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Characterization of a Gene Family of Outer Membrane Proteins (*ropB*) in *Rhizobium leguminosarum* bv. *viciae* VF39SM and the Role of the Sensor Kinase ChvG in Their Regulation▽

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The outer membrane of Gram-negative bacteria represents the interface between the bacterium and its external environment. It has a critical role as a protective barrier against harmful substances and is also important in host-bacteria interactions representing the initial physical point of contact between the host cell and bacterial cell. RopB is a previously identified outer membrane protein from *Rhizobium leguminosarum* bv. *viciae* that is present in free-living cells but absent in bacteroids (H. P. Roest, I. H. Mulders, C. A. Wijffelman, and B. J. Lugtenberg, Mol. Plant Microbe Interact. 8:576–583, 1995). The functions of RopB and the molecular mechanisms of *ropB* gene regulation have remained unknown. We identified and cloned *ropB* and two homologs (*ropB2* and *ropB3*) from the *R. leguminosarum* VF39SM genome. Reporter gene fusions indicated that the expression of *ropB* was 8-fold higher when cells were grown in complex media than when they were grown in minimal media, while *ropB3* expression was constitutively expressed at low levels in both complex and minimal media. Expression of *ropB2* was negligible under all conditions tested. The use of minimal media supplemented with various sources of peptides resulted in a 5-fold increase in *ropB* expression. An increase in *ropB* expression in the presence of peptides was not observed in a *chvG* mutant background, indicating a role for the sensor kinase in regulating *ropB* expression. Each member of the *ropB* gene family was mutated using insertional mutagenesis, and the mutants were assayed for susceptibility to antimicrobial agents and symbiotic phenotypes. All mutants formed effective nodules on pea plants, and gene expression for each *rop* gene in bacteroids was negligible. The functions of *ropB2* and *ropB3* remain cryptic, while the *ropB* mutant had an increased sensitivity to detergents, hydrophobic antibiotics, and weak organic acids, suggesting a role for RopB in outer membrane stability.

The outer membrane of Gram-negative bacteria represents the interface between the bacterium and its external environment. Consequently, it plays a critical role as a protective barrier against the entry of harmful substances (39) and is also important in host-bacteria interactions since it is the initial point of contact between the host cell and bacterial cell. For instance, the lipopolysaccharide (LPS) of the outer membrane is important for establishing a successful host infection in numerous Gram-negative bacteria (e.g., review reference 25) and protecting the cell from toxic compounds (e.g., review references 32 and 33). Although extensive research has been conducted on the role of LPS during plant infection by rhizobia (10), other components of the outer membrane such as the outer membrane proteins (OMPs) are relatively uncharacterized in *Rhizobium*.

A major protein component of the outer membrane of *Rhizobium leguminosarum* is the group II OMP RopB (11, 37). This OmpA-like protein is present in the outer membrane of free-living bacteria but is not found in membrane fractions of bacteroids isolated from *Pisum sativum* nodules (11). The specific trigger causing the decrease of RopB in bacteroids has not been found (38), and no further study of *ropB* gene expression in *R. leguminosarum* has been published to date. However, genes homologous to *ropB* have been studied in other members of the Rhizobiales, including *aopB* in *Agrobacterium tumefaciens* (20, 26), the *omp25-omp31* family in *Brucella* spp. (7, 28, 42, 48), and *ropB1* and *ropB2* in *Sinorhizobium meliloti* (3, 6). Mutations in some of these *ropB* homologs have affected host-microbe interactions. An *aopB* mutant of *A. tumefaciens* is attenuated in tumor formation when inoculated onto plants (20). A *Brucella abortus omp25* mutant is equally infective as the wild type, but after being internalized into host cells, the mutant is more sensitive to intracellular destruction (28). A *ropB1* mutant of *S. meliloti* exhibits a Fix^+ phenotype but is less competitive in nodulating alfalfa when coinoculated with the wild type (6). Some of these OMPs have also been implicated in outer membrane integrity. For example, a *S. meliloti ropB1* mutant is more sensitive to the detergent sodium deoxycholate (DOC) (6).

The regulation of OMP gene expression in the *Rhizobiales* has been best characterized in *A. tumefaciens* and *B. abortus*. Expression of *aopB* increases under acidic conditions, and this regulation is controlled through the two-component signal transduction system ChvI/ChvG (26). A homologous two-component signal transduction system in *B. abortus*, named BvrR/BvrS, regulates OMP gene expression, including that of *omp25* (16, 42).

It is not uncommon for rhizobial genomes to contain multiple *ropB*-like genes (3, 6, 7, 52). For example, four *ropB*

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference(s) or source
Strains		
E. coli		
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*argF-lacZYA*)*U169* ϕ80*lacZ* ΔM15	Invitrogen
S17-1	RP4 *tra* region, mobilizer strain, Sp^r	41
TOP10	F^- *mcr*A Δ(*mrr-hsdRMS-mcrBC*)ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139 galU galK* Δ(*ara-leu*)*7697 rpsL* (Str^r) *endA1 nupG*	Invitrogen
R. leguminosarum		
VF39SM	Biovar *viciae*, spontaneous streptomycin-resistant mutant of VF39, Sm^r	34
3841	Biovar *viciae*, JB300 derivative streptomycin resistant, genome sequenced	52
38EV27	3841 *fabF2 fabF1* mutant, deficient in 27-hydroxyoctacosanoate modified LPS	45
DF20	VF39SM, *chvG*ΩKm mutant, unable to grow on complex media, Sm^r Nm^r	This study
DF33	VF39SM, *ropB*ΩKm mutant, Sm^r Nm^r	This study
DF15	VF39SM, *ropB2*ΩKm mutant, Sm^r Nm^r	This study
DF42	VF39SM, *ropB3*ΩKm mutant, Sm^r Nm^r	This study
Plasmids		
pCRII-TOPO	TOPO TA cloning vector, Km^r Ap^r	Invitrogen
pCRS530	Vector containing CAS-GNm cassette, Ap^r Km^r	36
pDG71	Broad-host-range vector containing tryptophan promoter, Tc^r	14
pFus1par	Broad-host-range vector with promoterless *gusA* for transcriptional fusions, par-stabilized, Tc^r	36, 51
pHC41	IncP broad-host-range vector, Tc^r	9
pHP45ΩKm	Vector containing excisable Km^r gene cassette	12
pJQ200SK	Suicide vector, *mob*, *sacB*, Gm^r	35
pDF4	pFus1par with *ropB*::*gusA* transcriptional fusion, Tc^r	This study
pDF37	Broad-host-range vector pHC41 containing functional copy of *ropB*, Tc^r	This study
pDF38	Broad-host-range vector pDG71 containing functional copy of *chvG*, downstream of tryptophan promoter, Tc^r	This study
pDF40	pFus1par with *ropB2*::*gusA* transcriptional fusion, Tc^r	This study
pDF41	pFus1par with *ropB3*::*gusA* transcriptional fusion, Tc^r	This study

