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) (19). 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 amino-glycoside 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).
Rhizobial lipid A contains a very-long-chain fatty acid (VLCFA; 27-OH-28:0), attached as the secondary fatty acid of an acyl carrier protein. VLCFA is synthesized by the inner membrane-associated LpxXL (2, 42, 43), which is important for symbiosis. For S. meliloti, lpxXL encodes a dedicated acyl carrier protein. VLCFA is synthesized by the inner membrane-associated LpxXL (2, 42, 43), which is important for symbiosis. For S. meliloti, lpxXL encodes a dedicated acyl carrier protein. VLCFA is synthesized by the inner membrane-associated LpxXL (2, 42, 43), which is important for symbiosis. For S. meliloti, lpxXL encodes a dedicated acyl carrier protein. VLCFA is synthesized by the inner membrane-associated LpxXL (2, 42, 43), which is important for symbiosis. For S. meliloti, lpxXL encodes a dedicated acyl carrier protein. VLCFA is synthesized by the inner membrane-associated LpxXL (2, 42, 43), which is important for symbiosis. For S. meliloti, lpxXL encodes a dedicated acyl carrier protein. VLCFA is synthesized by the inner membrane-associated LpxXL (2, 42, 43), which is important for symbiosis. For S. meliloti, lpxXL encodes a dedicated acyl carrier protein. VLCFA is synthesized by the inner membrane-associated LpxXL (2, 42, 43), which is important for symbiosis. For S. meliloti, lpxXL encodes a dedicated acyl carrier protein. VLCFA is synthesized by the inner membrane-associated LpxXL (2, 42, 43), which is important for symbiosis. For S. meliloti, lpxXL encodes a dedicated acyl carrier protein. VLCFA is synthesized by the inner membrane-associated LpxXL (2, 42, 43), which is important for symbiosis. For S. meliloti, lpxXL encodes a dedicated acyl carrier protein. VLCFA is synthesized by the inner membrane-associated LpxXL (2, 42, 43), which is important for symbiosis. For S. meliloti, lpxXL encodes a dedicated acyl carrier protein. VLCFA is synthesized by the inner membrane-associated LpxXL (2, 42, 43), which is important for symbiosis. For S. meliloti, lpxXL encodes a dedicated acyl carrier protein. VLCFA is synthesized by the inner membrane-associated LpxXL (2, 42, 43), which is important for symbiosis. For S. meliloti, lpxXL encodes a dedicated acyl carrier protein. VLCFA is synthesized by the inner membrane-associated LpxXL (2, 42, 43), which is important for symbiosis. For S. meliloti, lpxXL encodes a dedicated acyl carrier protein. VLCFA is synthesized by the inner membrane-associated LpxXL (2, 42, 43), which is important for symbiosis. For S. meliloti, lpxXL encodes a dedica
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

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).
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 µM to induce NGR234 strains.

**Mutation of acp XL and bacA.** To delete acp XL, a 1.4-kb fragment containing acp XL and its flanking regions was amplified by PCR from NGR234 genomic DNA, using primers acp XLdelUP and acp XLdelDOWN (Table 2), and cloned into pBluescript II KS(−) (46) to create pKS-acp XL delDOWN, with pKS-acp XL as a template, resulted in a PCR product deleted of acp XL. This product was digested with EcoRI and then circularized to create pKS-Dacp XL. The accuracy of the PCR and the acp XL deletion were verified by sequencing of this plasmid. The 1.1-kb fragment containing the acp XL deletion was subcloned from pKS-Dacp XL into the suicide vector pK18mobHB (46) by use of BamHI and HindIII, generating pK18-DacpXL.

To mutate bacA, a 1.4-kb fragment containing bacA was amplified by PCR from genomic DNA, using primers bacA delUP and bacA delDOWN (Table 2), digested with BamHI and XhoI, and cloned into the corresponding sites of plpQ2005K to create pQO-bacA. An Ω cassette conferring resistance to spectinomycin was inserted into the unique Smal site located within the cloned bacA open reading frame (ORF), generating pQO-bacA ΔSp.

Triparental matings using the helper plasmid pRK2013 (20) were used to transfer the resulting suicide plasmids, pK18-DacpXL and pQO-bacA ΔSp, 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 primers acp XL/H9004 and BacA/H9024, to verify genetic integration of the transgene into the genome of NGR234 strains.

**Recombination reporter assay.** Transcriptional analyses with promoter-GFP fusions. The promoter regions of bacA and acp XL were amplified by PCR from genomic DNA of NGR234, using the primers bacA promrev and bacA promf for bacA and acp XL promrev and acp XL promf for acp XL (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-GFP (37). Plasmids were mobilized from E. coli DH5α into NGR234 and mutant derivatives by triparental matings, using the helper plasmid pRK2013 (20).

