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Isolation of *ropB*, a Gene Encoding a 22-kDa *Rhizobium leguminosarum* Outer Membrane Protein

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As judged from immunochemical detection, the levels of outer membrane antigen groups II and III of *Rhizobium leguminosarum* bv. *viciae* strain 248 decrease during bacteroid differentiation (R. A. de Maagd, R. de Rijk, I. H. M. Mulders, and B. J. J . Lugtenberg, J. Bacteriol. 171:1136-1142, 1989). Using a newly developed colony blot assay, a cosmid clone expressing the Mab8 epitope of the outer membrane antigen group II of *R. l.* bv. *viciae* strain 248 was selected in *Rhizobium meliloti* LPR2120. From this cosmid the gene encoding this epitope was cloned and characterized. An open reading frame of 636 nucleotides was found and predicted to encode a protein with a calculated molecular mass of 22.5 kDa. After subtraction of the predicted 23 amino acid signal peptide, a M_r of 20.3 kDa was calculated for the mature protein. This gene, designated *ropB*, was not active in *Escherichia coli* under the control of its own promoter. The C-terminal amino acid of the protein is a phenylalanine residue which is required for efficient translocation of outer membrane proteins. Membrane spanning amphipathic β-sheets are predicted to represent a major part of the secondary structure of the protein. A model of the topology of the protein is presented. We determined the start of transcription in order to analyze the promoter region. No homology was found with other known promoter sequences. The *ropB* gene appeared to be well-conserved in *R. leguminosarum* and *Agrobacterium tumefaciens* strains. An attempt was made to mimic the immunochemical decrease of RopB ex planta. Neither the various growth conditions tested nor the addition of nodule or plant extracts resulted in a reduction of the Mab8 epitope to bacteroid levels.

Bacteria of the genera *Rhizobium* and *Bradyrhizobium* can interact with leguminous plants. Under low nitrogen conditions this interaction can result in the formation of nodules on the plant roots in which a differentiated form of the rhizobia, called bacteroid, converts atmospheric nitrogen to ammonia which can be utilized by the host plant.

During differentiation into the bacteroid form, the bacterium undergoes a number of distinct morphological and biochemical changes. Changes in outer membrane protein patterns during bacteroid development have been studied in our laboratory. Outer membrane proteins of free-living *R. leguminosarum* bv. *viciae* strain 248 are divided into four major antigen groups, I through IV. The detection of the antigen groups II and III is severely reduced in bacteroids, whereas antigen groups I and IV remain present in normal amounts (de Maagd et al. 1989a). This appears to be a general phenomenon of all *R. leguminosarum* strains tested and is independent of nitrogen fixation (Roest et al. 1995). De Maagd et al. (1992) isolated a gene that encodes (part of) the antigen group III proteins and designated this gene *ropA*. In situ hybridization experiments showed that the expression of this gene is down-regulated in bacteroids at the level of transcription (De Maagd et al. 1994).

To be able to study the regulation of such outer membrane protein-encoding genes in more detail, we isolated the structural gene of the antigen group II protein of *R. l.* bv. *viciae* strain 248. The gene and the upstream promoter region were sequenced and analyzed.

RESULTS

Isolation of a cosmid encoding the antigen group II outer membrane protein.

The monoclonal antibody Mab8 recognizes an epitope of *R. l.* bv. *viciae* strain 248 outer membrane antigen group II. This epitope is present in other *R. leguminosarum* strains as well, but not in *R. meliloti* and *A. tumefaciens* strains (de Maagd 1989). *R. meliloti* and *A. tumefaciens* strains can express the antigen group III gene *ropA* of *R. leguminosarum*. When the *ropA* gene, under the control of its own promoter, is introduced in these strains, the outer membrane protein RopA can be detected with monoclonal antibody Mab38 (data not shown). This observation makes *R. meliloti* and *A. tumefaciens* strains potentially useful for the isolation of an antigen group II encoding cosmid. However, it was found that antigen group II proteins, in contrast to antigen group III proteins, cannot be detected on the surface of intact cells of *R. l.* bv. *viciae* strain 248 (de Maagd et al. 1989a). Therefore we attempted to improve the detection level of antigen group II by testing a number of additional treatments in a colony blot assay. Treatment with lysozyme and subsequent boiling in a β-mercaptoethanol/SDS solution indeed resulted in detection of antigen group II without introducing a reaction with *R. meliloti* or *A. tumefaciens* strains. To isolate a cosmid that encodes the outer membrane proteins(s) of antigen group II,

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the cosmid library of *R. l.* bv. *viciae* strain 248 (de Maagd et al. 1992) was introduced into *R. meliloti* strain LPR2120 and the resulting transconjugants were streaked on YMB-agar plates supplemented with the appropriate antibiotics. Approximately 2,000 colonies were tested in the colony blot assay for the production of the Mab8 epitope. Three positive transconjugants were identified. One of them did not contain a cosmid, although this strain was Mab8 positive and tetracyclin resistant. We assume that in this strain the cosmid has integrated into the chromosome through recombination. The two other transconjugants contained identical cosmids and one of these, designated pMP4002, was used for further investigations.

Nucleotide sequence, polarity, and genetic analysis of the open reading frame.

