JOURNAL OF BACTERIOLOGY, Apr. 1997, p. 2132–2140 Vol. 179, No. 7
0021-9193/97/$04.00+0

The *rkpGHI* and *-J* Genes Are Involved in Capsular Polysaccharide Production by *Rhizobium meliloti*

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Received 17 October 1996/Accepted 30 January 1997

The first complementation unit of the *fix-23* region of *Rhizobium meliloti*, which comprises six genes (*rkpABCDEF*) exhibiting similarity to fatty acid synthase genes, is required for the production of a novel type of capsular polysaccharide that is involved in root nodule development and structurally analogous to group II K antigens found in *Escherichia coli* (G. Petrovics, P. Putnoky, R. Reuhs, J. Kim, T. A. Thorp, K. D. Noel, R. W. Carlson, and A. Kondorosi, Mol. Microbiol. 8:1083–1094, 1993; B. L. Reuhs, R. W. Carlson, and J. S. Kim, J. Bacteriol. 175:3570–3580, 1993). Here we present the nucleotide sequence for the other three complementation units of the *fix-23* locus, revealing the presence of four additional open reading frames assigned to genes *rkpGHI* and *-J*. The putative RkpG protein shares similarity with acyltransferases, RkpH is homologous to short-chain alcohol dehydrogenases, and RkpJ shows significant sequence identity with bacterial polysaccharide transport proteins, such as KpsS of *E. coli*. No significant homology was found for RkpI. Biochemical and immunological analysis of Tn*5* derivatives for each gene demonstrated partial or complete loss of capsular polysaccharides from the cell surface; on this basis, we suggest that all genes in the *fix-23* region are required for K-antigen synthesis or transport.

Cell surface polysaccharides of gram-negative bacteria have been shown to play an important role in interactions with other living organisms. These molecules are surface antigens which are involved in both pathogenic (7) and symbiotic (15) processes, mediating resistance to important host defense mechanisms. A variety of polysaccharides, including extracellular polysaccharides (EPS), lipopolysaccharides (LPS), capsular polysaccharides (CPS), and cyclic β-(1,2)-glucans, are produced by rhizobia. Depending on the species and strains, some or all of these molecules have been shown to play a role in different steps of symbiotic nodule development (10). In *Rhizobium meliloti*, the symbiotic partner of alfalfa (*Medicago sativa*), the EPS were shown to play an important role in the root hair infection process and in infection thread development (17).

It has been demonstrated that genes of the *fix-23* region of *R. meliloti* 41 are required for the synthesis of a specific type of CPS (K polysaccharide [KPS]) and that the *fix-23* genes compensate for *exo* mutations, suggesting that KPS can provide the same functions as EPS during symbiotic nodule development (22, 24–26).

Although the complete structure of *R. meliloti* KPS has not been determined, available data indicate that it consists of a disaccharide repeating unit of α-keto-3,5,7,9-tetradeoxy-5,7-diaminononulonosic acid, which is a variant of 3-deoxy-D-*manno*-2-octulosonic acid (KDO) and aminohexuronic acid (26). The KPS preparation from strain AK631 (*R. meliloti* 41 *exoB*) showed a banding pattern that corresponds to the degree of polymerization which is affected by the *rkpZ* gene. The low-molecular-weight (LMW) form of KPS consists of 8 to 15 repeating units, and the high-molecular-weight (HMW) KPS is composed of 32 to 40 repeating units (26). This molecule is likely to be a surface component that binds bacteriophage 16-3 and is structurally analogous to the group II K antigens of *Escherichia coli* (22, 25). Such molecules have been identified in various microorganisms, such as in *E. coli* (29), *Neisseria meningitidis* (12), *Haemophilus influenzae* (16), and *R. fredii* (25).

Previous results showed that the *fix-23* region comprises four complementation units (24). Nucleotide sequence analysis of complementation unit I (Fig. 1) revealed six open reading frames exhibiting significant similarity to fatty acid synthase genes, including β-ketoacyl synthase, acyltransferase, dehydratase, enoyl reductase, keto reductase, and acyl carrier protein, respectively (22). These genes are likely to be involved in the production of a lipid carrier or the lipid anchor of a K-antigen-like polysaccharide structure present on the surface of *R. meliloti* 41 cells (22).

Here we report the nucleotide sequence of complementation units II, III, and IV of the *fix-23* region and the biochemical and immunological characterization of the KPS produced by its Tn*5* mutant derivatives.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, plasmids, culture conditions, and enzyme assays. Strain AK631 (3) is a Fix^+ Exo^- derivative of *R. meliloti* 41. PP428 is an *E. coli* strain harboring the *fix-23* region on cosmid clone pPP428 (24). Two subclones in plasmid pUC19 from this cosmid clone carrying complementation units II, III, and IV are described as pPP775 and pPP933 (this work). Rm5830 (31) is a Fix^- Exo^- derivative of AK631 carrying a Tn*5* insertion in the *rkpZ* gene. The *fix-23*::Tn*5* mutant strains and *fix-23*::Tn*10LK* derivatives (Fig. 1), as well as phage 16-3, have been described previously (24). *E. coli* JM109 was used as a host strain in cloning and sequencing procedures (32). Antibiotic concentrations and growth conditions for *E. coli* and *R. meliloti* strains have been described previously (23, 24). β-Galactosidase activities of the different translational Tn*10LK* fusions were measured as described by Miller (19).

