}	7,100.0
lpsB SmGC5	1.5×10^{-3}	800.0	1.0×10^{-1}	540.0
lpsC SmGC6	3.3×10^{-1}	3.6	21	2.6
lpsE SmGC8	5.9	0.2	7.9×10^{-3}	6,800.0
lpsE SmGC7	2.8×10^{-1}	4.3	6.9×10^{-2}	780.0
rkpK SmGC11	2.3×10^{-2}	52.0	5.8	9.3
greA SmGC14	1.2	1.0	3.2	17.0
SMc04209 GC21	8.3×10^{-2}	15.0	11	4.9
queA SmGC19	8.6×10^{-2}	14.0	35	1.5
SMb21425 GC13	2.1×10^{-1}	5.7	16	3.4
SMb21425 GC12	1.9×10^{-1}	6.3	31	1.7
SMc00644 SmGC18	20	0.06	9.4	5.8
ropB1 SmGC15	44	0.03	81	0.67
ddhB SmGC9	8.8×10^{-1}	1.4	22	2.5
ddhB SmGC10	3.0×10^{-1}	4.0	42	1.3
ND SmGC22	3.9×10^{-1}	3.1	15	3.6
ppiB SmGC20	5.1×10^{-1}	2.4	40	1.4
SMc20515 SmGC16	2.9×10^{-1}	4.1	31	1.7
SMc00984 SmGC17	3.9	0.3	35	1.5

[a] Sensitivity levels with a five-fold difference from that of the wild type were considered significant and are discussed in the text.

polymyxin B but have the same sensitivity to melittin as the wild type. The *lpsE* mutants have the most striking relative difference in sensitivity to polymyxin B and melittin of any of the mutants in this study.

(ii) *ddhB*. Mutants SmGC9 and SmGC10 both carry insertions in *ddhB*, a gene not previously implicated in *S. meliloti* LPS synthesis. The 356-amino-acid *ddhB* gene product is very similar to DdhB from *Yersinia pseudotuberculosis* (42% identity and 58% similarity over 340 amino acids) and *Salmonella enterica* (42% identity and 59% similarity over 340 amino acids). The DdhB enzyme is a CDP-glucose 4,6-dehydrogenase that catalyzes the synthesis of CDP-4-keto-6-deoxy-D-glucose from CDP-D-glucose, the second step in the pathway for making 3,6-dideoxyhexoses (DDH) (29, 44, 49). In nature, DDHs are uncommon monosaccharides that are found almost exclusively in LPS O antigens. In *S. enterica* and *Y. pseudotuberculosis*, 3,6-dideoxyhexose derivatives contribute to the O-antigen diversity among different strains. In *S. meliloti*, *ddhB* lies directly downstream of *ddhA* and may be within the same operon. DdhA catalyzes the synthesis from glucose-1-phosphate of the CDP-D-glucose substrate used by DdhB (29). Although we did not identify any other genes involved in the synthesis of DDH in the *S. meliloti* genome, the altered properties of the *ddhB* mutant suggest that a sugar derived from 4-keto-6-deoxy-D-glucose is important for the synthesis of wild-type *S. meliloti* LPS.

In a fashion similar to that of the *lpsE* mutants, the two alleles of *ddhB* appear to have differing degrees of alterations to their LPS when analyzed on gels in PAGE. SmGC9, the mutant containing the insertion closer to the start of the gene at amino acid 78, has a more severe phenotype and resembled an *lpsB* mutant on gels in SDS-PAGE in that both the rough and smooth LPS ran faster than that of the parental strain. It also exhibited sensitivity to the same range of phages as an *lpsB* mutant. In contrast, the *ddhB* mutant, SmGC10, with a more downstream insertion at amino acid 216, had less severe LPS alterations on gels in PAGE and a more modest shift in the migration speed of the smooth LPS and two distinct rough LPS bands. This may indicate that SmGC9 represents the null phenotype whereas SmGC10 retains partial activity. While both mutants were Fix^+ on alfalfa, they were less competitive on this species and were deficient at forming a nitrogen-fixing symbiosis with *M. truncatula*. Neither strain exhibits a strong sensitivity to cationic peptides.

