Role of BacA in Lipopolysaccharide Synthesis, Peptide Transport, and Nodulation by *Rhizobium* sp. Strain NGR234[▽]

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BacA of *Sinorhizobium meliloti* plays an essential role in the establishment of nitrogen-fixing symbioses with *Medicago* plants, where it is involved in peptide import and in the addition of very-long-chain fatty acids (VLCFA) to lipid A of lipopolysaccharide (LPS). We investigated the role of BacA in *Rhizobium* species strain NGR234 by mutating the *bacA* gene. In the NGR234 *bacA* mutant, peptide import was impaired, but no effect on VLCFA addition was observed. More importantly, the symbiotic ability of the mutant was comparable to that of the wild type for a variety of legume species. Concurrently, an *acpXL* mutant of NGR234 was created and assayed. In rhizobia, AcpXL is a dedicated acyl carrier protein necessary for the addition of VLCFA to lipid A. LPS extracted from the NGR234 mutant lacked VLCFA, and this mutant was severely impaired in the ability to form functional nodules with the majority of legumes tested. Our work demonstrates the importance of VLCFA in the NGR234-legume symbiosis and also shows that the necessity of BacA for bacteroid differentiation is restricted to specific legume-*Rhizobium* interactions.

Symbiotic interactions between rhizobia, Gram-negative soil bacteria, and leguminous plants result in the formation of new plant organs called nodules. Rhizobia penetrate inside nodules and differentiate into bacteroids, which reduce atmospheric nitrogen to compounds the plant can assimilate. A successful interaction requires the correct exchange of molecular signals, which also determine the number of partners with which a legume host or rhizobial strain can develop functional symbioses (6, 27). Certain rhizobia, such as *Sinorhizobium meliloti*, can nodulate only a few legumes, whereas others, such as *Rhizobium* sp. strain NGR234, have a broad host range and can nodulate more than 120 genera of legumes (40).

There are several parallels between the mechanisms used to establish and maintain a functional bacteroid and those used by pathogenic bacteria during infection of eukaryotic cells. In fact, a successful symbiosis requires an "acute infection" (rhizobia have to enter cells of the plant root) and also a "chronic infection" (persistence of nitrogen-fixing bacteroids within nodules)—stages often associated with intracellular bacterial pathogens (13, 22). Several molecular signals, such as modifications of bacterial surface components, have been described to be involved in these two phases (12, 14).

BacA was one of the first rhizobial proteins shown to be required for the establishment of a successful chronic infection (23). Initially, *bacA* was identified by screening *S. meliloti* mutants for symbiotic deficiencies (34). *bacA* mutants invade nodules normally but then lyse and die before differentiating into functional nitrogen-fixing bacteroids. This observation led to the suggestion that BacA is required for nodule maturation and persistence (23). BacA is also important for prolonged intracellular survival during host-bacterium pathogenic interactions, such as chronic infection by *Brucella abortus* (32). *S. meliloti bacA* mutants display pleiotropic phenotypes. In addition to impaired nodulation with *Medicago sativa* (19), they are more resistant to the glycopeptide bleomycin and to aminoglycoside antibiotics (e.g., gentamicin). On the other hand, the absence of BacA results in an increased sensitivity to sodium dodecyl sulfate (SDS), deoxycholate (DOC), and ethanol, which suggests that there could be alterations in membrane integrity. Indeed, the *S. meliloti bacA* mutant was shown to be affected in its lipopolysaccharide (LPS) composition (19).

Bacterial LPS is typically composed of three parts: a hydrophobic domain known as lipid A, a nonrepeating "core" oligosaccharide, and a distal (predominantly repeating) polysaccharide (or O antigen) which may or may not be present. The correct assembly of rhizobial LPS is important for symbiosis, as mutants impaired in LPS synthesis frequently do not form functional nodules (9–11, 28, 39). Furthermore, changes to both the core and O antigen can occur during the symbiotic interaction. *Rhizobium leguminosarum* LPS becomes more hydrophobic during bacteroid development (15, 29), while *Rhizobium etli* LPS is also modified in response to plant exudates (38). In the case of NGR234, a new LPS species, characterized by the presence of a rhamnose-rich "rhamnan" O antigen, is synthesized upon induction by flavonoids, the initiating molecular signals of host plants (5, 21, 44).

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TABLE 1. Bacterial strains and plasmids used for this study

Strain or plasmid	Relevant characteristics	NBRC no.	Reference or source
Strains			
Escherichia coli			
DH5α	*supE44 ΔlacU169*(φ80d*lacZ*ΔM15) *hsdR17 recA1 gyrA96 thi-1 relA1*		BRL, Bethesda, MD
Rhizobium strains[a]			
NGR234	Broad-host-range bacterium isolated from nodule of *Lablab purpureus*; Rif^r	101917	49
NGRΔ*acpXL*	NGR234 derivative with *acpXL* deleted; Rif^r	106209	This work
NGRΩ*bacA*	NGR234 derivative containing an Ω cassette inserted into the SmaI site of *bacA*; Rif^r Sp^r	106210	This work
Plasmids			
pBluescript II KS(+)	High-copy-number ColEI-based phagemid; Ap^r		Stratagene, La Jolla, CA
pKS-*acpXL*	pBluescript KS(+) derivative carrying a 1.4-kb PCR fragment containing the *acpXL* gene; Ap^r		This work
pKS-Δ*acpXL*	pKS-*acpXL* derivative in which the *acpXL* gene has been deleted; Ap^r		This work
pKS-*acpXL*pro	pBluescript KS(+) derivative carrying a 0.5-kb PCR fragment containing the *acpXL* promoter; Ap^r		This work
pKS-*bacA*pro	pBluescript KS(+) derivative carrying a 0.5-kb PCR fragment containing the *bacA* promoter; Ap^r		This work
pK18*mobsacB*	Suicide vector used to generate directed mutagenesis; Km^r		46
pK18-Δ*acpXL*	pK18*mobsacB* derivative containing the *acpXL* deletion; Km^r		This work
pJQ200SK+	Suicide vector used to generate directed mutagenesis; Gm^r		41
pJQ-*bacA*	pJQ200SK+ derivative carrying *bacA* of NGR234; Gm^r		This work
pJQ-*bacA*ΩSp	pJQ200SK derivative carrying the mutated *bacA*ΩSp fragment; Gm^r Sp^r		This work
pRK2013	Tra$^+$ helper plasmid		20
pPROBE-GT and pPROBE-GT′	Broad-host-range promoter probe vectors containing the GFP open reading frame; Gm^r		37
pGT′-*acpXL*	pPROBE-GT′ derivative containing the *acpXL* promoter region; Gm^r		This work
pGT′-*bacA*	pPROBE-GT′ derivative containing the *bacA* promoter region; Gm^r		This work
pGT-*fixF*	pPROBE-GT derivative containing the *fixF* promoter region; Gm^r		Antoine le Quéré, unpublished data

[a] NGR234 strains have been deposited at the NITE Database of Biological Resources (NBRC; http://www.nite.go.jp/) for long-term storage and distribution.