[a] Ap^r, Gm^r, Km^r, Nm^r, Sm^r, Sp^r, and Tc^r denote resistance to ampicillin, gentamicin, kanamycin, neomycin, streptomycin, spectinomycin, and tetracycline, respectively.

homologs are predicted in the genome of *R. leguminosarum* bv. *viciae* 3841 (52). Given the importance of OMPs in several of the *Rhizobiales* species, we were interested in further characterizing the genes in the *ropB* family. Insertional mutagenesis and *gusA* reporter gene transcriptional fusions were used to investigate the function and regulation of the *ropB* gene family members in *R. leguminosarum* bv. *viciae* VF39SM.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *R. leguminosarum* strains were grown on tryptone-yeast (TY) medium (5 g tryptone, 3 g yeast extract, 3.5 mM $CaCl_2$ per liter H_2O) or Vincent's minimal medium (VMM) (47) at 30°C, and *Escherichia coli* strains were grown on Luria-Bertani (LB) medium (40) at 37°C. Difco products supplied by Becton Dickinson were used for media preparation and included Bacto tryptone, Difco yeast extract, Difco casein digest, Bacto peptone, and Bacto soytone. When required, *R. leguminosarum* strains were grown in the presence of the following concentrations of antibiotics: gentamicin (Gm), 30 μg/ml; neomycin (Nm), 50 μg/ml; streptomycin (Sm), 500 μg/ml; spectinomycin (Sp), 100 μg/ml; and tetracycline (Tc), 5 μg/ml. *E. coli* strains were cultured in the following concentrations of antibiotics, where appropriate: ampicillin (Ap), 100 μg/ml; Gm, 15 μg/ml; kanamycin (Km), 50 μg/ml; Sp, 100 μg/ml; and Tc, 10 μg/ml.

Bioinformatic analysis. The genome sequence of *R. leguminosarum* 3841 (52) was used as a template to design PCR primers for the amplification of *ropB1*, *ropB2*, and *ropB3* genes from *R. leguminosarum* VF39SM. The Oligo program was used to select PCR primers. Previous genetic analysis indicated that the two genomes are very similar (15, 51). DNA sequencing to confirm the proper identity of the PCR amplicons was used. Amino acid sequences for proteins from rhizobial species were obtained from RhizoBase (http://bacteria.kazusa.or.jp/rhizobase/), and those for *Agrobacterium tumefaciens*, *Brucella melitensis*, and *Brucella abortus* were obtained from GenBank. Homologous sequences were analyzed using BLASTP (1). Protein sequences were aligned using the ClustalW program (24). NJplot was used to construct tree diagrams.

Conjugation. Insertional mutagenesis and reporter gene fusion constructs were mobilized from *E. coli* into *R. leguminosarum* using conjugation. Donor and recipient cells were cultured overnight. Conjugation was performed by mixing 0.5 ml of donor S17-1 cells (optical density at 600 nm [OD_{600}] of ~1.0) with 0.5 ml of recipient *R. leguminosarum* cells (OD_{600} of ~0.5) in a microcentrifuge tube. The mixture was pelleted by centrifugation at 9,632 × *g* for 5 min, the supernatant was removed, and the pellet was resuspended in 50 μl of sterile water. The cell suspension was spotted onto TY agar or VMM agar containing 0.5 mM proline and incubated at 30°C for 24 to 48 h, after which the cells were scraped off and resuspended in 1 ml of sterile water. A total of 100 μl of cells was plated on the appropriate selective media.

Insertional mutagenesis of *ropB*, *ropB2*, *ropB3*, and *chvG*. Insertional mutagenesis *via* allelic exchange was performed as described by Quandt and Hynes using the suicide vector pJQ200SK (35). Each gene to be mutated was amplified using PCR and cloned into the cloning vector pCRII-TOPO, according to the manufacturer's instructions (Invitrogen). The gene was inactivated by the insertion of an antibiotic cassette (12) into an internal restriction site. The inactivated gene was subsequently cloned into pJQ200SK and transformed into *E. coli* S17-1 cells for conjugation into *R. leguminosarum*. Putative double-crossover mutants were selected for growth on either TY or VMM containing the appropriate antibiotics and 5% (wt/vol) sucrose. Arising colonies were further screened for gentamicin sensitivity. Putative mutants found to be gentamicin sensitive were confirmed using Southern blotting and PCR analysis. The cloning details for construction of each individual construct are described below.

The primers RopBFor (GACGCCCTTGTAATGAGC) and RopBRev (CGC CAATCCCCCGAAAAC) were used to clone the *ropB* gene into the vector pCRII-TOPO. The construct pCRII::*ropB* was digested with BamHI/XhoI, and the fragment containing *ropB* was ligated into pJQ200SK. An internal SalI site

was used to interrupt *ropB* by inserting the ΩKm cassette from p1918ΩKm using SalI to create construct pDF33.

The *ropB2* mutant was created by cloning a 1.8-kb region containing the *ropB2* gene using the primers RopB2ProFor (5′-TCAAGACGCAGCAACTCAAGC A-3′) and RopB2R (5′-GCTATTATTTCATCGTGCCGACCC-3′) into pCRII-TOPO. The construct was digested with BamHI/XhoI, and the fragment containing *ropB2* was ligated into pJQ200SK. An internal SalI site was used to interrupt *ropB2* by inserting a ΩKm cassette excised from pHP45ΩKm to create the construct pDF15.

The construct used to mutate *ropB3* was created by amplifying the 5′ end of the *ropB3* gene using the PCR primers RopB3F5 (5′-CGGGAGCTCATGGCG AAGTATCAATCC-3′) and RopB3R5 (5′-CGGGATCCCGGTGCGTCATAA GAGGG-3′) and the 3′ end of the *ropB3* gene using the PCR primers RopB3F3 (5′-CGGGATCCCACGAGTGGAGGCATCAG-3′) and RopB3R3 (5′-CGGC TCGAGTGAGTGTCCAGTTCCGTG-3′). The 5′ fragment was digested with SstI/BamHI and ligated into similarly digested pJQ200SK. The resulting plasmid and the amplified 3′ fragment of the *ropB3* gene were then digested with BamHI/XhoI and ligated together. The *ropB3* gene was then interrupted with the ΩKm cassette from pHP45ΩKm at the internal BamHI site to create the construct pDF42.