(iii) *rkpK*. SmGC11 contains an insertion in *rkpK*. The RkpK protein is an UDP-glucose dehydrogenase that (according to mutant analysis in *S. meliloti* strain Rm41) is involved in the synthesis of both K antigen and LPS (6, 28). This mutant contains two rough LPS bands and appears to have an altered molecular weight distribution of its smooth LPS. The Rm41 *rkpK* mutants were selected as colonies resistant to the K antigen-specific phage 16-3 that does not infect strain Rm1021 (6, 28). It is interesting that this mutation in Rm1021 does not confer sensitivity to any of the phages we tested in our assay. It is also fairly DOC sensitive and has a mild sensitivity to low pH. This mutant was also found to be less competitive than the wild type and to have less nitrogenase activity during symbiosis with alfalfa and was found to be Fix^- when inoculated on *M. truncatula*. Perhaps the most striking phenotype of this mutant is its sensitivity to melittin. This mutant exhibited the second-highest sensitivity to melittin after the *lpsB* mutants. It also has some sensitivity to polymyxin B.

(iv) ORF SMc21425. Mutants SmGC12 and SmGC13 contain insertions in ORF SMc21425, encoding a conserved protein with one transmembrane helix. The function of the gene is unclear. However, it is downstream of SMc21426 and SMc21427, two ORFs predicted to be involved in carbohydrate synthesis, and may have a related function. The close proximity of these genes may indicate that they are in one operon. SMc21427 codes for a 168-amino-acid protein that has similarity to acetyl transferases, including a putative NodL protein from *Mesorhizobium loti* (34% identity over 138 amino acids). SMc21426 encodes a putative sugar nucleotide oxidoreductase-epimerase. These mutants have two distinct rough LPS bands by SDS-PAGE analysis, and the LPS seemed to purify less efficiently from these strains. These mutants are only resistant to the phage ϕM10 and have wild-type sensitivities to DOC and low pH. Both mutants are Fix^+ on alfalfa. However, SmGC12 is Fix^- on *M. truncatula* and SmGC13 is Fix^+ on this host plant. Both mutants are slightly sensitive to melittin but do not exhibit significant sensitivity to polymyxin B.

Insertions in genes with indirect roles in LPS synthesis. Interestingly, a large number of mutants had Tn*5* insertions in genes that are probably not directly involved in LPS synthesis but nevertheless affect the LPS structure.

(v) *greA*. SmGC14 was found to contain an insertion in a gene annotated *greA* on the basis of sequence similarity (32). *greA* functions have not been previously recognized as being important for symbiosis or cell surface characteristics. The *S. meliloti greA* gene product has significant sequence similarity to those of other bacterial GreA proteins which function as transcription elongation factors and shares two consensus signatures (Prosite PS00829 and PS00830) for prokaryotic transcription elongation factors present in all GreA proteins.

In *E. coli*, GreA induces the cleavage and removal of the 3′ proximal dinucleotide from stalled complexes and thereby allows transcription to resume (4). GreA, or an activity enhanced by GreA, also increases the fidelity of transcription through preferential cleavage of transcripts containing misincorporated residues (15). GreA lies directly upstream of *lpsB*, *-E*, *-D*, and *-C* and an ORF encoding a protein with similarity to another transcription factor, *lrp* (32), which is suggestive of a role for GreA in the proper expression of these LPS genes. Although *greA* is 122 bp upstream of *lpsB* and facing in the same direction, expression studies indicate that they have separate promoters (32), so it is unlikely that the LPS defect of this strain is due to a polar effect on *lpsB*. The SDS-PAGE phenotype of this mutant is distinct from the wild type in that it displays three prominent smooth LPS bands. This mutant was also very sensitive to DOC and low pH. Despite the obvious cell surface defects of this strain, it was only slightly resistant to the phages ϕM5 and ϕM9 and exhibited wild-type levels of sensitivity to melittin. It was somewhat sensitive to polymyxin B, however.