Subcloning of the fragments of pMP4002 into pMP92 revealed that a plasmid (pMP4003) with an insert of a single 2.6-kb *Eco*RI fragment was still able to express the Mab8 epitope (Fig.1A, lane 3). The restriction map of this fragment is shown in Figure 1B. To determine the fragment required for antigen group II production more precisely, a number of subclones of pMP4003 were made using internal restriction sites. The resulting plasmids were tested for the presence of the group II antigens after transconjugation to the recombination-deficient *A. tumefaciens* strain LBA4301 (Fig. 1A). This strain was used instead of *R. meliloti* strain LPR2120 to circumvent the possibility of recombination. After separation of cell envelope proteins using PAGE, the antigen group II consists of a number of protein bands. This is caused by β-mercaptoethanol-resistant multimers (see IIb in Fig. 1A) or the presence of peptidoglycan subunits (the two upper bands of region IIa in Fig. 1A). The lowest band with a M_r of approximately 22 kDa is the primary, unmodified version of antigen group II (de Maagd et al. 1989c). *A. tumefaciens* bacteria containing one of the plasmids pMP4003, pMP4007, and pMP4019 expressed the group II antigens in the same forms as *R. l.* bv. *viciae* strain 248 (Fig.1A, lanes 3, 4, 6, and 1, respectively). Since the possession of pMP4007 or pMP4019 is sufficient to express the Mab8 epitope, the approximately 1,400 nucleotides between the *Sac*I and the *Nru*I restriction site were sequenced in both directions (Fig. 2). Analysis of this sequence using the University of Wisconsin GCG software revealed only one complete open reading frame (ORF). The *Sal*I restriction site used to construct plasmid pMP4009 was located in this ORF (Fig. 1B). The ORF runs from nucleotide 604 to 1239 and contains a consensus ribosomal binding site seven nucleotides upstream of the ORF (Fig. 2, bold-faced type). No homology with *ropA* or any other known DNA sequence could be found in the Genbank and EMBL databases.

To verify the polarity of this gene, the *Sal*I site in the putative ORF was used for promoter studies. Two plasmids were constructed, pMP4018 and pMP4022, with fragments cloned in opposite orientations in pMP220. This plasmid contains a promoterless *lacZ* gene (Fig. 1B). The plasmids pMP220, pMP4018, and pMP4022 were transconjugated into *A. tumefaciens* strain LBA4301 and the transconjugants were tested for β-galactosidase activity. Only plasmid pMP4022 resulted in an increased β-galactosidase activity (920 U) as compared with the background activity of pMP220 (112 U). This result is in agreement with the deduced orientation of the gene. The β-galactosidase level of pMP4022 is also comparable to levels obtained with a *ropA–lacZ* fusion gene (de Maagd et al. 1992). In an *E. coli* strain harboring pMP4022, however, no increase of β-galactosidase activity was detected. This indicates that the *ropB* promoter, like the *ropA* promoter, is not active in *E. coli.*

The open reading frame encodes a protein of 211 amino acid residues with a calculated mass of 22.5 kDa. Analysis of the N-terminal region with the rules of von Heijne (1986) revealed a possible signal peptidase cleavage site, character-