The construct used to mutate *chvG* was created by cloning a 1.8-kb region containing the *chvG* gene into pCRII-TOPO using the PCR primers ChvGF (5′-TGTCGGAAATGTTGAGGGCT-3′) and ChvGR (5′-CGGTGATGTTTT CGGCTCTC-3′). The *chvG* region was removed by digesting it with BamHI/SphI and ligated into pBBRKG1 (15). The ΩKm cassette from pHP45ΩKm was inserted at an internal SstI site of *chvG* to interrupt it. pBBRKG1::*chvG*ΩKm was then digested with XbaI/SpeI, with the fragment containing *chvG*ΩKm ligated into pJQ200SK cut with XbaI to create pDF20.

Construction of *ropB*, *ropB2*, and *ropB3* promoter fusions. A transcriptional fusion for *ropB* was constructed by cloning the *ropB* promoter region into pFus1par. The primers RopBProFor (TCGAATTTCAGCCATGCGGC) and RopBProRev (AACGCCGTAAACGATCTGGC) were used to amplify a 442-bp fragment from VF39SM. This fragment included 169 bp upstream of the *ropB* start codon. This fragment was cloned into pCRII, according to the supplier's instructions, to create pCRII::*ropB*Pro. An EcoRI-digested fragment was then cloned into pFus1par in the proper orientation (verified by DNA sequencing) to create the transcriptional fusion pDF4.

A transcriptional fusion for *ropB2* was constructed by cloning the *ropB2* promoter region into pFus1par. The primers RopB2ProFor (TCAAGACGCAGC AACTCAAGCA) and RopB2ProRev (CGATGCCGTAGACGATATTTC CAG) were used to amplify a 1,194-bp fragment, including 924 bp upstream of the *ropB2* start codon. This fragment was cloned into pCRII, according to supplier's instructions. A 1.2-kb XhoI/KpnI fragment was cloned from this plasmid into XhoI/KpnI-digested pFus1par to create the transcriptional fusion pDF40, which was verified by DNA sequencing.

A transcriptional fusion for *ropB3* was created by using PCR primers RopB3ProF (5′-CGGGGTACCATGGCGAAGTATCAATCC-3′) and RopB3ProR (5′-CGGT GCGTCATAAGAGGG-3′) and by amplifying a 529-bp fragment containing the *ropB3* promoter. This fragment was cloned into pCRII-TOPO, according to manufacturer's instructions. The *ropB3* promoter region was liberated by digesting it with EcoRI/KpnI and ligated into a similarly digested pFus1par vector to create the *ropB3* transcriptional fusion pDF41, which was verified by DNA sequencing.

Assays for β-glucuronidase (*gusA*) activity. β-Glucuronidase was selected as the reporter gene to assay for *rop* gene expression (19). Assays for β-glucuronidase activity were carried out using a modified procedure originally described by Miller (30), with 3 mg/ml 4-nitrophenyl β-D-glucuronide replacing 4 mg/ml 2-nitrophenyl β-D-galactopyranoside. Reporter gene expression from free-living cells was measured using *R. leguminosarum* strains carrying the transcriptional fusions grown in the appropriate media at 30°C and harvested in late exponential phase. These data were calculated as Miller units using the following formula: $(A_{420} \times 1{,}000)/(\mathrm{OD}_{600} \times \text{time} \times \text{dilution})$. Reporter gene expression was also measured in the bacteroid state as described by Wang et al. (49). In brief, 10 to 15 nodules were harvested at 4 weeks postinoculation and crushed in a buffer containing 1 ml of 0.25 M mannitol and 0.05 M Tris-HCl (pH 7.5). After allowing the plant debris to settle, 100 μl of this bacteroid suspension was used to measure *gusA* activity. To confirm that the fusion plasmid was maintained in the nodule environment, 10 to 20 individual nodules were surface sterilized (as described below), crushed, and plated in duplicate on media containing Sm or Sm-Tc to confirm that the bacteria isolated from the nodule remain Tc resistant and therefore carry the fusion plasmid. In all cases, >95% plasmid retention was observed.

To determine if the mutation of a particular *ropB* gene family member altered gene expression of the other members of the *ropB* gene family, each gene fusion plasmid was mobilized into the corresponding *ropB1*, *ropB2*, or *ropB3* mutants via conjugation. Subsequent assays for β-glucuronidase were performed as described above.

Nodulation experiments. Pea seeds (*Pisum sativum* cv. Trapper) were surface sterilized in 2.625% sodium hypochlorite for 5 min and then in 70% ethanol for 5 min, followed by three washes in sterile water. The sterile seeds were then placed on 1.25% water agar plates and allowed to germinate in the dark at room temperature. After approximately 3 days, they were planted in modified magenta jars that were assembled to resemble Leonard jars (47). The magenta jars contained sterile vermiculite as the solid support medium and approximately 200 ml of Hoagland's plant medium (18). Two pea seeds were planted into each jar, and each seed was inoculated with 500 μl of an overnight culture (OD_{600} of ~1) of the appropriate *R. leguminosarum* strain resuspended in sterile water. The nodules were harvested after 4 weeks. The protocol described by Yost et al. (51) was used to assess the ability of the *ropB* mutant to compete against the wild type during nodulation.

Antimicrobial sensitivity assays. Sensitivity to detergents was evaluated by comparing the growth of strains on agar media, both with and without detergent. Serial dilutions of each *R. leguminosarum* strain being evaluated were plated on TY, TY containing 1.5 mM DOC, and TY containing 0.35 mM sodium dodecyl sulfate (SDS) plates and incubated for at least 4 days at 30°C. After this time, the numbers of CFU were counted, and the $\log_{10}$ reduction in growth due to the detergent was calculated. MIC assays were conducted for erythromycin and L-pyroglutamic acid (PGA) in TY and VMM broth. Growth was determined by measuring absorbance at OD_{600}.