Rhizobial lipid A contains a very-long-chain fatty acid (VLCFA; 27-OH-28:0), attached as the secondary fatty acid of an acyloxyacyl substituent on the distal glucosamine residue (24, 42, 43), which is important for symbiosis. For *S. meliloti*, *R. etli*, and *R. leguminosarum*, two genes involved in the addition of VLCFA to lipid A have been identified: *lpxXL*, which encodes a specific long-chain acyltransferase, and *acpXL*, which encodes a dedicated acyl carrier protein. VLCFA is synthesized attached to AcpXL in the cytoplasm and transferred onto the lipid A precursor by the inner membrane-associated LpxXL (2, 8, 17, 48). The VLCFA moiety is also found in a number of facultative intracellular pathogens that form chronic infections (e.g., *Bartonella henselae*, *Brucella abortus*, and *Legionella pneumophila*) (4, 54).

C_{28} fatty acids are not present in the lipid A fractions of *S. meliloti lpxXL* and *acpXL* mutants. As a consequence, the outer membrane is altered, and the cells are more sensitive to detergents (e.g., SDS and DOC). When these mutants are used to inoculate *Medicago* plants, they have reduced competitive abilities and take longer to induce nodules. Nevertheless, the nodules elicited on *M. sativa* and *Medicago truncatula* roots are normal and fix nitrogen, suggesting that VLCFA is important but not essential for chronic infection by *S. meliloti* (17, 48). LPS alterations in *S. meliloti bacA* mutants manifest as a significant decrease (about 50%) in the VLCFA content of lipid A (16, 19). This reduction in VLCFA on its own, however,

cannot explain the symbiotic deficiency of this mutant, as *acpXL*, *lpxXL*, and *acpXL lpxXL* mutants are able to establish successful symbioses with alfalfa. Moreover, *S. meliloti acpXL bacA* and *lpxXL bacA* double mutants lack VLCFA but are as symbiotically defective as the *bacA* mutant, suggesting that BacA must have additional functions required for chronic infection (17).

BacA is a homologue of SbmA of *Escherichia coli*, a transporter of peptide antibiotics, and thus could be involved in peptide uptake in rhizobia. Like SbmA, BacA is an integral inner membrane protein with seven transmembrane domains. Involvement of *S. meliloti* BacA in peptide uptake was recently confirmed using a truncated form of the eukaryotic antimicro-

TABLE 2. Oligonucleotide sequences used for this study

| Name	Sequence (5′–3′)[a]
acpXL$_{for}$	AAA<u>GGATCCC</u>ACGCCGCGAATGATGTTTGC
acpXL$_{rev}$	AAA<u>AGCTT</u>GGGGCCATCAATCCCACTTC
acpXL$_{del UP}$	AAA<u>GAATTC</u>ATGCACCGCAAAAGCCAATGC
acpXL$_{del DOWN}$	AAA<u>GAATTC</u>AAGGCCTGATCGCCTCTGC
acpXL$_{prom rev}$	AAA<u>GGTACCC</u>GCAAAAGCCAATGCCTCCC
bacA$_{for}$	CCAATCCTTCTTCCCGCAACC
bacA$_{rev}$	GGCCTTGATGAACTGCTGGTC
bacA$_{prom for}$	AAA<u>GGATCC</u>GCTGCTGGTCGAGGCTGTC
bacA$_{prom rev}$	AAA<u>GGTACC</u>GGGACGGCACTCGCTTTTCT

[a] Restriction sites are underlined.
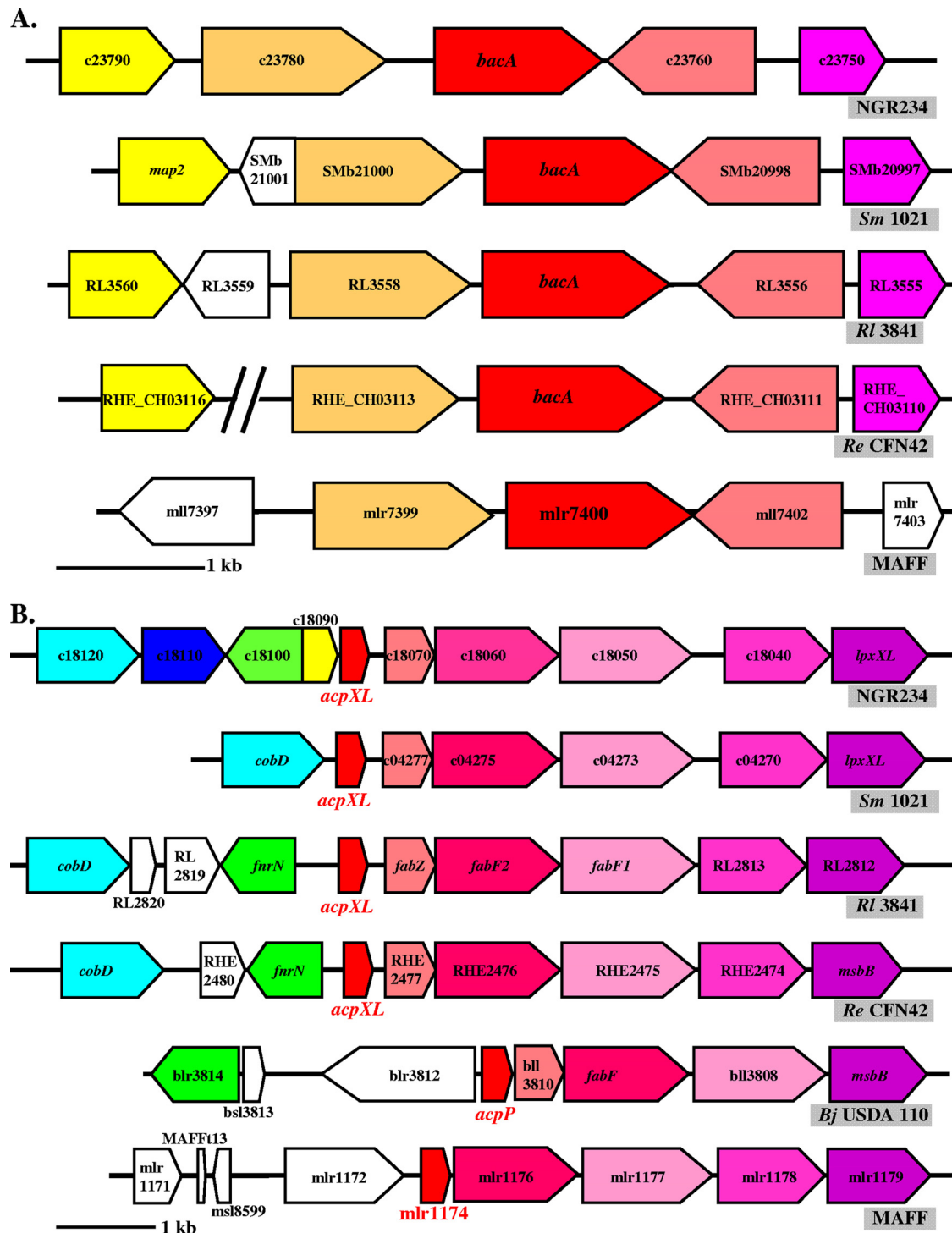
FIG. 1. *bacA* and *acpXL* loci of various rhizobia. Conservation of the *bacA* (A) and *acpXL* (B) loci is shown for NGR234, *S. meliloti* (*Sm*) 1021, *R. leguminosarum* bv. *viciae* (*Rl*) 3841, *R. etli* (*Re*) CFN42, and *M. loti* MAFF303099. In *B. japonicum* (*Bj*) USDA110, only a fragment of *bacA* is present and is surrounded by ORFs encoding putative transposases. Homologous genes are indicated by the same color. The genes are named according to their annotation in the published genomes (*S. meliloti* pSymB, GenBank accession no. NC_003078; *R. leguminosarum* bv. *viciae* 3841, NC_008380; *R. etli* CFN42, NC_007761; *B. japonicum* USDA110, NC_004463; and *M. loti* MAFF303099, NC_002678).