DNA manipulations. Plasmid and single-stranded DNA isolation, restriction enzyme digestions, agarose gel electrophoresis, purification of fragments, and cloning were done by standard methods compiled by Maniatis et al. (18). The enzymes applied in recombinant DNA experiments were used as recommended

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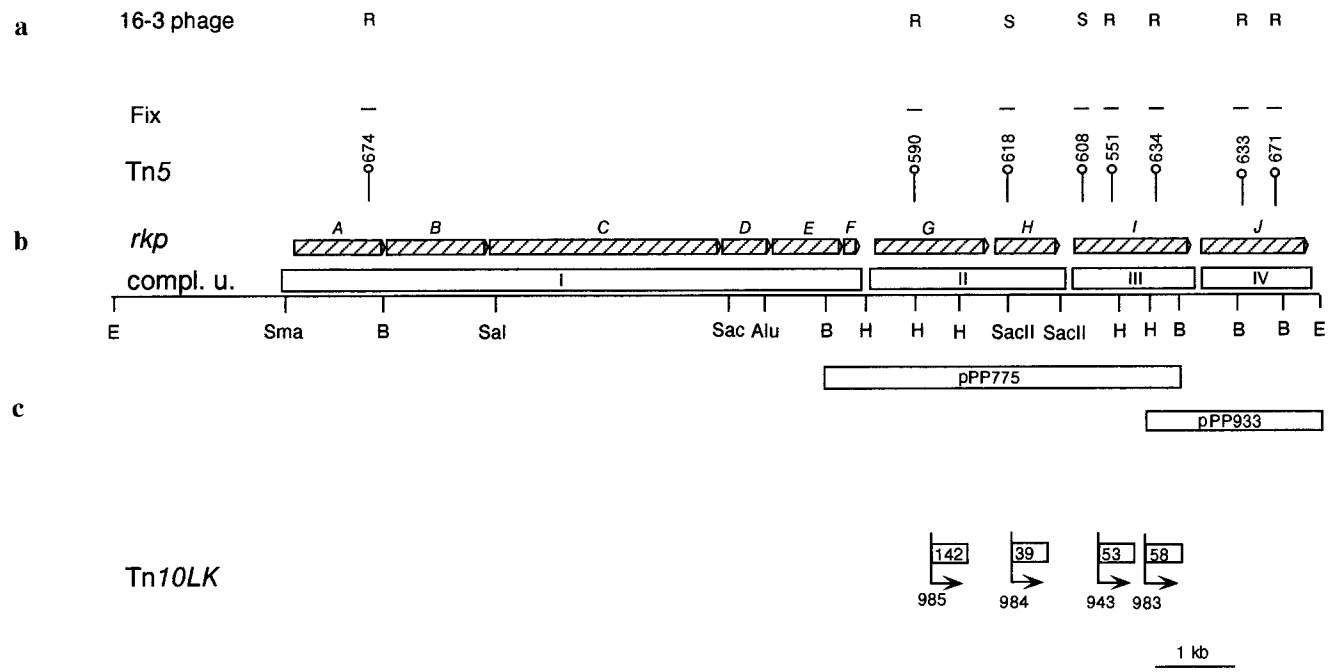

FIG. 1. Organization of the *fix-23* region. (a) Lollipops indicate Tn*5* insertions resulting in a Fix$^-$ phenotype in AK631. Most mutations also made the strain resistant to phage 16-3 (S, phage sensitive; R, phage resistant). (b) The genes and the four complementation units. Restriction sites (Alu, *Alu*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Sac, *Sac*I; SacII, *Sac*II; Sal, *Sal*I; Sma, *Sma*I) used in subcloning experiments are shown. (c) DNA fragments in plasmid clones used for subcloning. The boxed numbers at the bottom are the β-galactosidase activities of the different translational Tn*10LK* fusions in Miller units (18). Arrowheads indicate the orientation of the Tn*10LK* insertions.

by the suppliers. For sequencing, appropriate restriction fragments of the region were subcloned into vectors M13mp18 and M13mp19 (32). Determination of the DNA sequence was performed by the dideoxy-chain termination method (30), using [^{35}S]dATP and the Sequenase kit as described in the USB Corporation sequence protocols. Additional information was obtained by sequencing the Tn*5*-flanking regions of different insertion mutants with a Tn*5*-specific oligonucleotide primer (5′-GCAAAACGGGAAAGGTTCCG-3′).

Computer-assisted sequence analysis. DNA sequence analysis was done with computer programs from the PC/Gene software package (designed by Amos Bairoch, Intelligenetics Corp.). Amino acid homology searches were performed against the "nonredundant" database of the NCBI BLAST E-Mail Server by using the BLASTX program (1). Sequence alignments and codon preference analysis were done with the University of Wisconsin Genetics Computer Group program package for the VAX (8).