Isolation of cell envelope fractions and SDS-Tricine-PAGE analysis. Cells being used for cell envelope preparation were grown in either TY or VMM media using conditions described above. Cells were harvested by centrifugation at 4,000 × *g* for 10 min at 4°C and stored frozen at −70°C in membrane buffer (50 mM sodium phosphate buffer at pH 7.0, 7.5% [wt/vol] glycerol, 50 mM NaCl). The frozen cell slurry was thawed on ice, and phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 0.1 mM. The cell slurry was subjected to 3 rounds of 45 s of sonication (0.14 to 0.15 W) on ice using a Misonix Microson ultrasonic cell disruptor. The sonicated cell slurry was centrifuged at 11,000 × *g* for 10 min at 4°C to remove unbroken cells and subsequently centrifuged in a Beckman Coulter Optima L-90 K ultracentrifuge at 40,000 rpm for 90 min at 4°C in a Ti70 rotor to separate the membrane fraction from the cytosolic fraction. The membrane fraction was resuspended in membrane buffer, and total membrane protein amounts of each sample were determined using a modified Lowry assay (43) and frozen for storage at −70°C. SDS-Tricine-polyacrylamide gel electrophoresis (PAGE) was performed on isolated membranes from each sample. Membrane samples were resolved on either 12% or 16.5% SDS-Tricine-PAGE to resolve protein bands of <40 kilodaltons. All membrane protein samples were loaded onto the gel to final amounts of 2.5 μg or 5 μg. Membrane protein samples were loaded at either room temperature (22 to 25°C) or heated to 100°C before loading to resolve proteins of lower molecular weight. Proteins separated by SDS-Tricine-PAGE gels were visualized by conventional Coomassie blue staining and by trichloroethanol (TCE) staining. TCE was added to the gels during casting, at a final concentration of 0.5% (vol/vol) TCE, to visualize proteins with tryptophan residues by UV irradiation at 300 nm, according to the method described by Ladner et al. (23). The TCE staining technique increased the ability to visualize membrane proteins within the gel by ≥60% in comparison to conventional Coomassie blue staining, with no difference in protein migration. Coomassie blue staining was performed on all TCE-stained gels.

Nucleotide sequence accession numbers. The DNA sequences for *ropB1*, *ropB2*, and *ropB3* from *R. leguminosarum* VF39SM have been deposited in GenBank under the following respective accession numbers: FJ561446, FJ561447, and FJ561448.

RESULTS

The *R. leguminosarum* VF39SM genome has three genes encoding RopB paralogs. The genome of *R. leguminosarum* 3841 (52) has four *ropB*-like genes, based on a BLASTP search using the amino acid sequence of RopB from *R. leguminosarum* bv. *viciae* 248 (37). The gene RL1589, found on chromosome 3841, is annotated as *ropB* and encodes a protein which is 98% identical to the characterized RopB from *R. leguminosarum* 248 (37). Of the other three *ropB*-like genes, one is found on the chromosome (RL1307) and two are found on the