bial peptide Bac7 (35). BacA is essential for Bac7(1-16)-mediated cell death and for intracellular accumulation of fluorescently labeled Bac7(1-16). This phenotype is independent of the effect of BacA on lipid A. It is not known whether BacA is

directly or indirectly involved in peptide uptake (35). Peptides are produced in developing nodules of legumes, and it has been suggested that these peptides have a key function in *S. meliloti* bacteroid development (51). It has been proposed that

BacA-dependent peptide uptake could play a critical role in the chronic infection process of symbiotic and pathogenic bacteria (36).

BacA is essential for development of indeterminate pea nodules formed by *R. leguminosarum* bv. *viciae* but not of determinate bean nodules formed by *R. leguminosarum* bv. *phaseoli* and *R. etli*, although the *bacA* mutants of these strains show similar phenotypes under free-living conditions (30). Since the broad host range of NGR234 includes plants that form both determinate and indeterminate nodules, we tested the symbiotic phenotype of an NGR234 *bacA* mutant, as well as the role of BacA in LPS synthesis and peptide transport in NGR234.

MATERIALS AND METHODS

General microbiological and molecular techniques. Standard molecular cloning techniques were used throughout this study (1, 45). All strains and plasmids used are listed in Table 1, and all oligonucleotide primers are listed in Table 2. *E. coli* strains were grown at 37°C with Luria-Bertani medium (45). NGR234 and its derivatives were grown at 27°C with TY medium (3) or rhizobial minimal medium supplemented with succinate (RMS) (7). Ampicillin (Ap), gentamicin (Gm), kanamycin (Km), rifampin (Rif), and spectinomycin (Sp) were added at concentrations of 100, 20 (10 for *E. coli*), 50, 100, and 50 μg/ml, respectively. Where appropriate, the flavonoid apigenin was added at 10^{-6} M to induce NGR234 strains.

Mutation of *acpXL* and *bacA*. To delete *acpXL*, a 1.4-kb fragment containing *acpXL* and its flanking regions was amplified by PCR from NGR234 genomic DNA, using primers $acpXL_{for}$ and $acpXL_{rev}$ (Table 2), and cloned into pBluescript II KS(+) to create pKS-*acpXL*. A PCR using primers $acpXL_{delUP}$ and $acpXL_{delDOWN}$, with pKS-*acpXL* as a template, resulted in a PCR product deleted of *acpXL*. This product was digested with EcoRI and then circularized to create pKS-$\Delta acpXL$. The accuracy of the PCR and the *acpXL* deletion were verified by sequencing of this plasmid. The 1.1-kb fragment containing the *acpXL* deletion was subcloned from pKS-$\Delta acpXL$ into the suicide vector pK18*mobsacB* (46) by use of BamHI and HindIII, generating pK18-$\Delta acpXL$.

To mutate *bacA*, a 1.4-kb fragment containing *bacA* was amplified by PCR from genomic DNA, using primers $bacA_{for}$ and $bacA_{rev}$ (Table 2), digested with BamHI and XhoI, and cloned into the corresponding sites of pJQ200SK+ to create pJQ-*bacA*. An Ω cassette conferring resistance to spectinomycin was inserted into the unique SmaI site located within the cloned *bacA* open reading frame (ORF), generating pJQ-*bacA*ΩSp.

Triparental matings using the helper plasmid pRK2013 (20) were used to transfer the resulting suicide plasmids, pK18-$\Delta acpXL$ and pJQ-*bacA*ΩSp, into NGR234. Mutants with double recombination were selected by plating bacteria onto TY plates containing 5% sucrose and appropriate antibiotics. Putative mutants were confirmed by PCR and Southern blots of digested DNA, using standard procedures.

Transcriptional analyses with promoter-green fluorescent protein (GFP) fusions. The promoter regions of *bacA* and *acpXL* were amplified by PCR from genomic DNA of NGR234, using the primers $bacA_{promfor}$ and $bacA_{promrev}$ for *bacA* and $acpXL_{for}$ and $acpXL_{promrev}$ for *acpXL* (Table 2). The fragments obtained were cloned into pBluescript II KS(+) and sequenced to verify PCR fidelity. The promoter regions were then subcloned into the broad-host-range promoter probe vector pPROBE-GT' (37). Plasmids were mobilized from *E. coli* DH5α into NGR234 and mutant derivatives by triparental matings, using the helper plasmid pRK2013 (20).