**Bacterial strains.** 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 (OD600) of 0.1, to test gene induction by flavonoids or stress conditions, apigenin (10 µM), 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 25°C and a night temperature of 20°C. At least three independent inoculations were performed for each strain, and the number of nodules per plant was scored 6 weeks postinoculation.
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 agpin in which 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 Tri-HCl [pH 6.8], 2% [wt/vol] SDS, 4% [vol/vol] β-mercaptoethanol, 10% [vol/vol] glycerol, 0.05% [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 Tri-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 with 0.1% (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 rpm for 15 min at 4°C and then washed twice with 1% (wt/vol) NaCl and twice with distilled H₂O. Pellets were resuspended in 20 ml H₂O, heated at 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 H₂O was performed, and the two water phases were combined. The combined water phases were then dialyzed against H₂O 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 trimethylsilylation 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 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 OD₆₀₀ of 0.2. For the filter disk assay, 500 μl of culture was spread onto RMS plates. After 30 min, a paper disk (6-mm diameter; bioMérieux, Nu¨rtingen, 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 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 incubated at 80°C for 16 h in methanolic 1 M HCl, followed by trimethylsilylation 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 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.

**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* CNFN42, *Bradyrhizobium japonicum* USDA 110, and *Mesorhizobium loti* MAFF303099 and the homology (to NGR234) of proteins encoded by the ORFs are

| Table 4: Homology of translated ORFs in the acpYL locus of various rhizobia. | ORF (no. of aa encoded) | % Identity | % Similarity | ORF (no. of aa encoded) | % Identity | % Similarity | ORF (no. of aa encoded) | % Identity | % Similarity |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **bacA** | 378 | 65 (73) | 56 (62) | **bacA** | 378 | 65 (73) | 56 (62) | **bacA** | 378 | 65 (73) | 56 (62) |
| **bacA** | 378 | 65 (73) | 56 (62) | **bacA** | 378 | 65 (73) | 56 (62) | **bacA** | 378 | 65 (73) | 56 (62) |
| **bacA** | 378 | 65 (73) | 56 (62) | **bacA** | 378 | 65 (73) | 56 (62) | **bacA** | 378 | 65 (73) | 56 (62) |
| **bacA** | 378 | 65 (73) | 56 (62) | **bacA** | 378 | 65 (73) | 56 (62) | **bacA** | 378 | 65 (73) | 56 (62) |
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| **bacA** | 378 | 65 (73) | 56 (62) | **bacA** | 378 | 65 (73) | 56 (62) | **bacA** | 378 | 65 (73) | 56 (62) |
| **bacA** | 378 | 65 (73) | 56 (62) | **bacA** | 378 | 65 (73) | 56 (62) | **bacA** | 378 | 65 (73) | 56 (62) |
| **bacA** | 378 | 65 (73) | 56 (62) | **bacA** | 378 | 65 (73) | 56 (62) | **bacA** | 378 | 65 (73) | 56 (62) |
| **bacA** | 378 | 65 (73) | 56 (62) | **bacA** | 378 | 65 (73) | 56 (62) | **bacA** | 378 | 65 (73) | 56 (62) |
| **bacA** | 378 | 65 (73) | 56 (62) | **bacA** | 378 | 65 (73) | 56 (62) | **bacA** | 378 | 65 (73) | 56 (62) |
| **bacA** | 378 | 65 (73) | 56 (62) | **bacA** | 378 | 65 (73) | 56 (62) | **bacA** | 378 | 65 (73) | 56 (62) |
three replicate experiments. supplemented with 10 erless gfp (C) promoter regions. The promoter regions were fused to a promot-

analyses were performed with the bacA 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 NGRΔacpXL (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

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 ÕSpec cassette was inserted into the unique SmaI site of bacA (see Materials and Methods) to create NGRΔbacA.

We also mutated acpXL to create a VLCFA-negative strain to use as a comparison with NGRΔbacA. 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 NGRΔacpXL (see Materials and Methods).

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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 promot-

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

Plant (nodule type) and strain & No. of nodules & Nodule wt (mg) & Dry wt (mg)

<table>
<thead>
<tr>
<th>Plant (nodule type) and strain</th>
<th>No. of nodules</th>
<th>Nodule wt (mg)</th>
<th>Dry wt (mg)</th>
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<tr>
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<tr>
<td>Control</td>
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<td>700 ± 190</td>
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<tr>
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<td>1,020 ± 280</td>
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<td>0* ± 0</td>
<td>650* ± 140</td>
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<tr>
<td>NGRΩbacA</td>
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<td>450 ± 220</td>
<td>1,020 ± 350</td>
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<td>240 ± 40</td>
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<tr>
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<td>230 ± 50</td>
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<td>240 ± 90</td>
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<td>Tephrosia vogelii (I)</td>
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</tr>
<tr>
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<td>4 ± 2</td>
<td>120 ± 80</td>
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<tr>
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<td>85 ± 28</td>
<td>820 ± 130</td>
<td>1,240 ± 350</td>
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</table>

* D, determinate nodules; I, indeterminate nodules.

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 ≤ 0.01).
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 induction 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, NGR/H9004 acpXL, and NGR/H9024 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 NGR/H9024 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, NGR/acpXL, and NGR/bacA to SDS and DOC was tested on RMS plates. In the presence of detergents, the growth of...
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

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>14:0</th>
<th>16:0</th>
<th>18:0</th>
<th>28:0</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGR234</td>
<td>2.09</td>
<td>0.35</td>
<td>0.63</td>
<td>0.81</td>
</tr>
<tr>
<td>NGRΔacpXL</td>
<td>2.95</td>
<td>0.20</td>
<td>0.55</td>
<td>0.01</td>
</tr>
<tr>
<td>NGRΔbacA</td>
<td>2.82</td>
<td>0.30</td>
<td>0.50</td>
<td>0.83</td>
</tr>
</tbody>
</table>

* 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.
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.

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