Polysaccharide preparation. For electrophoresis and immunoblot analyses, bacterial pellets from 3-ml cultures were extracted by a modified hot phenol-water method; the water phases were dialyzed against distilled H_2O and freeze-dried. For large-scale preparations (5 to 15 liters), the extraction was followed by gel filtration chromatography over Sephadex G-150 superfine (0.2 M NaCl, 1 mM EDTA, 10 mM Tris base, 0.25% deoxycholic acid, pH 9.25), and the eluted fractions were assayed chemically for KDO by the thiobarbituric acid assay and for hexose with phenol-sulfuric acid. The fractions were also assayed for KPS and LPS by polyacrylamide gel electrophoresis (PAGE) (described below). These protocols have been described and cited previously (25).

PAGE and immunoblot analyses. Three-milliliter cultures of each bacterial strain were grown in liquid medium (22, 25), each to an optical density at 600 nm (OD_{600}) of 0.900. Each bacterial pellet, obtained by centrifugation, was extracted with hot phenol-water (22, 25) by using 500 μl each of 45% phenol and water. The water layer was obtained for each strain, and 1 μl of each water layer was used for PAGE and immunoblot analyses. The gels were either silver stained for LPS or Alcian blue (AB)-silver stained for KPS as previously described (25) but without the oxidation step. In this way, only KPS stains without interference from LPS; moreover, the intensity of staining is equal to that obtained with periodate oxidation. By this method, the crude KPS preparations (even though they do contain both LPS and KPS) stain in a manner identical to that of purified KPS. For serotype analysis, PAGE gels containing the cell extracts were blotted to Nytran+ (Schleicher & Schuell, Keene, N.H.) with a Trans-Blot SD apparatus (Bio-Rad, Richmond, Calif.), incubated with rabbit antibodies directed against *R. meliloti* 41, and developed with goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase. A stock of anti-Rm41 antibody was donated by Dale Noel (Marquette University, Milwaukee, Wis.). In some analyses, the antiserum was preadsorbed with an equal amount of whole cells of either mutant or wild-type *R. meliloti*. Antiserum development, preadsorption, and immunoblot protocols have already been described (22).

Nuclear magnetic resonance (NMR) analysis. ^{1}H NMR analysis was performed on a Bruker AM 250 in $2H_2O$. The measurement was made at 296 K.

ELISA. The enzyme-linked immunosorbent assay (ELISA) used was adapted from that of Fuhrmann and Wollum (13). Briefly, cell pellets from 3-ml cultures were washed and resuspended in coating buffer to an OD_{600} of 0.5. A 0.1-ml volume of the cell suspension was added to each well of a 96-well microtiter plate (Nunc), which was incubated overnight at 4°C. The plates were washed three times with phosphate-buffered saline (PBS)-Tween, 0.1 ml of a PP674-preadsorbed (22) anti-Rm41 suspension (1/1,000 dilution in PBS-Tween) was added, and the plates were left at room temperature for 3 h. The wash step was repeated, 0.1 ml of goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (diluted 1/1,000 in PBS-Tween) was added, and the plates were incubated overnight at 4°C. After a third wash step, 0.1 ml of substrate was added and color was allowed to develop. The OD_{492} was read in an ELISA reader. Three ELISAs were done in each of duplicate experiments for a total of six ELISAs per strain. The OD_{492} values were obtained after subtraction of the background value observed for strain PP674. The experiment was also done with anti-Rm41 that had not been preadsorbed with PP674 cells.

RESULTS

Nucleotide sequence analysis of complementation groups II, III, and IV of the *fix-23* region. The complete nucleotide sequences of the 4.3-kb *Bam*HI fragment from plasmid pPP775, containing complementation units II and III, and the 1.9-kb *Hin*dIII-*Eco*RI fragment from pPP933, harboring complementation unit IV (Fig. 1), were determined on both strands. For this purpose, overlapping restriction fragments were subcloned into M13mp18 and M13mp19 vectors and sequenced by using a universal primer. To complete and confirm the sequence data, additional oligonucleotides were derived from the sequences obtained and specific primers constructed for the ends of the Tn*5* transposon arms were used. The nucleotide sequence relevant to the present paper and our previous publication (22) appears in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession number X64131.