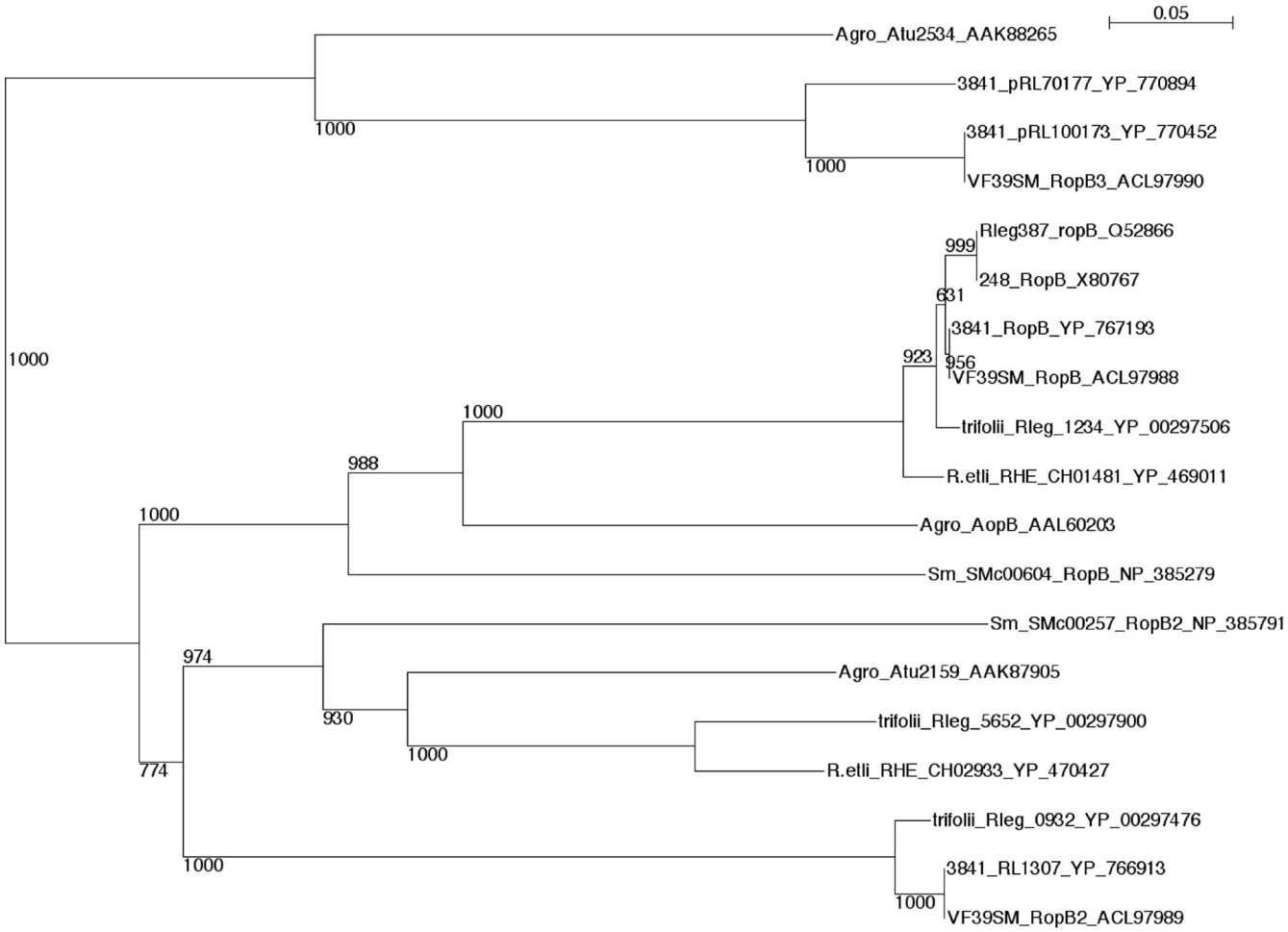

FIG. 1. A neighbor-joining tree derived from a ClustalW alignment of the amino acid sequences deduced from the *ropB* gene family is shown, with bootstrap values. Amino acid sequences were obtained from GenBank. Abbreviations: VF39SM, *R. leguminosarum* bv. *viciae* VF39SM; 3841, *R. leguminosarum* bv. *viciae* 3841; Agro, *Agrobacterium tumefaciens*; Sm, *Sinorhizobium meliloti*; *R.etli*, *Rhizobium etli* CFN42; trifolii, *R. leguminosarum* bv. *trifolii* WSM1325; 248, *R. leguminosarum* bv. *viciae* 248; Rleg387, *R. leguminosarum* bv. *viciae* 387. Protein names were included if they were available; otherwise, the GenBank locus tag was used to identify each protein. The GenBank accession numbers are listed following the protein name or locus tag.

plasmids pRl10 and pRl7 (pRL100173 and pRL70177). The DNA sequences of these genes were used to create PCR primers with the intention of amplifying homologous genes in the VF39SM genome. Genes homologous to RL1589, RL1307, and pRL100173 were amplified from the VF39SM genome, while a homolog of pRL70177 was not amplified. This is in agreement with data from a recently completed genome sequencing project of VF39SM (M. F. Hynes et al., unpublished data). Analysis of the genome indicates that the genetic region of pRL7 containing pRL70177 is absent from the VF39SM genome (Hynes et al., unpublished). Furthermore, primers that bind within the pRL70177 coding sequence did not amplify a fragment from VF39SM genomic DNA, even under low stringency conditions. The PCR amplicons containing the three *ropB*-like genes in VF39SM were subsequently sequenced to ensure they contained *ropB* homologs.

The predicted RopB-like proteins from VF39SM were identical to those found in strain 3841. The VF39SM homolog of RL1589 was named *ropB*, as it encodes a protein identical to RopB from strain 3841. The VF39SM homolog of RL1307 was named *ropB2* based on its corresponding amino acid sequence, sharing homology with RopB2 from *S. meliloti* 1021 (Fig. 1). As there has been no annotation of pRL100173 or related homologs, and since it encodes a predicted outer membrane protein that is homologous to RopB (26% identical and 43% similarity), we suggest that it be named *ropB3*. Figure 1 provides a phylogenetic tree of RopB homologs in the rhizobia.

Insertional mutagenesis of *ropB*, *ropB2*, and *ropB3*. Individual colonies of mutant strains DF33, DF15, and DF42 were isolated on TY agar media to evaluate any changes in colony morphology as a result of the mutations in *ropB*, *ropB2*, and *ropB3*, respectively. After approximately 4 days of incubation, wild-type VF39SM showed convex, circular colonies with perfect borders, while DF33 colonies had irregular edges and were flat. Complementation of DF33 with a plasmid-localized functional copy of *ropB* (pDF37) produced an intermediate phenotype where the colonies did not have perfectly round edges but were convex like the wild type. In addition, DF33 had a

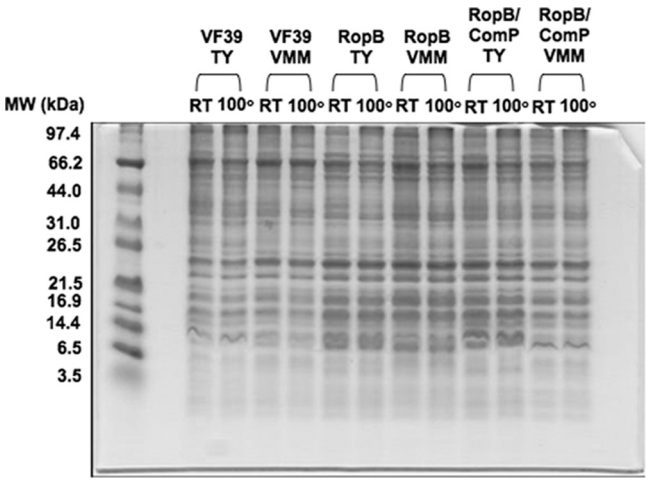

FIG. 2. Tricine-SDS-PAGE (16.5%) of isolated membranes from strains grown in TY and VMM. The strains tested were VF39SM (VF39), the *ropB* mutant (RopB), and the *ropB* mutant complemented with plasmid pDF37 (RopB/ComP). The lanes for each strain are indicated on the figure. A total of 5 μg of membrane protein was loaded into each lane of the gel and heated at either room temperature (RT) or to 100°C for 5 min. Coomassie blue gel staining was performed to visualize protein bands.

mucoid-like layer around the colony that was not present in VF39SM or the pDF37-complemented mutant. No changes in the colony morphology of the DF15 or DF42 mutant strains were observed. Furthermore, changes in colony morphology for the *ropB* mutant were not observed on VMM. The effect of a *ropB* mutation on the profile of membrane proteins was determined by fractionating cell envelope proteins and observing the protein profile on SDS-Tricine-PAGE gels. Mutation of *ropB* did not appear to cause any dramatic changes in the membrane protein profile of VF39SM (Fig. 2).

As stated previously, some rhizobial strains with outer membrane defects are impaired in the symbioses with their host plant (6). To evaluate whether the RopB family of OMPs participate in the plant-host relationship, pea plants were inoculated with the *ropB* family mutants, and their ability to form functional nodules was assessed. Plants inoculated with DF33, DF15, or DF42 all possessed large pink nodules that contained differentiated bacteroids when examined under a light microscope. In addition, the plants inoculated with these mutants exhibited healthy shoots similar to those exhibited by the control inoculated with the wild type. A *ropB* mutant in *S. meliloti* is less competitive for nodulation sites than the wild type when coinoculated on alfalfa (6). Therefore, we also examined the competitive abilities of the *ropB* mutant for competing against the wild type during nodulation. Three trials were completed using pea plants and a near to 1:1 ratio of the mutant and wild type. The subsequent ratio of mutant nodules to wild-type nodules recovered after 4 weeks of growth was only significantly (chi-square test; $P < 0.05$) reduced in one of the three trials, and this reduction was slight (data not shown). These results suggest that the *ropB* mutant in *R. leguminosarum* may not be substantially impaired in its ability to compete with the wild type during nodulation.