Bacterial strains carrying pPROBE constructs were grown in RMS supplemented with the appropriate antibiotics for 48 h. These precultures were used to inoculate 10 ml of RMS to an optical density at 600 nm (OD$_{600}$) of 0.1; to test gene induction by flavonoids or stress conditions, apigenin (10^{-6} M), polymyxin B (0.1 or 0.5 μg/ml), or NaCl (1% [wt/vol]) was added when appropriate. The optical density (600 nm) and fluorescence (excitation filter at 485 nm and emission filter at 528 nm) from 100-μl aliquots were recorded at 0, 6, 24, 48, and 72 h postinoculation, using a Synergy 2 multimode microplate reader (BioTek Instruments, Winooski, VT). At least three transcriptional assays were performed for each strain, and the fluorescence values obtained were normalized to the average optical density at each time point.

Nodulation tests. Legume seeds were obtained from the suppliers listed by Pueppke and Broughton (40). Nodulation tests were performed in Magenta jars as described by Skorpil et al. (48a). Plants were grown at a day temperature of

TABLE 3. Homology of translated ORFs in the *bacA* loci of various rhizobia

Rhizobium NGR234 ORF (no. of aa encoded)	Putative function of encoded protein	*S. meliloti* 1021 ORF (no. of aa encoded)	% Identity (% similarity)	*R. leguminosarum* 3841 ORF (no. of aa encoded)	% Identity (% similarity)	*R. etli* CFN42 ORF (no. of aa encoded)	% Identity (% similarity)	*M. loti* MAFF303099 ORF (no. of aa encoded)	% Identity (% similarity)
c23790 (257)	Methionine aminopeptidase (Map2)	SMb21002 (249)	92 (95)	RL3560 (252)	75 (88)	RHE_CH03116 (252)	74 (87)		
c23780 (414)	Major facilitator superfamily (MSF) protein	SMb21000 (377)	84 (88)	RL3558 (404)	59 (73)	RHE_CH03113 (372)	58 (71)	mlr7400 (401)	49 (65)
bacA (378)	Cell envelope modification; peptide uptake	*bacA* (420)	60 (75)	*bacA* (421)	65 (79)	*bacA* (421)	64 (77)	mlr7399 (419)	53 (69)
c23760 (334)	Hypothetical protein	SMb20998 (334)	83 (93)	RL3556 (328)	62 (75)	RHE_CH03111 (329)	60 (74)	mlr7402 (335)	53 (67)
c23750 (192)	Putative transmembrane protein	SMb20997 (194)	85 (93)	RL3555 (194)	60 (77)	RHE_CH03110 (194)	62 (78)		

28°C and a night temperature of 18°C, with a photoperiod of 16 h. Two plants were grown per Magenta jar, and each was inoculated with 10^7 bacteria. At harvest (6 weeks after inoculation for all plants, with the exception of *Leucaena leucocephala*, which was harvested at 7 weeks postinoculation), the aerial portion of the plant was removed and its dry weight recorded. Functional nodules were identified visually by their pink coloration due to the presence of leghemoglobin, an indicator of nitrogenase activity. The total number of active (pink) nodules and their fresh weight were determined for each replicate.

Polysaccharide analysis by SDS-PAGE and silver staining. Strains were grown in RMS for 40 h and supplemented with 10^{-6} M apigenin where required. Polysaccharides were obtained from cells collected by centrifuging 4 ml of culture, as described previously (25). Briefly, the cell pellets were resuspended in 30 μl lysis buffer (1 M Tris-HCl [pH 6.8], 2% [wt/vol] SDS, 4% [vol/vol] β-mercaptoethanol, 10% [vol/vol] glycerol, 0.03% [wt/vol] bromophenol blue) and boiled for 10 min. Lysed cells were treated with 10 μl protease K (2.5 mg/ml) at 60°C for 1 h, and then the samples were diluted by adding 80 μl of sample buffer (120 mM Tris-HCl [pH 6.8], 3% [wt/vol] SDS, 9% [vol/vol] β-mercaptoethanol, 30% [vol/vol] glycerol, 0.03% [wt/vol] bromophenol blue). Polysaccharides were separated by SDS-PAGE (18% acrylamide), and the gels were stained specifically for LPS or capsular polysaccharides (KPS) as described previously (31).

LPS extraction and analysis of hydroxy fatty acids. Strains were grown in 1 liter of RMS for 40 h. Cells were harvested by centrifugation at 5,000 × g for 15 min at 4°C and then washed twice with 1% (wt/vol) NaCl and twice with distilled H_2O. Pellets were resuspended in 20 ml H_2O, heated to 65°C, and then mixed with the same volume of hot phenol (at 65°C). Samples were incubated at 65°C for 15 min, and then the water and phenol phases were separated by centrifugation at 13,000 rpm for 15 min at 10°C. A second extraction with the same volume (20 ml) of H_2O was performed, and the two water phases were combined. The combined water phases were then dialyzed against H_2O and lyophilized. To determine the content of lipid A hydroxy fatty acids, this material was incubated at 80°C for 16 h in methanolic 1 M HCl, followed by trimethylsilylization of the hydroxyl groups (53). The resulting derivatives were analyzed by gas chromatography with a 30-m SPB-1 column (Supelco; Sigma-Aldrich, St. Louis, MO). After injection, the oven temperature was raised 2°C min⁻¹ from 150 to 180°C, 1°C min⁻¹ from 180 to 200°C, and 5°C min⁻¹ from 200 to 305°C and then maintained at 305°C for 25 min. The identity and relative molar response of each fatty acid were determined by comparison to parallel analyses of purified LPS of *Salmonella enterica* and *R. etli* strains whose lipid A structures and average compositions are known.

Sensitivity assays. To test membrane integrity, bacteria were grown in liquid RMS for 48 h. The cells were then centrifuged, washed, and resuspended in fresh RMS to an OD_{600} of 0.2. For the filter disk assay, 500 μl of the resuspended culture was spread onto RMS plates. After 30 min, a paper disk (6-mm diameter; bioMérieux, Nürtingen, Germany) was applied to the center of the plate, and 5 μl of 10% (wt/vol) SDS was applied. At least three plates were prepared for each test. The plates were incubated for 72 to 96 h at 27°C, and then the diameters of the growth inhibition zones were recorded. For the DOC assay, RMS plates containing 0 mM, 2 mM, or 10 mM DOC were prepared, and 20 μl of culture was streaked for each strain to be tested. The plates were incubated at 27°C for 72 h. To test the sensitivity of strains to polymyxin B, the strains were precultured for 48 h in RMS and then washed and resuspended to an OD_{600} of 0.05 in RMS containing polymyxin B at different final concentrations. Growth was monitored by measuring the OD_{600} for the next 72 to 96 h.