SmGC14, the *greA* mutant, had an unusual phenotype on plants. At 3.5 weeks, the nodules looked normal, being pink and elongated and containing wild-type levels of bacteria (G. R. O. Campbell and G. C. Walker, unpublished data). However, the plants were short and yellow, resembling uninoculated plants, and acetylene reduction assays indicated that these plants had reduced nitrogenase activity. By 4.5 weeks, the

nodules had lost most of their color. Possibly GreA function becomes more important for transcription after the bacteroids have been with the plant cells for a prolonged period and have been exposed to various forms of stress.

(vi) *ropB1*. Mutant SmGC15 contains an insertion in *ropB1*. The *ropB1* gene product is a 212-amino-acid, 22-kDa protein with high sequence similarity to RopB from *Rhizobium leguminosarum* (43% identity and 57% similarity over 168 amino acids) and Omp25 from the related mammalian pathogen *Brucella abortus* (35% identity and 48% similarity over 170 amino acids). The Omp25 protein is highly conserved among *Brucella* species (9, 10). So far this class of outer-membrane proteins has not been found outside the α-2 subgroup of proteobacteria. RopB from *R. leguminosarum* was originally detected as a major antigenic determinant of the bacterial outer membrane. It was found that expression of the *R. leguminosarum* RopB protein decreases in bacteroids (46), but no function has been found for this protein. The SmGC15 mutant has a less obvious LPS phenotype by our analysis on SDS-PAGE but may have one or two additional rough LPS bands. In addition, it is resistant to the phages ϕM1, ϕM5, ϕM6, and ϕM9, is highly sensitive to DOC and low pH, and has the unusual phenotype of being resistant to melittin. Since RopB1 is an outer-membrane protein, it is possible that the insertion in SmGC7 disrupts the outer membrane, resulting in deoxycholate sensitivity and alterations to the LPS. This mutant is also fairly slow growing and is temperature sensitive when grown at 37°C. Although it is Fix^+ on both alfalfa and *M. truncatula*, it is much less competitive than the wild-type strain when coinoculated on alfalfa. This phenotype might be due to its slow growth rate in addition to, or instead of, its altered LPS phenotype.

(vii) ORF SMb20515. SmGC16 contains an insertion in an interesting multidomain protein, ORF SMb20515. The gene product of this ORF has been annotated as being a chemotaxis methyltransferase protein in the *S. meliloti* database because it contains regions of similarity to the methyltransferase domains of CheR and the methyl esterase domain of CheB (13). In addition, it contains a sensor histidine kinase domain and displays strong similarity to predicted sensor histidine kinases from *Methanosarcina acetivorans*, *Anabaena* species, *Rhodobacter capsulatus*, and *Agrobacterium tumefaciens*. Perhaps members of this gene family encode proteins involved in the regulation of genes involved in LPS synthesis. SmGC16 has clear LPS alterations by SDS-PAGE, displaying four distinct smooth LPS bands and two distinct rough LPS bands. In addition, it is resistant to the phages ϕM1, ϕM5, and ϕM6 and has a slight resistance to ϕM9. It exhibits wild-type sensitivity levels to DOC, cationic peptides, and pH, however. This mutant is Fix^+ on alfalfa but Fix^- on *M. truncatula*.

(viii) ORF SMc00984. SmGC17 contains an insertion in the ORF SMc00984, whose gene product has sequence similarity to the Sua5 protein in yeast (31% identity and 43% similarity over 374 amino acids). Furthermore, this protein contains a Sua5/YciO/YrdC family signature domain (Prosite PS01147). This sequence has similarity to that of the HypF hydrogenase maturation domain (ProDom PD002209). The function of the Sua5 protein in yeast is unclear. However, the crystal structure of the Sua5/YciO/YrdC domain has been solved for the YrdC protein and has been found to display a novel folding configuration featuring a large concave surface on one side with a positive electrostatic potential suggestive of a nucleic acid binding site (48). This mutant appears to have slightly faster migrating rough LPS by SDS-PAGE, so perhaps in *S. meliloti* this protein plays a role in regulating genes involved in LPS synthesis. The LPS alterations revealed by PAGE analysis are fairly modest, but it is possible that a noncarbohydrate component of the LPS is being altered, for example, a lipid A fatty acid. In addition, the mutant is sensitive to the full panel of phages tested and has wild-type levels of sensitivity to DOC and both cationic peptides but is sensitive to low pH. This mutant has the distinction of being Fix^- on both alfalfa and *M. truncatula*.