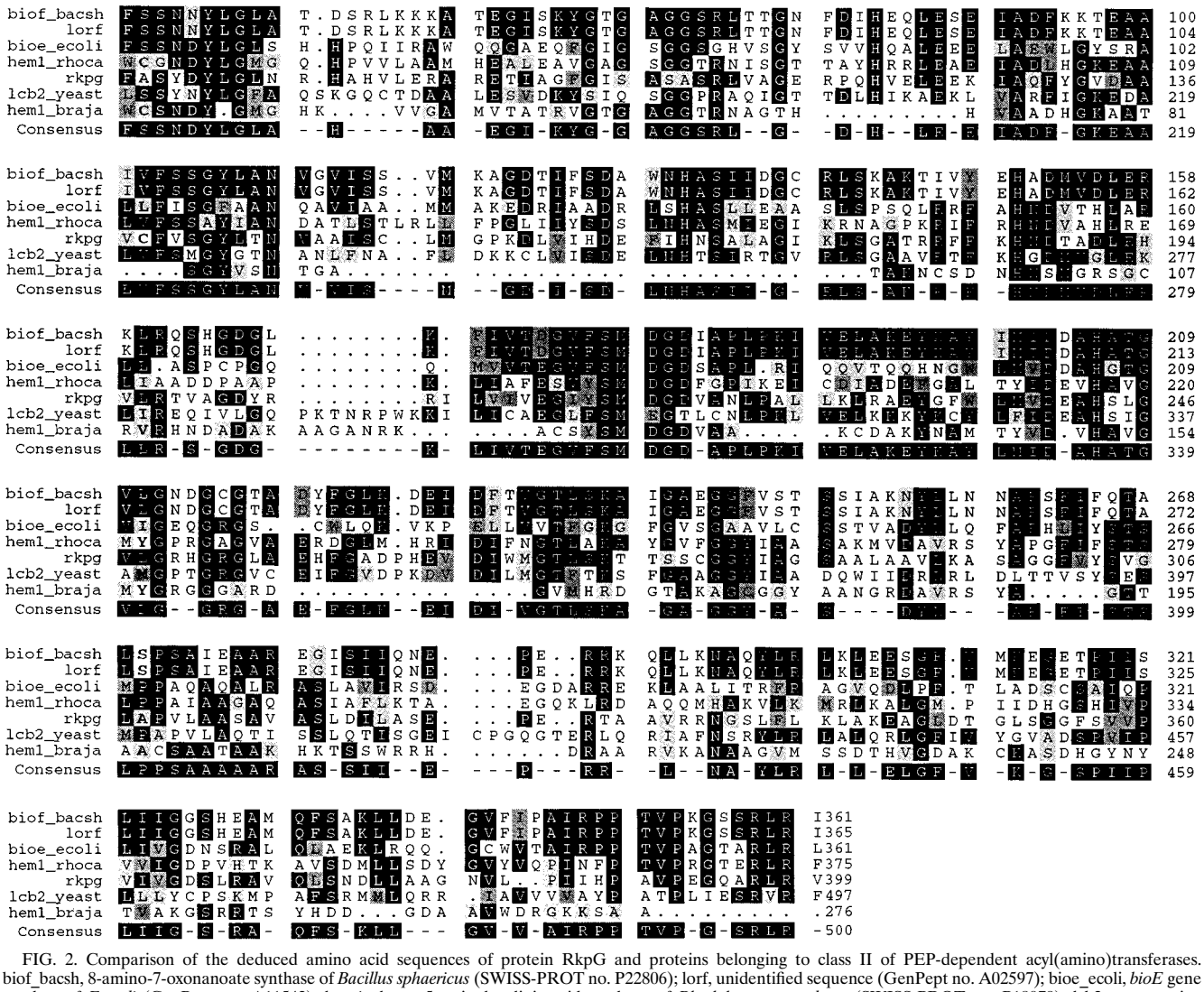

FIG. 2. Comparison of the deduced amino acid sequences of protein RkpG and proteins belonging to class II of PEP-dependent acyl(amino)transferases. biof_bacsh, 8-amino-7-oxonanoate synthase of *Bacillus sphaericus* (SWISS-PROT no. P22806); lorf, unidentified sequence (GenPept no. A02597); bioe_ecoli, *bioE* gene product of *E. coli* (GenPept no. A11542); hem1_rhoca, 5-aminolevulinic acid synthase of *Rhodobacter capsulatus* (SWISS-PROT no. P18079); lcb2_yeast, serine palmitoyltransferase 2 of *Saccharomyces cerevisiae* (SWISS-PROT no. P40970); hem1_braja, 5-aminolevulinic acid synthase of *Bradyrhizobium japonicum* (SWISS-PROT no. P08262). The complete nucleotide sequence of the *fix-23* region is in the EMBL database under accession number X64131. Black boxes represent amino acid identity, and gray boxes show amino acid similarity; the consensus sequence of the alignments is in the bottom line.

Computer-aided analysis with the PC/Gene program package revealed four open reading frames (ORFs). Codon preference analysis (8) confirmed that the codon usage in these ORFs is in accordance with most of the *R. meliloti* genes sequenced so far. Neither typical −10 and −35 promoter nor typical terminator sequences were observed. Recently, the designation *rkp* was proposed for genes involved in KPS production by rhizobia (26). Therefore, the previously identified genes of the *fix-23* region were designated *rkpABCDEF*. Consequently, the newly determined genes were termed *rkpG*, *rkpH*, *rkpI*, and *rkpJ*. To confirm the direction of transcription in complementation units II, III, and IV of the *fix-23* region, *lac* fusions (22) were generated by site-directed Tn*10LK* transposon mutagenesis of cosmid clone pPP428 and the β-galactosidase activities were determined. The orientation of the active *lac* fusions (30 to 150 Miller units) that mapped within the complementation units was left to right, while insertions with the opposite orientation exhibited background levels of β-galactosidase activity (under 10 Miller units), supporting the notion that the four genes were transcribed in the same orientation, from left to right (Fig. 1).