To test the sensitivity of NGR234 to Bac7, strains were grown in liquid TY to early stationary phase and then diluted to an OD_{600} of 0.05 in fresh TY medium containing Bac7 at different final concentrations. Growth was monitored by measuring the OD_{600} for the next 72 to 96 h. The tests were repeated at least three times for each strain and stress condition.

RESULTS

Identification and mutation of *acpXL* and *bacA* in NGR234. Homology searches using *bacA* of *S. meliloti* 1021 located a single *bacA* locus on the chromosome of NGR234 (Fig. 1A). *bacA* of NGR234 encodes a predicted protein of 378 amino acids belonging to a subfamily of the ABC transporter family (SbmA-BacA). The *bacA* loci from *S. meliloti* 1021, *R. leguminosarum* bv. *viciae* 3841, *R. etli* CFN42, *Bradyrhizobium japonicum* USDA 110, and *Mesorhizobium loti* MAFF303099 and the homology (to NGR234) of proteins encoded by the ORFs are

TABLE 4. Homologies of translated ORFs in the *acpXL* loci of various rhizobia

Rhizobium NGR234 ORF (no. of aa encoded)	Putative function of encoded protein	S. meliloti 1021		R. leguminosarum 3841		R. etli CFN42		B. japonicum USDA110		M. loti MAFF303099	
		ORF (no. of aa encoded)	% Identity (% similarity)	ORF (no. of aa encoded)	% Identity (% similarity)	ORF (no. of aa encoded)	% Identity (% similarity)	ORF (no. of aa encoded)	% Identity (% similarity)	ORF (no. of aa encoded)	% Identity (% similarity)
c18120 (326)	Cobalamin biosynthesis protein	*cobD* (327)	78 (86)	*cobD* (326)	65 (73)	*cobD* (326)	63 (73)				
c18110 (264)	Hypothetical protein (linked to cobalamin synthesis?)										
c18100 (246)	Transcriptional regulator (Fnr/CRP family)			*fnrN* (240)	62 (80)	*fnrN* (239)	64 (81)	blr3814 (282)	24 (44)		
c18090 (113)	Hypothetical protein										
acpXL c18080 (92)	Acyl carrier protein	*acpXL* (95)	98 (100)	*acpXL* (92)	95 (97)	*acpXL* (92)	95 (97)	*acpP* (96)	76 (90)	mlr1174 (93)	82 (92)
c18070 (158)	Hydroxy-myristoyl acyl carrier protein dehydratase	SMc04277 (158)	90 (95)	*fabZ* (160)	80 (87)	RHE_CH2477 (160)	81 (87)	bll3810 (156)	48 (63)		
c18060 (399)	Beta-ketoacyl-ACP synthase II	SMc04275 (400)	90 (95)	*fabF2* (401)	73 (84)	RHE_CH2476 (401)	73 (84)	*fabF* (401)	44 (62)	mlr1176 (396)	55 (67)
c18050 (427)	Beta-ketoacyl-ACP synthase II	SMc04273 (427)	96 (98)	*fabF1* (428)	85 (91)	RHE_CH2475 (428)	86 (92)	bll3808 (425)	58 (72)	mlr1177 (416)	67 (79)
c18040 (342)	Alcohol dehydrogenase protein	SMc04273 (342)	95 (98)	RL2813 (342)	93 (97)	RHE_CH2474 (342)	94 (97)			mlr1178 (342)	84 (92)
lpxXL (c18030) (309)	Acyltransferase	*lpxXL* (312)	91 (94)	RL2812 (311)	74 (86)	*msbB* (288)	77 (86)	*msbB* (310)	38 (56)	mlr1179 (328)	59 (76)

shown in Fig. 1A and Table 3, respectively. The *bacA* region is well conserved in the rhizobia we examined, with the exception of *B. japonicum* USDA110 (Fig. 1A and Table 3). Despite this conservation, predicted gene functions give little insight into any possible symbiotic roles of the proteins encoded by this locus (Table 3). To mutate *bacA*, an ΩSpec cassette was inserted into the unique SmaI site of *bacA* (see Materials and Methods) to create NGRΩ*bacA*.

We also mutated *acpXL* to create a VLCFA-negative strain to use as a comparison with NGRΩ*bacA*. The *acpXL-lpxXL* locus of NGR234 was identified by homology searches using *acpXL* and *lpxXL* of *S. meliloti* 1021 (Fig. 1B). The region between *acpXL* and *lpxXL* includes four other ORFs that are also (based upon their homologies) probably involved in lipid A synthesis (Table 4). *acpXL-lpxXL* loci show preserved synteny, and the ORFs therein are highly conserved among different rhizobia, whereas the region upstream of *acpXL* is variable (Fig. 1B and Table 4). An in-frame deletion of *acpXL* was created, generating NGRΔ*acpXL* (see Materials and Methods).

Measurement of *acpXL* and *bacA* expression in response to flavonoids. To determine whether the expression of *acpXL* and *bacA* is regulated by flavonoids, the promoter regions of the two genes were cloned upstream of GFP in the broad-host-range vector pPROBE-GT′ (see Materials and Methods). Fluorescence of NGR234 strains carrying the promoter-GFP fusions was measured in the presence or absence of flavonoids. The promoter region of a gene (*fixF*) similarly cloned upstream

FIG. 2. Measurement of *bacA* and *acpXL* expression. Expression analyses were performed with the *bacA* (A), *acpXL* (B), and *fixF* (C) promoter regions. The promoter regions were fused to a promoterless *gfp* gene and assayed at different time points in RMS and RMS supplemented with 10^{-6} M apigenin. The values for the empty vector control were subtracted from the actual readings, and data were normalized according to the OD_{600}. Numbers shown are the averages for three replicate experiments.