(ix) *ppiB*. SmGC20 has an insertion in a putative *ppiB* homologue coding for a peptidyl-prolyl *cis-trans* isomerase of the cyclophilin family (26). These enzymes catalyze the *cis-trans* isomerization of peptide bonds to which proline contributes the nitrogen (41). Mutants lacking *ppiB* would be expected to have global effects on cellular physiology and may be indirectly involved in LPS synthesis by assisting in the folding of a protein involved in LPS production. Alternatively, this mutation may have a polar effect on *queA*, the gene directly downstream of it (described in the next section), which has a very similar phenotype. This mutant has quite dramatic changes of its LPS profile on gels in SDS-PAGE, displaying a series of additional faster-moving smooth LPS bands and two rough LPS bands. It is only slightly resistant to the phages ϕM1 and ϕM6, however, and is resistant to DOC and low pH. It has less nitrogenase activity than the wild-type strain on both alfalfa and *M. truncatula* and is out-competed by the wild-type strain on alfalfa in coinoculation experiments.

(x) *queA*. SmGC19 has an insertion in a gene coding for a protein with similarity to QueA of other species. These are enzymes with *S*-adenosylmethionine:tRNA-ribosyltransferase-isomerase activity and are involved in the synthesis of the modified tRNA base queuine (45). By affecting tRNA structure, insertions in *queA* may have global effects on cellular physiology, extending to production of LPS. Alternatively, it is possible that this protein adds modifications to the LPS structure in *S. meliloti*. This gene is 180 bp downstream of *ppiB*, and the two genes are separated by two RIME elements, repetitive elements found throughout the Rm1021 genome (40). Nevertheless, SmGC19 and SmGC20 have very similar phenotypes. The LPS profile of this mutant looks very similar to that of SmGC20, it has the same phage resistance profile, and it has wild-type resistance levels to DOC and low pH. However, SMGC19 is somewhat sensitive to melittin, while SMGC20 is not. It would be interesting to find out whether these two genes that carry out very different functions in the cell are coregulated. This mutant has wild-type levels of nitrogenase activity but is less competitive in coinoculation experiments with the wild-type strain on alfalfa and has reduced nitrogenase activity on *M. truncatula*.

(xi) ORF SMc00644. The mutant SmGC18 contains an insertion in ORF SMc00644. This ORF is predicted to encode a protein containing a membrane-spanning domain and a P-loop ATP/GTP binding motif but with little similarity to other reported protein sequences. The LPS phenotype of this mutant appears to be quite modest by SDS-PAGE, displaying several additional smooth LPS bands. Furthermore, this mutant is sensitive to all the phages in our panel. This mutant does have

a strong sensitivity to DOC, however, suggesting a significant disruption of the outer envelope. Like *ropB1*, SMGC18 is resistant to melittin, but this strain is slightly sensitive to polymyxin B. It is less competitive when coinoculated on alfalfa and exhibits reduced nitrogenase activity on *M. truncatula*.

(xii) ORF SMc04209. SmGC21 has an insertion in ORF SMc04209, which has similarity only to uncharacterized hypothetical proteins in GenBank. The LPS profile contains two major species of rough LPS and a somewhat reduced level of smooth LPS composed of at least three distinct bands. This mutant is slightly resistant to all the phages in the test panel. It has wild-type sensitivity to DOC and low pH, but it is somewhat sensitive to melittin and slightly sensitive to polymyxin B. SmGC21 has wild-type levels of nitrogenase activity, but it is less competitive in coinoculation experiments with the wild-type strain on alfalfa and has reduced nitrogenase activity on *M. truncatula*.