Three possible ATG start codons were found for *rkpG* (complementation unit II) at nucleotide positions 7599, 7635, and 7671, respectively. A likely Shine-Dalgarno (SD) sequence was found 13 bp 5′ to the third start codon, suggesting that the ORF starts at position 7671 and terminates at position 9011. *rkpG* contains 1,341 nucleotides determining a protein of 446 amino acid residues with a predicted molecular mass of 48.2 kDa. Computer-aided structural analysis (9) revealed five hydrophobic amino acid motifs predicted to be membrane-associated helices. Two of them coincide with the transmembrane helices found by the method of Argos and Rao (2) and might act as membrane anchors for this protein.

The predicted product of the *rkpG* gene showed a high degree of similarity to a subfamily of acyltransferases (Fig. 2). The most definitive of these similarities was with the HemA proteins of different organisms involved in δ-aminolevulinic acid synthesis. The highest homology found (36% amino acid

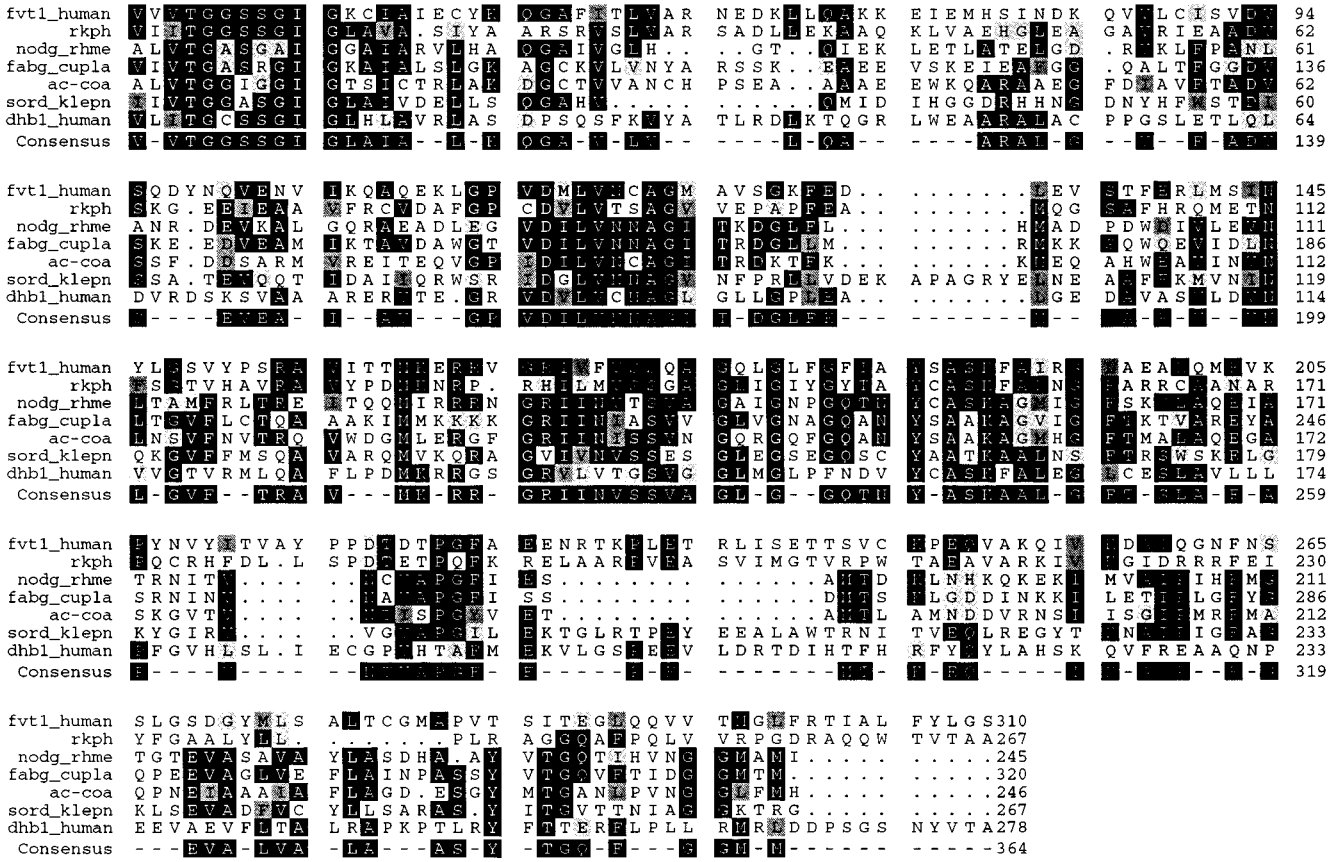

FIG. 3. Alignment of the predicted *rkpH* product and proteins belonging to the ribitol-type dehydrogenase family. fvt1_human, follicular variant translocation protein 1 precursor (SWISS-PROT no. Q06136); nodg_rhme, NodG protein of *R. meliloti* (GenPept no. Y00604); fabg_cupla, 3-ketoacyl-acyl carrier protein reductase of *Cuphea lanceolata* (SWISS-PROT no. P28643); ac-coa, acetoacetyl coenzyme A reductase of *Chromatium vinosum* (PIR no. S29279); sord_klepn, sorbitol-6-phosphate-2-dehydrogenase of *Klebsiella pneumoniae* (SWISS-PROT no. P37079); dhb1_human, 17-β-hydroxysteroid dehydrogenase (SWISS-PROT no. P14061).

identity, 56% amino acid similarity) was to the HemA protein of *Bradyrhizobium japonicum*. These enzymes require a pyridoxal phosphate (PEP) prosthetic group; a segment (GTLSKT TSS) exhibiting strong sequence similarity to PEP-binding sites was found between the putative membrane-spanning regions.