TABLE 5. Nodulation of *Lablab purpureus*, *Leucaena leucocephala*, *Tephrosia vogelii*, and *Vigna unguiculata* by NGR234 and derivative mutants

Plant (nodule type)[a] and strain	Mean ± SD[b]		
	No. of nodules	Nodule wt (mg)	Dry wt (mg)
Lablab purpureus (D)			
Control	0	0	700 ± 190
NGR234	10 ± 4	520 ± 160	1,020 ± 280
NGRΔ*acpXL*	0* ± 0	0* ± 0	650* ± 140
NGRΩ*bacA*	10 ± 5	450 ± 220	1,020 ± 350
Leucaena leucocephala (I)			
Control	0	0	90 ± 15
NGR234	15 ± 3	140 ± 30	240 ± 40
NGRΔ*acpXL*	11 ± 3	130 ± 20	230 ± 50
NGRΩ*bacA*	13 ± 2	140 ± 60	240 ± 90
Tephrosia vogelii (I)			
Control	0	0	150 ± 30
NGR234	5 ± 4	200 ± 110	260 ± 100
NGRΔ*acpXL*	0* ± 0	0* ± 0	150* ± 30
NGRΩ*bacA*	4 ± 2	120 ± 80	200 ± 70
Vigna unguiculata (D)			
Control	0	0	90 ± 20
NGR234	80 ± 18	740 ± 150	1,320 ± 270
NGRΔ*acpXL*	0* ± 0	0* ± 0	60* ± 10
NGRΩ*bacA*	85 ± 28	820 ± 130	1,240 ± 350

[a] D, determinate nodules; I, indeterminate nodules.
[b] The figures listed are per plant. Only pink (i.e., functional) nodules were scored. Nodulation tests were repeated at least twice, with 6 to 8 plants being tested each time. Statistical analyses (Student's *t* test) were performed to compare each mutant to the wild-type strain. *, significant difference ($P \leq 0.01$).
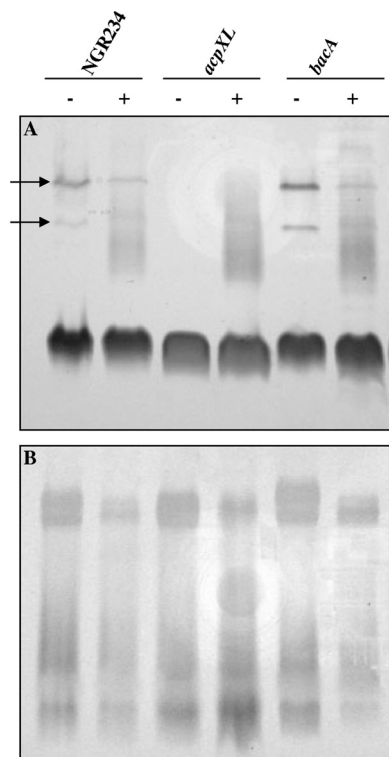
FIG. 3. LPS and KPS profiles of NGR234 and its mutants. SDS-PAGE analysis was performed to detect LPS (A) and KPS (B) synthesized by NGR234 and the *acpXL* and *bacA* mutants grown in the absence (−) or presence (+) of apigenin. The arrows indicate the low-mobility bands present in noninduced samples from NGR234 and NGRΩ*bacA* that are absent in NGRΔ*acpXL*.

FIG. 4. Detection of VLCFA in NGRΔ*acpXL* and NGRΩ*bacA*. Gas chromatograms for NGRΔ*acpXL* (A) and NGRΩ*bacA* (B) LPS extracts show where 27OH-C28:0 emerges and, as a loading control, where 3OH-C18:0 peaks. The relative absence of 27OH-C28:0 in NGRΔ*acpXL* is indicated by the arrow in panel A.

of GFP in pPROBE-GT′ (and known to be induced by flavonoids) was used as a positive control. Expression of both *acpXL* and *bacA* was low and constitutive (Fig. 2). Fluorescence of each mutant was also measured under stressful conditions (osmotic stress or polymyxin B), but no changes in expression were observed for either gene (data not shown).

Nodulation tests. To investigate their symbiotic proficiency, the *acpXL* and *bacA* mutants were inoculated onto various plants belonging to different tribes and forming determinate or indeterminate nodules. No symbiotic differences were observed between the *bacA* mutant and NGR234 after inoculation onto *Lablab purpureus* (Phaseoleae), *Leucaena leucocephala* (Mimoseae), *Tephrosia vogelii* (Millettieae), or *Vigna unguiculata* (Phaseoleae) (Table 5). We tested more NGR234 host plants—*Cajanus cajan*, *Macroptilium atropurpureum*, *Pachyrhizus tuberosus* (all Phaseoleae), *Lotus japonicus* (Loteae), and *Crotalaria juncea* (Crotalarieae)—but again found that the *bacA* mutant caused no impairment in symbiosis with these legumes (relative to the wild type) (data not shown). In contrast, the *acpXL* mutant did not form functional (pink) nodules on *L. purpureus*, *T. vogelii*, or *V. unguiculata*, although numerous white (pseudo-)nodules were induced. *L. leucocephala* was efficiently nodulated by the *acpXL* mutant (Table 5), however.

LPS and KPS profiles. To check whether the production of rhamnan, an O-antigen species produced upon flavonoid in-
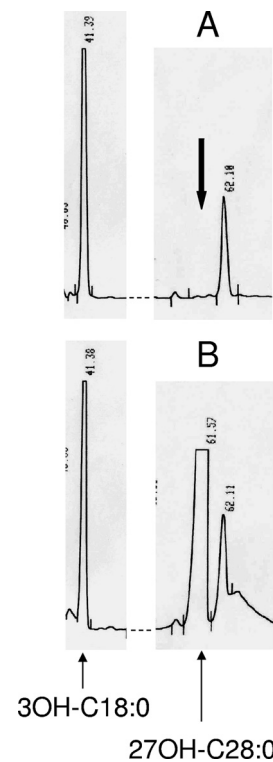
duction of NGR234, was affected in the mutant strains, all three (NGR234 as well as the *acpXL* and *bacA* mutants) strains were grown in minimal (RMS) medium (with or without flavonoids), and their polysaccharide (LPS and KPS) profiles were analyzed by SDS-PAGE (see Materials and Methods). LPS and KPS profiles of the *bacA* mutant were indistinguishable from those of NGR234 (Fig. 3). Similarly, the *acpXL* mutant showed the same KPS profile as NGR234 and made rhamnan in the presence of flavonoids, but the low-mobility bands usually seen in the absence of apigenin (indicated by arrows in Fig. 3A) were absent.

Fatty acid composition of LPS. Crude LPS extracts of NGR234, NGRΔ*acpXL*, and NGRΩ*bacA* were analyzed to determine the contents of hydroxy fatty acids characteristic of lipid A. Only traces of VLCFA (27-OH-28:0) were present in the *acpXL* mutant, but its content of 3-hydroxy fatty acids (Fig. 4 and Table 6) was similar to that of NGR234. No differences in the VLCFAs as well as the major 3-hydroxy fatty acids of NGRΩ*bacA* and NGR234 were found (Fig. 4 and Table 6).