Conclusion. In an attempt to determine which *S. meliloti* genes are necessary for proper LPS production and to what extent LPS structural changes affect symbiosis, we have carried out a more detailed study of the putative LPS mutants isolated by Clover et al. (11). This included determining the sites of the transposon insertions in these strains, an effort made possible by the recent completion of the *S. meliloti* Rm1021 genomic sequence (3, 8, 19, 21). Moreover, we have analyzed the LPS from these mutants on gels in SDS-PAGE and have performed a more detailed analysis of their symbiotic phenotypes. Although the original paper by Clover et al. (11) has been widely referenced as evidence that LPS does not have a symbiotic effect in *S. meliloti* symbiosis, we have found through a more extensive characterization of these mutants that most of them are less effective at forming a symbiosis with their host plants. These results are consistent with more recent studies of *lpsB* mutants of *S. meliloti* (5, 37) and of mutants resulting in LPS defects from other *Rhizobium* species (27) in which LPS alterations have been found to frequently result in symbiotic defects. In agreement with Clover et al. (11), we found that most of these mutants were Fix^+ on alfalfa. However, many of the mutants showed reduced levels of nitrogen fixation and competition assays indicated that many are less proficient than the wild-type strain in nodule invasion. In addition, most of the mutants were found to show no, or reduced, acetylene reduction activity compared to that of the parental strain when in symbiosis with *M. truncatula*. This might indicate that consistent with the findings of Niehaus et al. for an *lpsB* mutant (37), certain aspects of *S. meliloti* LPS are important for symbiotic host range determination. Some of the mutations in this collection, such as those in *lpsC*, *lpsE*, and *ddhB*, probably directly affect LPS structure. It should be pointed out, however, that many of the others (for example, those in *greA* and *ropB1*) may cause pleiotrophic phenotypes in addition to their effects on LPS structure. It is possible that these other effects contribute at least partially to the symbiotic defects exhibited by these strains.

The mutants in this collection exhibited a striking complexity of phenotypes on LPS gels. One interesting characteristic shared by the majority of the LPS mutants was that they had more than one prominent band in the region corresponding to the rough LPS of the wild-type strain. Only one prominent rough LPS band was visible in the wild-type strain. The positions of these extra bands, and the relative amount of material within each band, differed between mutants. For example, SmGC21 (SMc024209) has three bands in the rough LPS portion of the gel, one darkly staining band and two fainter bands. In contrast, the lanes with the LPS samples from the mutants SmGC12 and SmGC13 (containing insertions in ORF21425) contain two bands of equal intensity. The *lpsC* mutant had four distinct bands running in the rough LPS region, the most of any of the strains. Most of the other mutants had their own unique banding patterns in the rough LPS section of the gel.

The smooth LPS also differed dramatically between strains in terms of the distinctness of the bands, the relative amounts, and the molecular weight distribution. For example, the strains SmGC8 (*lpsE*), SmGC19 (*queA*), SmGC20 (*ppiB*), SmGC11 (*rkpK*), SmGC16 (SMb20515), and SmGC14 (*greA*) all displayed a prominent ladder of faster-moving smooth LPS bands. In contrast to the wild-type strain, which appears to synthesize mostly high-molecular-weight O antigen, these mutants make more of the mid- to small-size-range O antigen. These phenotypes are reminiscent of those of *wzz* (formerly known as *rol* or *cld*) mutants in enteric bacteria (44, 54). *wzz* mutants have unregulated O-antigen chain lengths, causing banding patterns by PAGE analysis that are distinct from the modal distribution of bands in the wild-type strain. It is possible that a similar mechanism for regulating O-antigen chain length also exists in *S. meliloti* that is unable to perform its function properly on the altered LPS synthesized in these mutant backgrounds. An additional explanation might be that the additional smooth LPS bands with different mobilities resulted from the O antigen being added to additional core species that are usually minor species in these strains.