The *rkpH* gene (complementation unit II) contains 840 bp, from nucleotide residues 9048 to 9887. Translation of this sequence predicted a protein of 279 amino acid residues with a calculated molecular mass of 29.7 kDa. No significant SD sequence was found 5′ to the initiation codon. Analysis of the hydropathy plot revealed three potential hydrophobic domains alternating with flexible hydrophilic motifs. The predicted protein product of *rkpH* exhibits similarity to the ribitol-type dehydrogenases (Fig. 3) belonging to the short-chain alcohol dehydrogenase superfamily. The highest amino acid identity (32%) and similarity (54%) found were to the human FVT-1 protein participating in the tumoral process of follicular cells (28). Two regions that typify these enzymes are also conserved in the putative amino acid sequence of RkpH: a glycine-rich cluster (TGGSSGIG) that might be responsible for binding of NAD(P)H (14) and a YCASKFALNGF sequence that is known to be a signature of ribitol-type alcohol dehydrogenases (21), in which amino acids around the positively charged lysine residue from the active center of the molecule. The active sites were predicted to be in the hydrophobic part of the molecule.

The RkpI (complementation unit III) protein consists of 445 residues determined from base 9922 to base 11535 having a calculated molecular mass of 61.1 kDa. A likely SD sequence was found at 12 bp 5′ to the GTG start codon. Six transmembrane helices were predicted in the first half of the amino acid sequence. The molecule is mainly hydrophobic with few hydrophilic domains. Our attempts to find homology to the *rkpI* gene were unsuccessful.

The *rkpJ* gene (complementation unit IV) is 1,239 bp long (from base 11560 to base 12798), encoding a predicted protein of 412 amino acids with a molecular mass of 45.8 kDa. A putative SD sequence was found 8 bp upstream of the ATG start codon. Hydropathy analysis revealed a mainly hydrophilic protein lacking transmembrane- or membrane-associated helices. The predicted amino acid sequence of RkpJ does not contain a typical N-terminal signal sequence either. Comparison of the derived protein sequence of RkpJ with data banks showed significant amino acid similarity (32% identity, 55% similarity) to the KpsS enzyme of *E. coli* (20), and 32% amino acid identity to the LipB protein of *N. meningitidis* was found (Fig. 4). These enzymes are involved in the transport of CPS to the cell surface, possibly through a prerequisite phospholipid substitution step (12).

PAGE analysis of extracted polysaccharides. The cell extracts from derivatives of AK631, carrying mutations in the four *rkp* genes described above, were analyzed by PAGE for KPS production (Fig. 5). Both this study and previous studies have shown that mutations in the *fix-23* region do not affect the LPS of the mutants (22, 26). The PAGE gels were prestained