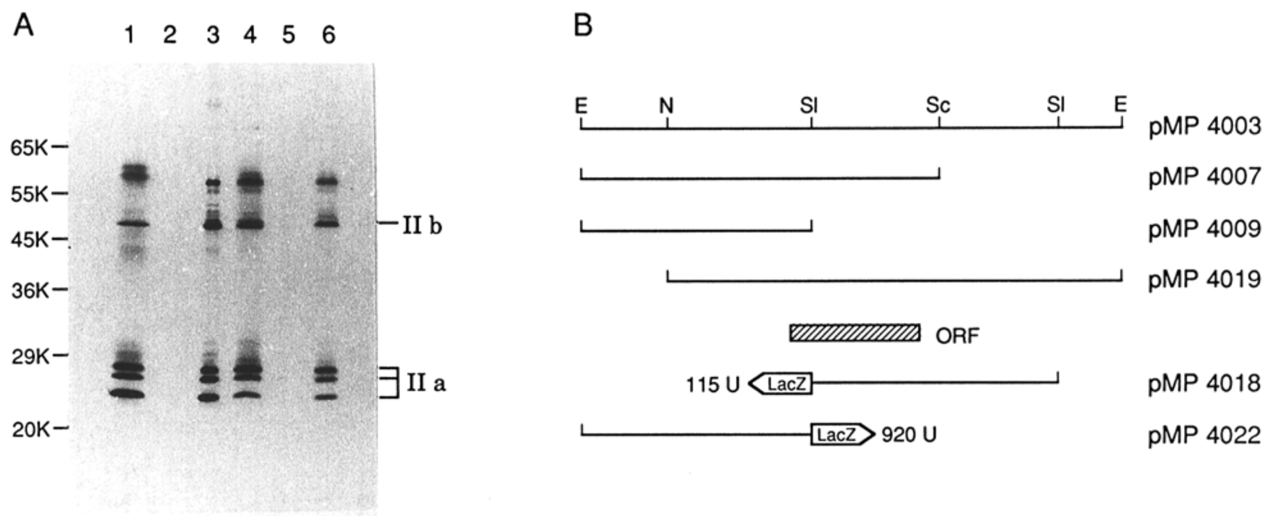

Fig. 1. Expression of group II antigen by various subcloned plasmids. **A,** Western blot of cell envelopes incubated with Mab8. Lanes represent cell envelopes of *Rhizobium leguminosarum* bv. *viciae* strain 248 (1), *Agrobacterium tumefaciens* LBA4301 containing pMP92 (2), pMP4003 (3), pMP4007 (4), pMP4009 (5), and pMP4019 (6). Molecular weight markers are indicated on the left. **B,** Restriction map of the 2.6-kb *Eco*RI fragment of pMP4003. The shaded rectangle indicates the position of *ropB* in this fragment. The arrows of pMP4018 and pMP4022 indicate the fusions to the promoterless *lacZ* gene in pMP220 with their respective β-galactosidase activity expressed in Miller units in *A. tumefaciens*. Abbreviations: E, *Eco*RI; N, *Nru*I; Sl, *Sal*I; Sc, *Sac*I.

istic for exported proteins, between amino acid residues 23 and 24. The predicted mature protein consists of 188 amino acid residues with a calculated mass of 20.3 kDa, which corresponds well with the 22 kDa estimated from SDS-polyacrylamide gel electrophoresis (de Maagd et al. 1989a).

The antigen group II is located in the outer membrane of *R. l.* bv *viciae* strain 248 (de Maagd et al. 1989a). To test whether the isolated gene indeed is the structural gene for antigen group II, the method of Kyte and Doolittle (1982) was used to draw the hydropathy plot of the deduced amino acid sequence of RopB (Fig. 3). No large stretches of hydrophobic amino acids were found, indicating that RopB does not contain any regions to span the cytoplasmic membrane. The amino acid sequence did reveal a number of membrane-spanning β-sheets of no more than 10 amino acids that have an amphipathic character and that are characteristic for outer membrane proteins (Vogel and Jähnig 1986). These membrane-spanning regions are indicated in Figure 3 by solid bars. In analogy with *ropA* (*R*hizobium *o*uter membrane *p*rotein) we propose the name *ropB*.

Using the criterion of the membrane spanning amphipathic β-sheets together with other criteria governing the folding of outer membrane proteins (Tommassen 1988), we predict the topology of RopB to be as shown in Figure 4.

```
     NruI
   1 TCGCGAGCCTTGCAACCAACGCGCCCGTTTCGCCGAGATCGCTAAGATCG

  51 GCCTGCAGCGCGACCGCCCTTTGTCCCTTTCGCCGCAATTCCGCCACCAG

 101 CTCTTCGGCCTCACCGATGGAGCCGTTCGCGTGGATCGCAACGGAAAAGC

 151 CATTTGCAGCAAGGTCTTCGGCGATTGCCCGGCCTATTCTTTTAGCGGCC

 201 CCTGTTATAAGGGCCGAGCGAAGTCTTTTGTGGTTCAAAATCTTGCCTTC

 251 TGATCCCGCGAGTCTGTCCGCAGACTATATAGGGGCCGACGGAGATTATA

 301 TAAATAGGCCGTTGCGGTTGCGTCCGAGAGCCGGATATTCTATGTATTAT

 351 TTAAGAATGCGATTTCCTAAAATAGGTATTTTAAGTTTAACTCTTCGGTA

 401 GGGTTAACAAAGTGTATGTTGCGCCAAAGCAACATCGAATTTCAGCCATG

 451 GCGCAGCCACAATGATATCACAATTTGGCTCTTTCAAATCCGCATTTCAG
                    *                          *
 501 TTCCAATTTCAATCTCGATCCGGAAGGGACATCTGGCAATATCCCGCCAA

 551 TGCTTCACTGATAGACAGATGGTAAGCGGCGCGGGACTGAAAGGAGACTA

 601 AGTATGCGTGTACTCATTGCTGGCCTCATGGCCTCCGTTTTTGCAATTGC
        M  R  V  L  I  A  G  L  M  A  S  V  F  A  I  A
                                          SalI
 651 GGGCGTCTCGGCAGCTCAGGCGGCCGATCGCGTCGACCAGGTTCCGGAAG
      G  V  S  A  A  Q  A ↑A  D  R  V  D  Q  V  P  E  A

 701 CACCGGTCGCCCAGGAAGCTCCGGTCAAGCCGGCCGGCAGCTGGGAAGGC
       P  V  A  Q  E  A  P  V  K  P  A  G  S  W  E  G

 751 TTCTACCTCGGCGGCGCCGGCACCTATAACATGGGTGACTTCGGTTCCGA
     F  Y  L  G  G  A  G  T  Y  N  M  G  D  F  G  S  D

 801 TCGCCACACCTACGGTTTCGGCGGCCAGGTCTTCACCGGCTACAACTGGC
      R  H  T  Y  G  F  G  G  Q  V  F  T  G  Y  N  W  Q

 851 AGCAGGGCCAGATCGTTTACGGCGTTGAATCCGATCTCGGCTACAGCGGC
       Q  G  Q  I  V  Y  G  V  E  S  D  L  G  Y  S  G

 901 GACGACGTCTCCTCGGGCGGTGTCGAGAACAAGTATGGCTGGAACGGCTC
     D  D  V  S  S  G  G  V  E  N  K  Y  G  W  N  G  S

 951 CGTCCGTGGCCGCGTCGGCTACGACATGAACCCCTTCCTGCTCTACGGCA
      V  R  G  R  V  G  Y  D  M  N  P  F  L  L  Y  G  T

1001 CGGCCGGTCTTGCCATCGGCGACGTCAAGGTTTCCGACGACACTTCGGAC
       A  G  L  A  I  G  D  V  K  V  S  D  D  T  S  D

1051 GAAAGCAAGACGAACTTCGGCTATACGGTCGGCGCCGGCGTCGAAGCCTT
     E  S  K  T  N  F  G  Y  T  V  G  A  G  V  E  A  F

1101 CGTGACCAACAACATCACGACGCGCCTCGAATATCGCTACACCGACTACC
      V  T  N  N  I  T  T  R  L  E  Y  R  Y  T  D  Y  Q

1151 AGAGCAAGGATTACGACCTCGACTCCGGCAGCTTCTCGCGCGGTTACGAC
       S  K  D  Y  D  L  D  S  G  S  F  S  R  G  Y  D

1201 GAGAACAGCGTCAAGCTCGGTATCGGCGTCAAGTTCTAAGCCGACCGAAT
     E  N  S  V  K  L  G  I  G  V  K  F

1251 ATCCTGATATGGGAAAGCCGGGCACGTCGCCCGGCTTTTTGCTGTCCGCC
                  ------------>    <------------
                                               SacI
1301 TGTCTCTCCAGACGCTGAGCCCGCTCAACCCTCAGACTTGAGCTC
```