Sensitivity assays. Bacterial mutants affected in LPS in general, and lipid A in particular, are generally sensitive to various stresses. As an example, agents that affect membranes, such as detergents or polymyxin B (an antimicrobial peptide which binds to LPS and permeabilizes the cell membrane), can be used to assay membrane integrity. The sensitivity of NGR234, NGRΔ*acpXL*, and NGRΩ*bacA* to SDS and DOC was tested on RMS plates. In the presence of detergents, the growth of

TABLE 6. Hydroxy fatty acid compositions of NGR234 and derivatives normalized to glucosamine

Strain	Relative amt of hydroxy fatty acid[a]			
	14:0	16:0	18:0	28:0
NGR234	2.09	0.35	0.63	0.81
NGRΔacpXL	2.95	0.20	0.55	0.01
NGRΩbacA	2.82	0.30	0.50	0.83

[a] The relative molar contents of 3-hydroxymyristic acid (14:0), 3-hydroxypalmitic acid (16:0), 3-hydroxystearic acid (18:0), and 27-hydroxyoctacosanoic acid (28:0) per 2.0 mol of glucosamine are shown.

the *acpXL* mutant was significantly inhibited compared to that of NGR234, whereas the *bacA* mutant was not affected (Fig. 5). Sensitivity to polymyxin B was tested in liquid cultures. In the presence of 0.25 μg/ml polymyxin B, the growth of the *acpXL* mutant was significantly slower than the growth of NGR234, and it stopped completely at concentrations of ≥0.5 μg/ml, while the growth of NGR234 was only slightly reduced at these concentrations. As in the case of detergents, the *bacA* mutant displayed the same phenotype as NGR234 (Fig. 6).

BacA as a peptide transporter. BacA of *S. meliloti* is involved in peptide transport, and a *bacA* mutant is more resistant than the parent strain to the antimicrobial peptide Bac7 (which has been shown to inhibit DnaK as well as other unknown targets [47]) and to the glycopeptide antibiotic bleomycin (18, 26, 32). Since NGR234 is naturally resistant to bleomycin, this assay could not be used, but the *bacA* mutant was similarly resistant, implying that bleomycin resistance is expressed through a mechanism that does not involve BacA (data not shown). On the other hand, Bac7 inhibited the growth of NGR234, but NGRΩbacA was significantly less susceptible. The sensitivity of NGR234 and NGRΩbacA to Bac7 was tested by measuring the growth inhibition in liquid TY medium in the presence of different concentrations of Bac7 (Fig. 7). Furthermore, the sensitivity of the *acpXL* mutant to Bac7 was also tested, but this mutant showed the same phenotype as NGR234 (data not shown).

DISCUSSION

Our data show that *bacA* influences neither the VLCFA content nor the LPS of NGR234. This is in stark contrast to the *S. meliloti bacA* mutant, which induces only nonfunctional nodules and produces lipid A with reduced quantities of VLCFA. Since *S. meliloti acpXL* and *lpxXL* mutants lack VLCFA but remain symbiotically proficient (16, 17, 33), BacA must have other functions critical for chronic infection in nodules. BacA of *S. meliloti* is involved in the uptake of synthetic peptides (35). Thus, BacA may have a role in the recognition/transport of plant peptides required for bacteroid differentiation within the indeterminate nodules formed specifically by galegoid legumes (e.g., *Medicago* and *Vicia* species), but not with phaseoloid legumes forming determinate nodules (30, 51). BacA is important in the symbiosis between *Mesorhizobium huakuii* 7653R and *Astragalus sinicus*, which also belongs to the Galegeae. An *M. huakuii* 7653R *bacA* mutant is defective in nitrogen fixation during symbiosis. The same mutant under free-living conditions is also sensitive to cell envelope-disrupting agents, is resistant to bleomycin and has reduced amounts
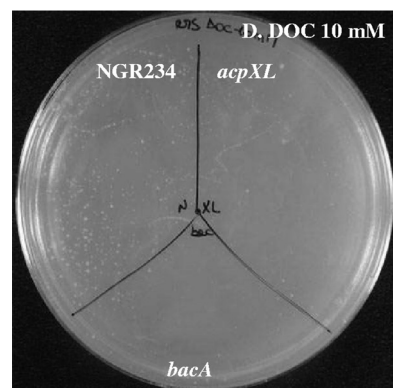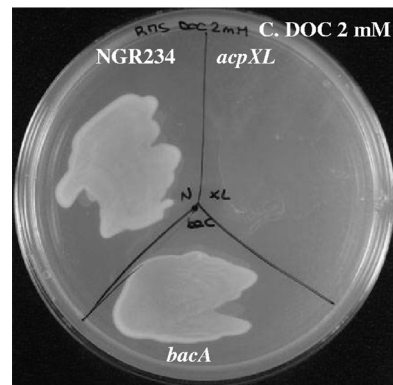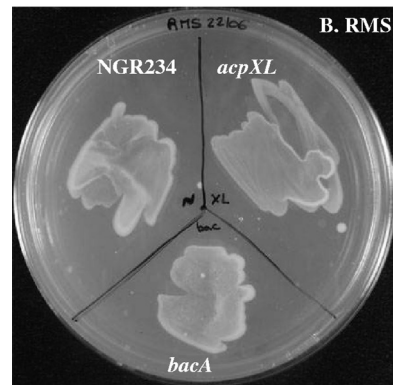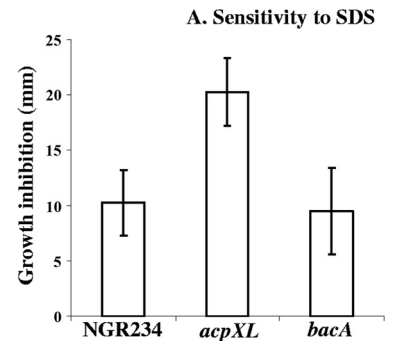