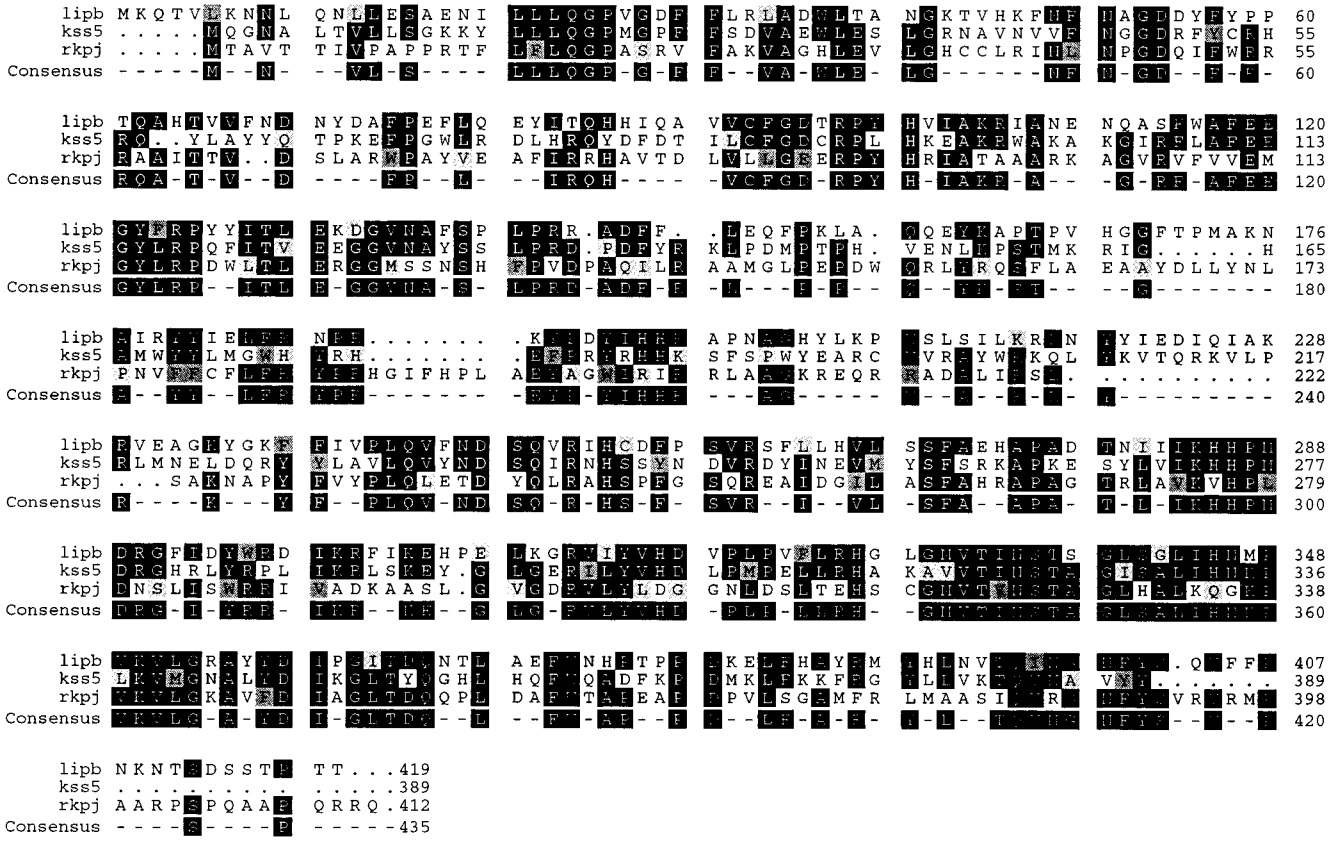

FIG. 4. Comparison of the predicted protein encoded by the *rkpJ* gene (rkpj) with CPS export protein KpsS of *E. coli* (kss5; SWISS-PROT no. P42218) and CPS modification protein LipB of *Neisseria meningitidis* (lipb; SWISS-PROT no. Q05014).

with AB, which is a cationic dye that specifically binds the acidic polysaccharides, and then silver stained without the oxidation step. This allowed maximum staining of KPS without interference from LPS. It has previously been shown that the banding pattern of strain AK631 in Fig. 5, lane 1, is identical to that of purified KPS (22, 25). Thus, the AB staining patterns observed in Fig. 5 are due to KPS.

Each well was loaded with 1 μl of the water layer from phenol-water extraction of a small bacterial culture (see Materials and Methods). Each bacterial culture was grown to an OD_{600} of 0.900 prior to extraction. Since all of the wells contained the same amount of material from each mutant, the differences in the visual intensities of staining reflect differences in the levels of KPS produced by the various strains.

The *rkpH* mutant (PP618) and strain PP608, with a Tn*5* insertion between the *rkpH* and *rkpI* genes, released more KPS upon extraction (lanes 4 and 5) than did mutants affected in the *rkpG rkpI*, and *rkpJ* genes (lanes 2, 3, and 6), as well as those that carry the Tn*5* insertion in the first complementation unit (22). This is shown by more intense AB-specific staining of KPS in PAGE gels (Fig. 5). In addition, the *rkpH* mutant (PP618) produces polysaccharide that is polymerized to a greater degree (i.e., higher molecular weight), as shown by the presence of AB-specific PAGE bands with lower mobility (lane 5) than those obtained with the other mutants (Fig. 5). In contrast, the *rkpJ* mutant (lane 6) was similar to complementation unit I mutant PP674 (22) in that there was limited production of LMW KPS which was not exported (discussed further below).

The facts that PP618 produced the most HMW KPS and that PP608 exhibited less HMW KPS than did PP618 but more than did the others are of particular interest, as these two *fix-23* mutant strains retained partial sensitivity to phage 16-3 (24), whereas all of the other mutants were resistant to this phage. This phage-sensitive phenotype indicates that the KPS produced by these strains is structurally similar to the AK631 polysaccharide and that significant amounts of the polysaccharide are exported to the cell surface.

Immunochemical analyses. Previous studies (22) have shown that PP674 produces a limited amount of LMW KPS and that preadsorption of anti-Rm41 with whole cells of PP674 resulted in complete removal of LPS antibodies and retention of antibodies against KPS. Thus, the LPS was both unchanged by the mutation in PP674 and located on the cell surface, whereas PP674 did not produce or export HMW KPS. For a further determination of polysaccharide structure and export in the phage-sensitive mutants, the cell extracts were analyzed by immunoblotting with anti-Rm41 serum (Fig. 6). PP618 KPS (lane 5) effectively bound anti-Rm41 serum, indicating structural similarity to AK631 KPS. There was minor staining of the small amount of HMW KPS produced by PP608 (visible on the original immunoblot but not readily visible in Fig. 6 [lane 4]) and no staining of the KPS from the other mutants. However, LMW KPS, even that from the parent AK631, does not readily stain with anti-Rm41 during the immunoblotting procedure (22), even though composition and NMR analyses have indicated that both LMW and HMW KPS have identical or very similar structures (26). Since all of the mutants except PP608 and PP618 produce exclusively LMW KPS, this lack of staining may be due to the LMW of these polysaccharides, which makes