Fig. 2. Nucleotide sequence and derived predicted amino acid sequence of *ropB* in the *Nru*I–*Sac*I fragment of pMP4003. One-letter symbols for amino acids are used and are placed under every first nucleotide of a triplet. The ribosomal binding site (AGGAG) is indicated in bold type print. Asterisks above bold typed letters mark the two transcriptional start sites. The vertical arrow indicates the predicted cleavage site of the signal peptide. An inverted repeat, functioning as a rho-independent transcription terminator, is indicated by the horizontal arrows downstream of the open reading frame. The underlined part of the nucleotide sequence designates the primer sequence used for the detection of the transcription start site.

Determination of the transcriptional start site and characterization of the region upstream of the transcriptional start.

To analyze the promoter in the upstream region of the *ropB* gene we determined the 5′ end of the mRNA of *ropB*. For this experiment we used a synthetic primer complementary to nucleotides 619–634 (see Fig. 2, underlined) and the start of transcription was determined by primer extension. This re-

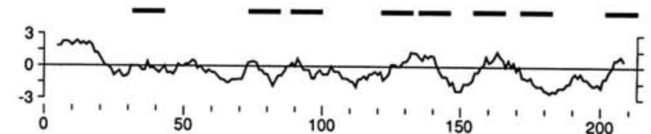

Fig. 3. Hydrophobicity plot of the predicted amino acid sequence of RopB determined by the method of Kyte and Doolittle (1982) with a window of nine amino acids. Positive values indicate hydrophobic regions, negative values indicate hydrophilic regions. The solid bars indicate the amphipathic β-sheets.

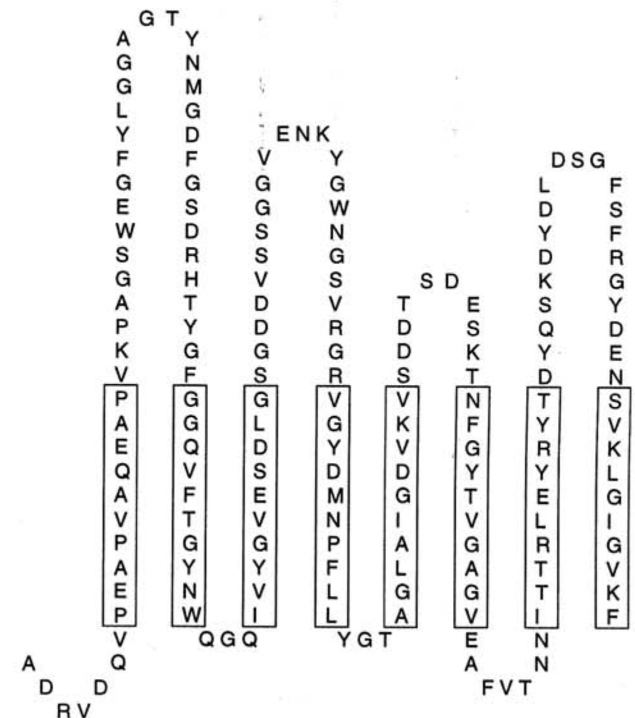

Fig. 4. Topological model of RopB, containing eight transmembrane segments (see Fig. 3) in a consecutive hydrophobic amino acid pattern.

vealed two transcriptional start sites (Fig. 5). One *ropB* transcript starts at a residue 87 nucleotides upstream of the initiation codon, and a less efficient second start site was found at position 538, 21 nucleotides downstream of the first and main transcription start. These positions are indicated in Figure 2 by asterisks. No clear –10 and –35 promoter sequence for RNA polymerase could be observed for either transcriptional start site.

Transcription is probably terminated in a rho-independent manner (Platt 1981) via an inverted repeat of 10 nucleotides between position 1264 and 1288 (Fig. 2, horizontal arrows), 24 nucleotides downstream from the *ropB* gene.