In light of the variety of LPS defects exhibited in these strains, it is of particular interest that all of the *S. meliloti* SU47 mutants in this collection isolated by Clover et al. (11) or in other studies retained some form of O antigen. This contrasts strikingly with studies of lipopolysaccharide mutants with *E. coli*, *Brucella* species, and other *Rhizobium* species (1, 42, 53). One explanation for this might be that the loss of O antigen is lethal for *S. meliloti*, but this seems highly unlikely since this is not the case with LPS mutants in other bacterial species. An alternative explanation is that *S. meliloti* makes more than one species of core that carries O antigen. Thus, mutations eliminating one species of core would not result in mutants lacking O antigen, especially if loss of one species of LPS results in a compensating increase in the numbers of one of the other species. Such an explanation is consistent with the discovery by Campbell et al. that *S. meliloti lpsB* mutants synthesize an LPS that has a core with dramatically different sugar composition than the parental strain (5). LpsB mutants have a characteristic shift in both the smooth and rough LPS species. We saw a similar, albeit more modest, phenotype displayed by the *lpsE* mutant SmGC7 and possibly in the *ddhB* mutant SmGC9. Determining the basis of the shifts in these mutants would require more extensive analysis of their LPS composition.

Consistent with the complexity of LPS phenotypes observed on gels in PAGE, a significant diversity of phenotypes was seen when these mutants were tested for phage resistance and sensitivity to DOC, low pH, and cationic peptides. Phage resistance did not seem to correlate with either DOC or acid sensitivity. With the exception of SmGC17, DOC sensitivity seems

to correlate with sensitivity to low pH, suggesting that similar defects can lead to these phenotypes. It is particularly interesting, however, that phage resistance and cationic peptide sensitivities do not appear to correlate with the other phenotypes we tested, suggesting that the true diversity in the surface defects of these mutants is even broader than what is exhibited on PAGE gels.

A few mutants had pronounced sensitivities to cationic peptides, while only two, *ropB1* and SMGC18, had greater resistance (to melittin) than the wild type. The *lpsE* mutants were unusual in that they had a high level of sensitivity to polymyxin B (comparable to that of *lpsB* mutants) but no sensitivity to melittin. Polymyxin B and melittin have quite different amino acid sequences and different structures. Polymyxin B has a cyclic amphipathic structure (47), while melittin is linear and has two amphipathic α-helices (51). The precise mechanisms of antimicrobial activity of different structural classes of cationic peptides are still being defined (14, 51). In gram-negative bacteria, polymyxin B has been shown to cause outer-membrane blebbing while melittin forms channels in membranes (25, 35, 52). The composition of LPS influences the efficiency of binding and the antimicrobial activity of both of these peptides. Hydrophobicity as well as a negative charge for the outer membrane can have complex effects on the adsorption of polymyxin B (47). *Brucella* species rough mutants that lack O antigen bind polymyxin B and melittin more efficiently than smooth strains and are more sensitive to these peptides (20). In *S. enterica* serovar Typhimurium, mutants in *waaP*, whose gene product is a kinase of the LPS inner core, are sensitive to polymyxin B and have an outer membrane with decreased stability (55). Also, however, lipid A modifications in *S. enterica* serovar Typhimurium are associated with resistance to polymyxin B (24, 43). Likewise, in *S. meliloti*, changes in lipopolysaccharide fatty acid composition are associated with the increased resistance of the *bacA* mutant to cationic peptides (17). Although *bacA*, unlike *lpsB*, is more resistant to cationic peptides, it is similar to *lpsB* in its sensitivity to agents that may be indicative of outer-membrane instability (17, 43). Thus, the relative contributions to cationic peptide sensitivity of the efficiency of adsorption of these compounds to the outer membrane and stability of this membrane have yet to be determined.

Our expectation prior to sequencing the transposon insertion sites of these mutants was that they would be in genes encoding enzymes predicted to be involved in carbohydrate biosynthesis and other functions necessary for proper LPS structure. To our surprise, the majority of these mutants had insertions in genes whose products are not predicted to have any relation to LPS or carbohydrate synthesis. Instead, these genes encode proteins whose functions are predicted to be as diverse as transcriptional regulation, chemotaxis, and tRNA base modification. These mutants are sure to open up new avenues of research into the factors contributing to LPS structure for many years to come.

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