Defects in the outer membrane are also known to cause increased sensitivity to antimicrobial compounds, such as detergents, cationic peptides, and antibiotics (17). The detergents sodium dodecyl sulfate (SDS) and sodium deoxycholate (DOC) were added to TY agar plates at concentrations of 0.35 mM and 1.5 mM, respectively. The *ropB2* and *ropB3* mutants did not have an increased sensitivity to either of the detergents (Table 2). Mutation of *ropB*, on the other hand, increased sensitivity to both of the detergents, resulting in a >3-log reduction in growth in the presence of the detergents (Table 2). Complementation of the *ropB* mutant with the pDF37 plasmid restored the mutant's ability to grow in the presence of detergents. MIC assays revealed that DF33 is also more sensitive to the hydrophobic antibiotic erythromycin and the weak organic acid L-pyroglutamic acid, while mutants DF15 and DF42 showed no difference compared to the wild type.

Increased expression of *ropB* in EDTA. Addition of EDTA to the growth media chelates the calcium present, and removal of calcium decreases the stability of the outer membrane (32). We hypothesized that the addition of EDTA to VMM might increase expression of *ropB*, since phenotypic data suggested a role for RopB in outer membrane stability. Gene expression of strains grown in VMM containing 1 mM EDTA was compared to expression of those in VMM alone. The presence of EDTA caused a >2-fold increase in *ropB* expression (Table 3). The *ropB2* and *ropB3* fusions did not exhibit an increase in expression of a similar magnitude (Table 3).

Expression of the *ropB* gene family in free-living and bacteroid cells. The absence of RopB in the outer membranes of bacteroids has been observed previously using Western blot-

TABLE 2. Effect of antimicrobial compounds on growth of the *ropB* family mutants

Strain	Log growth reduction[a]		MIC[b]			
			PCA		Erythromycin	
	1.5 mM DOC	0.35 mM SDS	TY	VMM	TY	VMM
VF39SM	0.191 ± 0.057	0.263 ± 0.112	5.5	6.0	8.83×10^{-3}	0.027
DF33 (*ropB* mutant)	4.85 ± 1.22*	3.86 ± 1.21*	4.5	5.5	1.36×10^{-3}	0.010
DF33 (*ropB* mutant) + pDF37	0.351 ± 0.163	0.708 ± 0.386	5.5	6.0	8.83×10^{-3}	0.027
DF15 (*ropB2* mutant)	0.103 ± 0.179	0.101 ± 0.355	5.5	6.0	8.83×10^{-3}	0.027
DF42 (*ropB3* mutant)	0.123 ± 0.251	0.026 ± 0.131	5.5	6.0	8.83×10^{-3}	0.027

[a] Growth reduction values were calculated by subtracting the number of log CFU/ml grown on TY plus detergent from the number of log CFU/ml grown on TY. Data are presented as the average results ± standard deviations from at least three trials. An asterisk indicates a significant difference in growth between the wild type and the mutant (*t* test; *P* of <0.01).

[b] Strains were grown in either TY or VMM broth with various concentrations of PCA or erythromycin. MICs are presented in mM and are representative values from a minimum of three separate replicates.

TABLE 3. Promoter activity of *ropB*, *ropB2*, and *ropB3* in VF39SM under various growth conditions

Plasmid	*gusA* reporter fusion	*gusA* activity[a]			
		Strains grown in TY	Strains grown in VMM	Strains grown in VMM + 1 mM EDTA	Bacteroids
pDF4	*ropB*::*gusA*	$5.37 \times 10^3 \pm 1.40 \times 10^3$	690 ± 279*	$1.61 \times 10^3 \pm 47.8$	233 ± 62.4*
pDF40	*ropB2*::*gusA*	240 ± 27.8	194 ± 32.3	139 ± 62.6	116 ± 30.0
pDF41	*ropB3*::*gusA*	417 ± 61.9	199 ± 30.0	307 ± 53.9	114 ± 11.9

[a] Data are expressed as the means ± standard deviations from at least three assays, and values are expressed as Miller units (27). The average background level of *gusA* activity for TY-grown cells carrying pFus1 is approximately 250 ± 27.2 Miller units. An asterisk indicates a significantly different mean expression compared to expression in TY for each respective gene (*t* test; *P* of <0.005).

ting (38). To determine if RopB disappearance is correlated to transcriptional downregulation of *ropB*, the *ropB*::*gusA* transcriptional fusion pDF4 was created. The promoter activity of *ropB* was measured in free-living cells grown in complex TY media and bacteroids isolated from pea plants. There was a 23-fold decrease in *ropB* expression in the nodule compared to that in TY-grown cells (Table 3). This is consistent with the findings of Roest et al. (38) and suggests that diminished RopB in bacteroids is due to decreased rates of gene transcription. A growth condition in TY that reduced *ropB* expression to the level of expression observed in bacteroid cells could not be identified, but during these investigations, an 8-fold reduction in *ropB* expression was observed when the fusion strain was grown in VMM (Table 3).

Both the *ropB2* and *ropB3* fusions were close to background levels in bacteroid cells, suggesting negligible transcriptional expression in bacteroids. Free-living expression of *ropB2* and *ropB3* were observed at levels below *ropB* expression in both TY medium and VMM (Table 3). The expression of the *ropB2* fusion was close to background levels, indicating that it is unlikely that this gene is expressed under the conditions assayed. In contrast, expression of *ropB2* in *S. meliloti* is increased in bacteroids compared to that in free-living cells grown in complex media (3). The *ropB3* fusion had modest expression in TY and expression close to background levels in VMM.

Increased expression of *ropB* in peptide-rich media. The increased expression of *ropB* in TY relative to VMM was investigated further. Addition of either tryptone or yeast extract to VMM resulted in an increase of *ropB* expression, while additional $CaCl_2$ had no effect (Table 4). Inclusion of both tryptone and yeast extract in VMM had an additive effect on *ropB* expression (Table 4). The components of VMM did not cause a decrease in *ropB* expression when added to TY (data not shown). Tryptone and yeast extract are rich in peptides; therefore, other peptide sources were tested to determine if they caused an increase in *ropB* expression. The addition of hydrolyzed casein, meat peptone, or soytone to VMM all produced a similar increase in *ropB* expression (Table 4). Amino acids do not increase *ropB* expression, since the addition of individual or groups of amino acids to VMM did not increase *ropB* expression (data not shown).