Conservation of *ropB* in other bacteria.

From preliminary experiments with *ropA* we know that strains that do not have the Mab38 epitope do contain sequences that hybridize strongly with *ropA* (data not shown). Hence we wished to establish whether *ropB* was conserved among various gram-negative bacteria. We used strains of which the presence or absence of the Mab8 epitope has been determined previously (de Maagd 1989) and a number of which the presence or absence of the Mab8 epitope was not known. We performed a genomic Southern blot with a number of gram-negative bacterial strains. Genomic DNA was digested with *Eco*RI, electrophoresed on 1% agarose, blotted, probed, and washed at medium stringency. As a probe we used a PCR fragment from nucleotides at position 532 until 1346. In this way we used a probe with the complete coding region of *ropB* and an additional 82 and 106 nucleotides 3′ and 5′ from the coding region, respectively. The probe hybridized strongly with all the tested *R. leguminosarum* strains. All three *R. meliloti* strains (Fig. 6, lanes 7–9) and also *Rhizobium* sp. NGR234 and *A. tumefaciens* hybridized (Fig. 6, lanes 10 and 12, respectively). No hybridization was visible with *B. japonicum*, *P. fluorescens*, and *E. coli* (Fig. 6, lanes 11, 13, and 14, respectively). From this experiment we conclude that *ropB* is not only present in strains that express the Mab8 epitope, like the *R. leguminosarum* strains, but that a well-conserved sequence is present in other *Rhizobium* species and in *A. tumefaciens* as well.

Regulation of RopB decrease as detected with Mab8.

The environmental conditions inside a nodule differ substantially from that outside (Witty et al. 1986). For instance, bacteroids depend on the supply of dicarboxylic acid as their main energy source (McRae et al. 1989). In an attempt to find factor(s) involved in the decrease of the Mab8 epitope of RopB we varied a number of growth conditions. The parameters that were varied include: carbon sources (mannitol, succinate, fumarate, or malate), nitrogen sources (NH_4^+ instead of NO_3^-), pH (5.0, 5.5, 6.0, 7.0, 7.5, or 8.0), low oxygen (5, 2.5, or 1%), Ca^{2+} concentrations (0 or 5.5 mM) or combinations of parameters. Cultures were maintained under such conditions for approximately 10 generations of growth, as tested by monitoring the optical density at 660 nm. Cell envelopes were isolated, electrophoresed, transferred to nitrocellu-

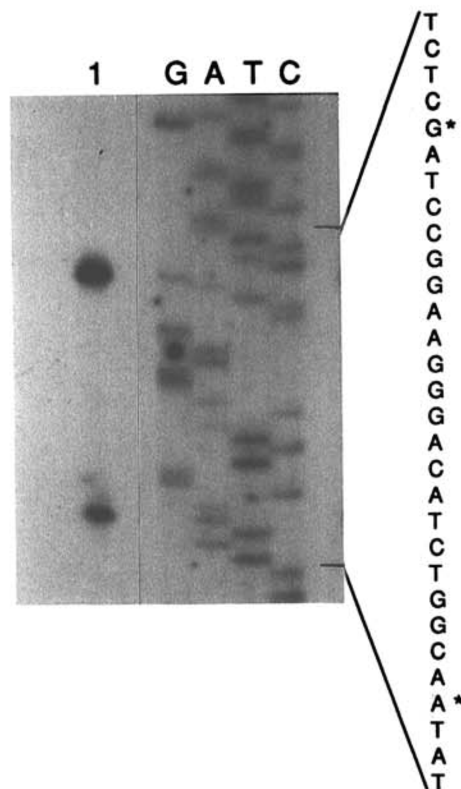

Fig. 5. Determination of the transcriptional start site by primer extension. The primer used is indicated in Figure 2. The sequence obtained with this primer (lanes G, A, T, and C) was run alongside the products of the extended primer (lane 1). The sequence lanes were exposed to X-ray film for 36 h, the lane with the extension products for 16 h. The transcription start sites are indicated on the right with asterisks.

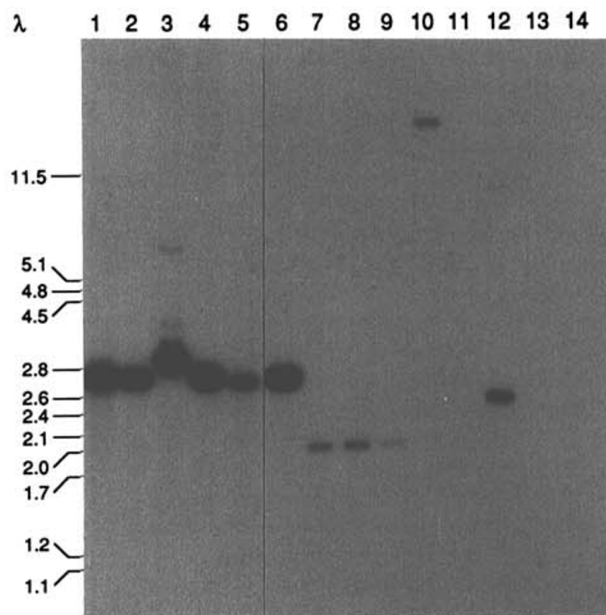

Fig. 6. Southern blot analysis of *Eco*RI-digested genomic DNA from *Rhizobium leguminosarum* bv. *viciae* strains 248 (1), RBL1471 (2), TOM (3), and RBL5523 (4), biovar *trifolii* strain ANU843 (5), biovar *phaseoli* strain 8401 (6), *R. meliloti* strains 1021 (7), AK631 (8) and 102F34 (9), *Rhizobium* sp. NGR234 (10), *B. japonicum* strain USDA110 (11), *A. tumefaciens* strain LBA201 (12), *P. fluorescens* strain WCS365 (13) and *E. coli* strain KMBL1164 (14). The high molecular weight forms observed with strains TOM and RBL5523 are due to incomplete *Eco*RI digestion. On the left the fragment sizes in kbp of *Pst*I digested phage lambda DNA are indicated.