FIG. 5. Sensitivity to detergents. (A) NGR234, NGRΔacpXL, and NGRΩbacA were plated on RMS agar, and 5 μl of 10% (wt/vol) SDS was added on a paper disk. The diameters of growth inhibition were recorded after 72 to 96 h at 27°C. (B to D) Sensitivities of NGR234, NGRΔacpXL, and NGRΩbacA to RMS (B), RMS + 2 mM DOC (C), and RMS + 10 mM DOC (D).
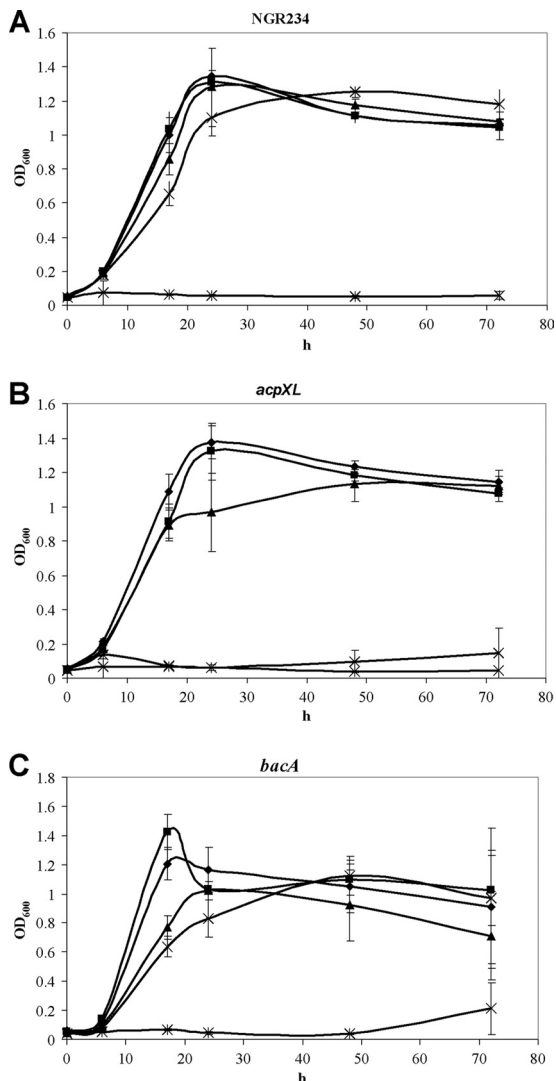
FIG. 6. Sensitivity to polymyxin B. Growth of NGR234 (A), NGRΔ*acpXL* (B), and NGRΩ*bacA* (C) was measured in liquid RMS (◆) and RMS supplemented with polymyxin B at different final concentrations (■, 0.1 μg/ml; ▲, 0.25 μg/ml; ×, 0.5 μg/ml; ∗, 1 μg/ml). The values shown are the averages for three replicate experiments.

FIG. 7. NGRΩ*bacA* has increased resistance to Bac7. Growth was measured for NGR234 (A) and the *bacA* mutant NGRΩ*bacA* (B) in liquid TY containing different concentrations of Bac7. The graphs represent the means (± SD) for three different experiments.

of VLCFA in its lipid A (50). *R. leguminosarum* bv. *phaseoli* and *R. etli bacA* mutants display similar phenotypes in their free-living states, but they are able to establish functional symbioses (30). Our data show that BacA of NGR234 might be involved uniquely in peptide uptake (but not to permit bacteroid differentiation) and has no role in VLCFA synthesis or transport.

The NGR234 *acpXL* mutant did not possess VLCFA in its lipid A. Although we cannot rule out the possibility that the nature of the mutation affected downstream genes, this absence of VLCFA had detrimental effects on the ability of this strain to nodulate several legumes. The fact that the NGR234 *acpXL* mutant was not able to nodulate most of the plants tested was also unexpected, since *acpXL* mutants of *S. meliloti* and *R. leguminosarum*, although less efficient, still induce functional nodules (17, 48, 53). In the case of *R. leguminosarum*, the
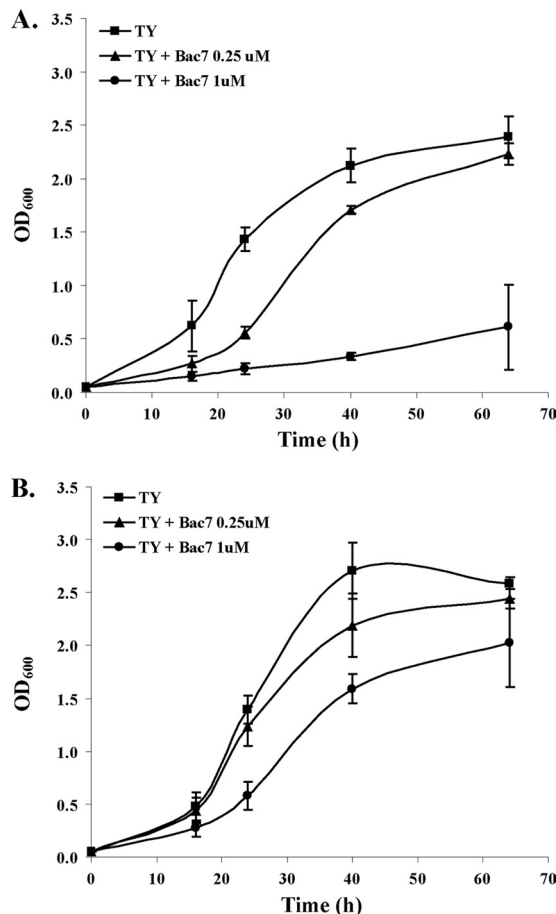
acpXL mutant lacks VLCFA *in vitro*, but lipid A extracted from *acpXL* mutant-containing bacteroids of *R. leguminosarum* possessed VLCFA. This suggests that in this strain, there is another *acp* gene that is activated during symbiosis which is able to replace AcpXL function and add VLCFA to lipid A (52). One possible reason for the disparity in phenotypes between the NGR234 and *R. leguminosarum acpXL* mutants is that NGR234 does not possess functional *acp* homologues.

Why is VLCFA important for symbiosis, and particularly so for NGR234, even though expression of *acpXL* in NGR234 is independent of flavonoids? Possible explanations were revealed by studying the *acpXL* mutant under free-living conditions. The absence of VLCFA compromises membrane integrity, as shown by the mutant's heightened sensitivities to membrane-disrupting agents (detergents and polymyxin B). The fact that VLCFA is needed for maximal outer membrane stability is likely to be particularly acute during rhizobial uptake into plant cells as well as in rhizobial persistence within the cortical cells as bacteroids. At these stages of the symbiosis, rhizobia, especially at their outer surfaces, are subjected to osmotic and possibly oxidative stresses as well as plant defense reactions. In a similar vein, it has also been suggested that VLCFA can span the

whole outer bacterial membrane, significantly contributing to its stability (53).

We have shown that BacA is not necessary for bacteroid development by NGR234 in nongalegoid legumes that form either determinate or indeterminate nodules. BacA is responsible for resistance to Bac7 but does not affect the VLCFA content of LPS or membrane stability. On the other hand, the presence of VLCFA in the lipid A of NGR234 is essential for successful symbiotic interactions with most plants tested.

ACKNOWLEDGMENTS

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