Regulation of *ropB* by the sensor kinase ChvG. Expression of *ropB* homologs in other *Alphaproteobacteria* is regulated by a two-component signal transduction system. For example, ChvI/ChvG regulates *aopB* expression in response to acidic pH (20, 26) in *A. tumefaciens*. A homologous system in *B. abortus* named BvrR/BvrS regulates OMP gene expression, including *omp25-omp31* (16), and mutation of the *bvrR-bvrS* genes results in decreased virulence (42). *R. leguminosarum* has a two-component signal transduction system that is homologous to those found in *A. tumefaciens* and *B. abortus*. The sensor kinase of this system, also denoted *chvG* in the *R. leguminosarum* 3841 genome, was cloned and mutated in VF39SM in order to determine if it plays a role in *ropB* regulation.

The VF39SM *chvG* mutant DF20 was unable to grow on complex media, similar to the *chvG* mutant of *A. tumefaciens* (8). The growth of the *chvG* mutant was substantially delayed compared to the growth of the wild type, and the mutant does not form effective nodules on pea plants. DF20 was cultured routinely on VMM agar because the mutant grew very poorly in VMM broth. The poor growth of this mutant echoes the difficulty in obtaining loss-of-function *chvG* (*exoS*) mutants in *S. meliloti* (9), and the *chvI* and *chvG* mutants were obtained only recently in *S. meliloti* (4). In order to determine if ChvG plays a role in regulating expression of the *ropB* family, the transcriptional fusions pDF4, pDF40, and pDF41 were introduced into the *chvG* mutant background. We wanted to compare *ropB* expression in VMM and TY between the *chvG* mutant and wild type. However, this comparison was not pos-

TABLE 4. Promoter activity of *ropB* when grown in the presence of peptides

Medium	GusA activity assayed using pDF4[a]
TY	$5.37 \times 10^3 \pm 1.40 \times 10^3$*
VMM	690 ± 279
VMM + CaCl2[b]	789 ± 135
VMM + yeast extract[b]	$2.66 \times 10^3 \pm 128$*
VMM + tryptone[b]	$2.74 \times 10^3 \pm 94.9$*
VMM + tryptone + yeast extract[b]	$4.50 \times 10^3 \pm 278$*
VMM + 1% hydrolyzed casein	$3.48 \times 10^3 \pm 570$*
VMM + 0.5% meat peptone	$3.03 \times 10^3 \pm 132$*
VMM + 0.5% soytone	$3.43 \times 10^3 \pm 94.2$*
VMM + 2.5 mM IQY[c]	436 ± 109
VMM + 2.5 mM YVL[c]	445 ± 11.3
VMM + 2.5 mM FSDKIAK[c]	467 ± 28.8

[a] *ropB* promoter activity was assayed using *gusA* fusion plasmid pDF4 in a VF39SM wild-type background. Data are expressed as the means ± standard deviations from at least three assays, and values are expressed as Miller units (27). The background level of *gusA* activity for TY grown cells is approximately 250 Miller units. *, growth in TY or in VMM amended with peptide-rich medium components resulted in a statistically significant increase in *ropB* expression compared to that in growth in VMM (*t* test; *P* of <0.001).

[b] Consists of 0.5% tryptone, 0.3% yeast extract, and 3.4 mM $CaCl_2$ used at the same concentration as TY.

[c] Synthesized peptides with sequences specified by the single-letter amino acid code were added to VMM.

TABLE 5. Effect of a *chvG* mutation on *ropB* expression in the presence of tryptone

Strain	Expression[b]		
	VMM + $CaCl_2$[a]	VMM + tryptone + $CaCl_2$[a]	Fold induction
VF39SM	310 ± 58.7	1.59×10^3 ± 380	5.11 ± 0.260
chvG mutant	430 ± 9.50	665 ± 100	1.54 ± 0.210*

[a] Strains were cultured on VMM agar with 3.4 mM $CaCl_2$ added and 0.5% tryptone, as indicated.

[b] Expression data is expressed as the means ± standard deviations from at least three assays, and values are expressed as Miller units (27). Fold induction is measured by comparing the expression in the presence and absence of 0.5% tryptone. An asterisk indicates that a significant difference in the effect of tryptone on the increase in *ropB* expression between the *chvG* mutant and the wild type was observed (*t* test; *P* of <0.0001).

TABLE 6. Expression of the *ropB* gene family fusions in a mutant deficient in 27-hydroxyoctacosonate-modified LPS

Plasmid	Fusion	GusA activity for strain[a]:	
		3841	38EV27
pDF4	*ropB*::*gusA*	6.25×10^3 ± 1.74×10^3	235 ± 35.7*
pDF40	*ropB2*::*gusA*	201 ± 21.7	196 ± 8.11
pDF41	*ropB3*::*gusA*	381 ± 44.6	480 ± 139

[a] Strains were grown in TY broth and assayed for GusA activity using the modified Miller assay described in Materials and Methods. Data are expressed as the means ± standard deviations from at least three assays, and values are expressed as Miller units (27). An asterisk indicates a significant difference in *ropB* gene expression between the wild type and lipid A mutant (*t* test; *P* of <0.0001).

sible due to the inability of the *chvG* mutant to grow in TY broth. Further investigation into growth phenotypes of the *chvG* mutant led to the discovery that the *chvG* mutant can grow on VMM agar supplemented with 0.5% tryptone when 3.4 mM $CaCl_2$ is present. However, the mutant was incapable of growing under similar conditions in broth culture. In light of this, VMM agar with 3.4 mM $CaCl_2$ and VMM with 0.5% tryptone and 3.4 mM $CaCl_2$ were used to study the role that ChvG plays in the expression of the genes in the *ropB* family.

The 5-fold increase in expression of *ropB* in the wild-type background in the presence of tryptone was abolished in the *chvG* mutant background (Table 5). The mutation of *chvG* had no effect on the regulation of *ropB2*, as the expression of *ropB2* remained very low when grown on either type of media and regardless of the background. The expression patterns of *ropB3* were similar in both the wild-type and *chvG* mutant backgrounds. The inability of the *chvG* mutant to grow in broth culture precluded us from determining the role of ChvG in upregulating *ropB* under Ca^{2+}-limiting conditions.