lose and RopB was detected using Mab8. Under all these tested (combinations of) conditions no decrease was detected.

To test whether the decreased detection is caused by a factor of plant origin, cells were grown in the presence of aquaeous and chloroform extracts of pea plant roots or nodules. Also this approach did not result in a diminished RopB detection.

DISCUSSION

The results presented in this study describe the isolation and characterization of a gene that is involved in the production of the antigen group II proteins of *R. l.* bv. *viciae* strain 248. The gene could only be obtained by developing a novel method. This method allowed the detection of the Mab8 epitope in *R. meliloti* and *A. tumefaciens* after treatment of whole cells with lysozyme and boiling in β-mercaptoethanol and SDS. Figure 1 shows that only *ropB* is necessary for expression of the antigen group II in these bacteria, since all protein bands detected with Mab8 are expressed when this gene is present.

RopB shows all properties of an outer membrane protein. RopB has a well-defined signal sequence of 22 amino acids for transport across the cytoplasmic membrane (von Heijne 1986). This results in a mature protein of 20.3 kDa which is in agreement with the M_r of the antigen group II primary protein band. It contains several β-sheets and at the C-terminal end a phenylalanine residue is present that is considered essential for an efficient translocation of proteins in the outer membrane (Struyvé et al. 1991). It therefore is clear that *ropB* is not a regulatory gene, but the structural gene of the outer membrane antigen group II.

RopB is transported and integrated in the outer membrane in a similar way in *A. tumefaciens* as it is in *R. leguminosarum* as shown in Western blots (Fig. 1). This suggests a similar linkage of RopB to the peptidoglycan layer of *A. tumefaciens*. RopB could not be detected in *E. coli* when *ropB* was under the control of its own promoter.

Eight transmembrane spanning amphipathic β-sheets of about 10 alternating hydrophobic amino acid residues were identified in the amino acid sequence of RopB. Based on these membrane-spanning regions we propose a topology as shown in Figure 4. Since no homology with any known sequences was found, this model as well as the function of RopB remains to be resolved.

After isolating the gene we determined the start and stop of transcription. The transcription appears to start at two different positions. Whether the transcription start at position 538

Table 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
R. leguminosarum		
248	Wild type bv. *viciae*	Josey et al. 1979
TOM	Wild type bv. *viciae*. Nodulates Afghan peas	Lie 1978
RBL1471	class I LPS mutant of strain 248	de Maagd et al. 1989b
RBL5523	bv. trifolii cured of pSym5, with Sym plasmid pRL1JI	Priem et al. 1984
ANU843	Wild type bv. *trifolii*	Rolfe et al. 1980
8401	bv. *phaseoli* cured of its Sym plasmid	Lamb et al. 1982
R. meliloti		
LPR2120	RCR2001, *rif*	Hooykaas et al. 1982
AK631	*exoB* mutant of strain Rm41	Kondorosi et al. 1989
1021	SU47, *str*	Meade et al. 1982
102F34	Wild type *R. meliloti*	S. Long, Stanford, CA
Rhizobium sp.		
NGR234	Wild-type strain, nodulates tropical legumes	Trinick 1980
B. japonicum		
USDA110	Wild-type strain	Nieuwkoop et al. 1987
A. tumefaciens		
LBA201	C58 wild-type strain, contains Ti plasmid	Hooykaas et al. 1982
LBA4301	Ach5, cured of its Ti-plasmid, *rif recA*	Klapwijk et al. 1979
E. coli		
JM101	Δ(*lac-proAB*) *supE thi* (F′*traD36 proAB lacI*^{q}ZΔM15)	Yanish-Perron et al. 1985
KMBL1164	Δ(*lac-pro*) *supE thi*	P. v.d. Putte, Leiden
Pseudomonas		
WCS365	Wild type *P. fluorescens*	de Weger et al. 1986
Plasmids		
pIC20H	Multicopy cloning vector, IncColE1	Marsh et al. 1984
pRK2013	Helper plasmid for triparental matings	Ditta et al. 1980
pLAFR1	IncP, Mob$^+$ cosmid vector	Friedman et al. 1982
pMP92	IncP cloning vector	Spaink et al. 1987
pMP220	IncP expression vector containing a promoterless *lacZ* gene	Spaink et al. 1987
pMP4002	pLAFR1 clone with an insert of genomic DNA from strain 248	This work
pMP4003	2.6-kb *Eco*RI subclone of pMP4002 in pMP92	This work
pMP4004	2.6-kb *Eco*RI subclone of pMP4003 in pIC20H	This work
pMP4007	1.8-kb *Eco*RI-SacI fragment of pMP4003 in pMP92	This work
pMP4008	*Sal*I deletion of pMP4004	This work
pMP4009	1.1-kb *Sal*I fragment of pMP4008 as a *Hin*dIII fragment in pMP92	This work
pMP4014	1.2-kb *Sal*I fragment of pMP4003 in pIC20H	This work
pMP4018	1.2-kb *Eco*RI–*Pst*I fragment of pMP4014 in pMP220	This work
pMP4022	1.1-kb *Hin*dIII fragment of pMP4008 in pMP220	This work

is just less efficient or, for example, caused by an intermission in the reverse transcriptase activity is not known.

The transcription termination of *ropB* is probably rho-independent.

Detection of antigen group II with Mab8 is severely decreased in bacteroids as compared to free-living bacteria (de Maagd et al. 1989a). The exact cause of this decrease is yet to be determined. For RopA, another outer membrane protein that is severely decreased during bacteroid development, it is shown that transcription of this gene in mature bacteroids is reduced to a very low level (de Maagd et al. 1994). This might also be the case for RopB, since RopB is invisible in bacteroid preparations and also not detectable with a polyclonal antiserum (de Maagd et al. 1989a). The region upstream from the major transcription start site was screened for promoter sequences that might be involved in down regulation during symbiosis (de Maagd et al. 1992). No consensus homology was found. Like in the *ropA* promoter a consensus sequence for the integration host factor (IHF) was detected. This factor, which has been shown to play a role in a number of regulatory processes (Friedman 1988), might be involved in the down regulation of *R. leguminosarum* outer membrane proteins. We identified a possible IHF binding site at nucleotides 378–390. This site, however, was not conserved in nucleotide 386 and 388 and was located in a moderate AT-rich region. The facts that the *ropB* promoter is not active in *E. coli* and that *ropB* homologs were only detected in *Rhizobium* and *Agrobacterium* suggest that the outer membrane antigens that decrease during bacteroid development are under the control of a regulatory element(s) that is specific for the latter two bacterial species. Based on recent data, however, it is not likely that antigen group II and III are regulated in the same way (Roest et al. 1995). The type of nodule that is infected strongly determines the down regulation of the different outer membrane antigen groups.

Apart from direct evidence that there is an anaerobic environment inside the nodule (Witty et al. 1986), not much is known about the conditions inside a root nodule. In addition, bacteroids are surrounded by a peribacteroid membrane, thus creating an environment that is even more difficult to understand. Changes identical to those observed in bacteroids could be induced ex planta. Variation in the growth conditions, such as succinate as a carbon source or a change in pH, resulted in the production of the bacteroid form of LPS (Kannenberg and Brewin 1989; Tao et al. 1992). Providing dicarboxylic acids as the carbon source results in induction of the dicarboxylic acid transport (*dct*) system (Ronson and Astwood 1985), and anaerobic conditions are a prerequisite for the expression of *nifA*, the regulatory gene of the cascade *nif* and *fix* genes (Gussin et al. 1986). Our attempts to detect physiological conditions or signals responsible for the decreased detection of RopB with Mab8 antibodies were not successful.

Theoretically, modification of RopB during bacteroid development would also explain the immunochemical decrease in RopB detection. In fact, a weak additional band was found in bacteroid lysates that reacted with the Mab8 antibodies (Roest et al. 1995). This band was, however, not observed in bacteroids from every host plant tested. It might therefore represent a completely new protein that is expressed after bacteroid differentiation in only a number of host plant species or it may simply represent a degradation product.

MATERIALS AND METHODS

Strains, plasmids, and media.

The strains and plasmids used in this study are listed in Table 1. Tetracyclin (*tc*) was added to a concentration of 20 μg/ml for *E. coli*, 5 μg/ml for *R. meliloti*, and *A. tumefaciens* and 2 μg/ml for *R. leguminosarum*. Rifampicin (*rif*), streptomycin (*str*), and spectinomycin (*spc*) were added to a final concentration of 20, 500, and 200 μg/ml, respectively. B^- medium was used in most of the experiments (van Brussel et al. 1977) with mannitol as the carbon source in a concentration of 25.65 mM. The effects of various carbon sources on the expression of outer membrane antigen groups was investigated using B^-suc, B^-fum, and B^-mal containing 25.65 mM of succinate, fumarate, or malate, respectively, instead of mannitol. To prevent a metabolic pH shift during this experiment the media were supplemented with 40 mM 2-(*N*-morpholino)ethanesulfonic acid (MES, pH 6.0). In experiments where the influence of the pH was tested, B^- was supplemented with the following buffers at a concentration of 40 mM: piperazine at pH 5.0; MES at pH 5.5 and at pH 6.0; 3-(*N*-morpholino)propanesulfonic acid (MOPS) at pH 7.0; *N*-(2-hydroxyethyl)piperazine-*N′*-ethanesulfonic acid (HEPES) at pH 7.5 and *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) at pH 8.0. All buffers were obtained from Sigma (St. Louis, Missouri). To test the effect of different Ca^{2+} concentrations on antigen expression, the amount of $Ca(NO_3)_2.4H_2O$ in B^- was varied and the loss of nitrate was compensated by adding KNO_3. In some experiments NH_4^+ was used as the nitrogen source instead of NO_3^-. In such cases $Ca(NO_3)_2.4H_2O$ and KNO_3 were omitted and replaced by equimolar amounts $CaSO_4.2H_2O$ and K_2SO_4, respectively. For experiments under various oxygen concentrations TY medium (Beringer 1974) was used to culture the bacteria. The experimental setup used was as described by Kannenberg and Brewin (1989). Oxygen/nitrogen gas mixtures of 5%/95%, 2.5%/97.5%, and 1%/99% were purchased from Hoekloos (Amsterdam, The Netherlands) and the supplied gas volume was regulated with a Sho-Rate Flowmeter (Brooks Instrument, Veenendaal, The Netherlands).