TY-dependent upregulation of *ropB* is abolished in an LPS mutant lacking 27-hydroxyoctacosanoate-modified lipid A. The LPS of *R. leguminosarum*, and of other members of *Rhizobiaceae*, is modified by the addition of 27-hydroxyoctacosanoate (also referred to as very long chain fatty acid) to the 2′ position of lipid A (13, 46). We recently isolated a mutant of *R. leguminosarum* 3841 where the lipid A is lacking the 27-hydroxyoctacosanoate modification (45). The *ropB*, *ropB2*, and *ropB3* transcriptional fusion plasmids were mated into this mutant as well as into the 3841 wild-type strain. *gusA* gene expression from pDF4 was measured in both wild-type and mutant strains grown in TY or VMM. A 4-fold upregulation of *ropB* expression in TY broth relative to VMM broth was observed in wild-type 3841 carrying pDF4, similar to the upregulation observed in VF39SM (data not shown). Notably, the increased expression in the *ropB* fusion that is typical when the fusion strain is grown in TY broth was not evident in the LPS mutant (Table 6). The expression patterns in the *ropB2* and *ropB3* fusions were unchanged between the wild type and the LPS mutant (Table 6).

DISCUSSION

Phenotypic and gene expression data from this study support the hypothesis that RopB, like OmpA (32) of *E. coli*, is an important structural component of the outer membrane, while the roles of RopB2 and RopB3 remain cryptic. Mutations that lead to structural defects in the outer membrane often increase outer membrane permeability and increase a cell's sensitivity to hydrophobic antimicrobials (17, 44). Of the *rop* mutants, only the *ropB* mutant was sensitive to detergents, the hydrophobic weak organic acid pyroglutamic acid (50), and the hydrophobic antibiotic erythromycin. The contribution of RopB to outer membrane stability may involve an interaction with LPS. Other eight-stranded β-barrel OMPs with secondary structure similar to that of RopB have been proposed to interact with LPS, including Omp21 from *Comamonas acidovorans* (2), OprH from *Pseudomonas aeruginosa* (5), and Omp25 from *Brucella* (7). A possible interaction between RopB and LPS is supported by the fact that *ropB* expression is significantly reduced in a LPS lipid A mutant of *R. leguminosarum* 3841 (Table 6). The reduction of *ropB* expression in the lipid A mutant may contribute to the increased sensitivity to detergents and other membrane-associated stressors observed in LPS mutants lacking 27-hydroxyoctacosanoate-modified lipid A (13, 45, 46). Outer membrane protein interaction with LPS at the divalent cation binding sites has been suggested for the *Pseudomonas aeruginosa* OMP OprH. Increased *oprH* expression is thought to stabilize the outer membrane under Mg^{2+}-limiting growth conditions by compensating for the decrease in divalent cations (5, 31, 53). The possibility that RopB functions similarly to OprH is supported by the increased expression of *ropB* when VF39SM is grown in media containing EDTA (Table 3), which chelates 98% of the Ca^{2+} present in VMM (H. Weger, personal communication). A role of calcium in rhizobial OMP gene regulation has been noted previously. Kim and colleagues (22) have observed that in *Sinorhizobium fredii* USDA257, expression of Omp22, an OMP with homology to RopB, increases under calcium-limiting growth conditions. They also noted that the majority of Omp22 was found in the extracellular fraction when cells are calcium limited, suggesting that calcium is required for the structural integrity necessary to maintain its localization in the outer membrane (22). de Maagd and colleagues (11) have noted that LPS is also released into the growth media under calcium-limiting growth conditions. It would be interesting to ascertain if the RopB is also found to be associated with the LPS excreted into the growth medium.

The functions of *ropB2* and *ropB3* in VF39SM remain elusive, as we could not identify any phenotypic changes in these

mutants, and we were unable to identify conditions under which gene expression was increased. Expression of *ropB2* did not increase in a *ropB* or *ropB3* mutant background, suggesting it does not compensate for the loss of *ropB* or *ropB3*. Furthermore, *ropB3* expression was not increased in the *ropB* mutant background, suggesting that RopB3 does not compensate for loss of RopB.

The outer membrane of *Rhizobium leguminosarum* undergoes structural changes during bacteroid maturation, including a transition in the LPS from a hydrophilic to a predominantly hydrophobic nature (21), and the downregulation of the *ropB* genes in mature bacteroids emphasizes the changes that occur to the cell envelope during bacteroid maturation. The data also confirm the earlier Western blot data of Roest et al. (38), indicating that the absence of these OMPs in mature bacteroids is due to downregulation of gene expression. It is notable that the difference in *ropB* gene expression between cells grown in VMM and bacteroids is not large, especially compared to *ropB* expression in TY (Table 3). Therefore, interpretation of the physiological significance of a change in gene expression from a free-living to bacteroid state should be considered in the context of the growth parameters used for the free-living cells.

A role for ChvG in the regulation of *ropB* expression is consistent with the ChvG-dependent OMP gene regulation observed in other *Rhizobiales* (16, 26, 29, 42). However, ChvG-dependent induction of OMP gene expression due to the presence of growth components rich in peptides has not been previously reported. Regulation of OMPs in response to growth in complex media has been reported for Omp21 from *C. acidovorans*, which is present in cells grown in complex media and absent in cells grown in minimal media (2). Given the direct correlation between peptide-rich media and *ropB* expression, we attempted to identify specific peptides that may be involved in regulation using synthetic peptides derived from the peptide sequences resulting from an enzymatic hydrolysis of casein. These sequences were selected based on their reported high antimicrobial activity in *E. coli* (27). However, these peptides did not induce *ropB* gene expression (Table 4). Identification of a particular peptide can be daunting, given the number of diverse peptides generated from the enzymatic hydrolysis of casein. It is also possible that peptides indirectly induce expression of *ropB* through ChvG, and this requires a mixture of several peptides. It is interesting to note that a *chvG* mutant is incapable of growing on TY and that *ropB*, which is regulated by *chvG*, is strongly induced in TY. Although a *ropB* mutant is still able to grow in TY, it does exhibit colony morphology that is different from that of the wild type (data not shown). The role of ChvG in inducing *ropB* expression under Ca^{2+}-limiting growth conditions remains to be elucidated. A major hurdle in this experiment is the poor growth of the *chvG* mutant in the presence of EDTA and also in broth media.

Future experiments are planned to define in greater detail how ChvG induces expression of *ropB* and the possible mechanism for reduced *ropB* expression in a lipid A mutant. We will also continue our characterization of *ropB2* and *ropB3* in an effort to assign functions to these genes. Understanding the regulation and function of outer membrane proteins will further define the important role of the outer membrane in both Gram-negative symbioses and pathogenesis.

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