Growth in the presence of plant extracts.

Equal amounts of fresh weight pea nodules and roots were extracted according to the method of Bligh and Dyer (1959). Both the water/methanol extracts and the chloroform extracts were evaporated and stored at –20°C. Cells were grown in TY medium supplemented with 1% (v/v) of these extracts for 16 h.

DNA manipulations and bacterial matings.

Recombinant DNA techniques were carried out essentially as described by Sambrook et al. (1989). Plasmids were transferred from *E. coli* to *Rhizobium* or *Agrobacterium* strains using the triparental mating described previously (Ditta et al. 1980) with pRK2013 as the helper plasmid. PCR was performed on a PREM III apparatus from Wessex (Andover, England) with heat-stable PFU DNA polymerase (Stratagene, La Jolla, Calif.) in a 100-μl reaction volume.

Cell envelopes, electrophoresis, and Western blotting.

Cell envelopes were isolated as described earlier (Roest et al. 1995). SDS-polyacrylamide gel electrophoresis was per-

formed on 11% polyacrylamide gels as described previously (Lugtenberg et al. 1975). Cell envelope components separated on SDS-polyacrylamide gels were transferred to nitrocellulose by electroblotting using a LKB Multiphor 2117 (Pharmacia). Immunodetection with monoclonal antibodies was carried out as described previously (de Maagd et al. 1989a).

Colony blotting.

To detect cosmids that express the Mab8 antigen, bacteria of *R. meliloti* strain LPR2120 containing cosmids of the *R. l.* bv. *viciae* strain 248 genomic library (de Maagd et al. 1992) were grown on YMB medium supplemented with tetracyclin (5 μg/ml) and rifampicin (20 μg/ml). Colonies were transferred to nitrocellulose (Schleicher & Schull BA85) as described elsewhere (de Maagd et al. 1989b). The blots were treated with lysozyme (1 mg/ml) at 37°C for 30 min and rinsed with demineralized water. Subsequently the blots were boiled in a solution of 2% SDS (w/v) in 0.1 M Tris (pH 7)/5% β-mercaptoethanol for 3 min. Immunodetection was performed as described by de Maagd et al. (1989a) with the exception that peroxidase-conjugated rabbit anti-mouse IgG (Sigma) was used as the second antibody and 5,5′,3,3′-tetramethylbenzidine as the substrate.

DNA sequencing.

DNA sequencing was performed according to Sanger et al. (1977) using either M13 vector Tg130 (Kieny et al. 1983) or pIC20H (Marsh et al. 1984) plasmids with (α-^{35}S)dATP (Amersham International, Little Chalfont, England). The primers used were synthesized in a DNA synthesizer model 392 (Applied Biosystems, Maarssen, The Netherlands). Sequenase (U.S. Biochemicals, Cleveland, Ohio) and T7-polymerase (Pharmacia LKB, Woerden, The Netherlands) were used according to the manufacturer's guidelines. The nucleotide sequence of the *ropB* gene has been deposited in the Genbank/EMBL library under number X80767.

Primer extension.

mRNA from TY-grown *R. l.* bv. *viciae* strain 248 was isolated as described by van Slogteren et al. (1983). The primer was labeled with (γ-^{32}P)dATP (Amersham) using polynucleotide kinase (Pharmacia) as described by Sambrook et al. (1989). Primer extension was performed using avian myeloblastosis virus reverse transcriptase (Pharmacia) annealed on approximately 20 μg of RNA at 45°C. To prevent self-copying of labeled primer actinomycin D was added to the reaction in a final concentration of 50 μg/ml.

Predictions based on computer models.

A possible signal sequence cleavage site was calculated using the standard weight-matrix approach of von Heijne (1986). The nonpolar transbilayer helices in amino acid sequences of membrane proteins were predicted with the method of Kyte and Doolittle (1982) using a window of nine amino acids.

Southern blot analysis.

Digested genomic DNA was separated in 1% (w/v) agarose gels and transferred to Genescreen filters (New England Nuclear, Boston, Mass.) by standard methods (Sambrook et al. 1989). Hybridization was performed at 42°C in 50% formamide—5× SSPE (1× SSPE is 150 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA)—5% (w/v) SDS. The blots were washed at 65°C for 15 min with 2× SSPE/0.1% SDS, followed by washings in 1× SSPE/0.1% SDS and 0.1× SSPE/0.1% SDS for the same period. Probes were nick-translated with (α-^{32}P)dCTP (Amersham).

β-Galactosidase assay.

Promoter activity was measured assaying the β-galactosidase activity of *lacZ* fusions as described by Miller (1972).

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