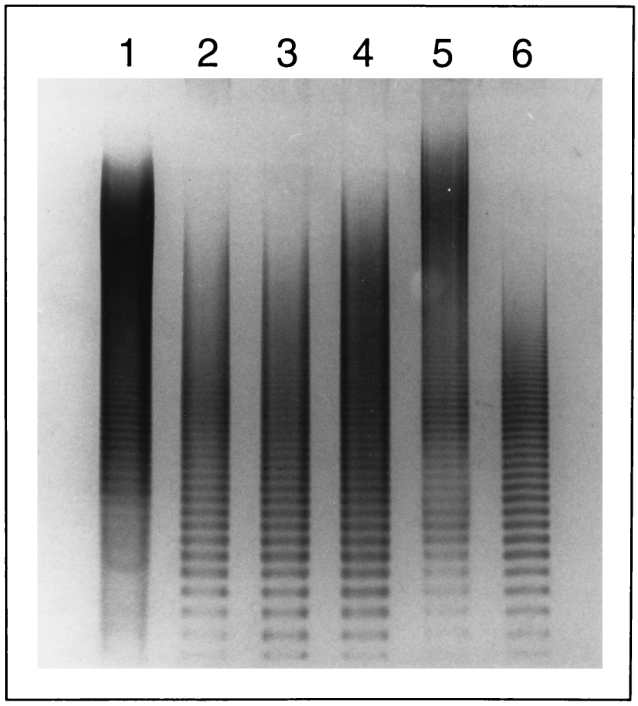

FIG. 5. PAGE analysis of KPS. The gel was AB-silver stained, without oxidation, for KPS-specific analysis. Each lane contained 1 μl of the water layer from the phenol-water extract prepared from equal amounts of strains AK631 (lane 1), PP590 (lane 2), PP551 (lane 3), PP608 (lane 4), PP618 (lane 5), and PP671 (lane 6). The ladder pattern may result from sequential degradation of the KPS during extraction, due to the lability of the glycoside bond of 2-keto-3,5,7,9-tetradeoxy-5,7-diaminononulosonic acid, one of the two sugars in the repeat unit (24).

transfer from the PAGE gel to the membrane inefficient, or may be due to a subtle structural difference in these LMW KPS.

Another experiment showed that the AK631-like KPS produced by PP618 is located on the cell surface. Aliquots of anti-Rm41 serum were separately preadsorbed with equal amounts of cells from three different strains, AK631 (positive control), PP674 (negative control), and PP618 (the phage-sensitive *rkpH* mutant); unadsorbed antiserum was also used. The different antiserum preparations were then tested on immunoblots by using AK631 extracts (Fig. 7). The results obtained with AK631 and PP674 were identical to previous results (22); i.e., the AK631-adsorbed antiserum bound nothing on the blot (lane 1), indicating that all LPS- and KPS-specific antibodies were adsorbed out of the serum, whereas the PP674-adsorbed antiserum stained KPS (lane 3) with an intensity equal to that of the unadsorbed control preparation (lane 2). This result confirmed the finding of the previous study (22) that PP674 did not produce or export significant quantities of KPS found on the surface of AK631. The LPS antibodies were removed, showing that no change in the LPS is associated with the *fix-23* mutation. In contrast to PP674-adsorbed antiserum, PP618-adsorbed antiserum showed KPS staining that was significantly reduced but well above that of the AK631-adsorbed positive control (lane 4). This indicates that PP618 is capable of exporting some level of structurally similar KPS to its cell surface, which also explains the phage sensitivity phenotype. As with PP674, there was no apparent difference in the LPS.

ELISAs of whole cells using PP674-adsorbed anti-Rm41 (i.e., KPS specific) showed that AK631 and Rm41 yielded the same response (Fig. 8), indicating that the cells exported nearly identical amounts of KPS to the cell surface (the wild-type response would then be 100%). The others yielded lesser responses (PP618, 50%; PP608, 35%; PP633, 5%). These relative response levels were the same at every concentration of primary antiserum tested. These data, together with the PAGE and immunoblot results, suggest that PP608 and PP618 may export certain levels of KPS to their cell surfaces.

Rm5830, the *rkpZ* mutant of AK631, was also included in this assay and yielded an ELISA response some 20% higher than that of the wild-type strains (120% of the wild-type level). The *rkpZ* gene has significant homology to *kpsC*, a gene involved in K-antigen biosynthesis in *E. coli*. Previous studies had found that this mutant produces a much higher-molecular-weight form of KPS than does Rm41 or AK631 (26). Perhaps this increase in molecular weight results in more sites for antibody binding in this assay. Most importantly, the results show that the mutation in the *kpsC* homolog does not negatively affect the level of KPS expression; hence, the functions of KpsC and RkpZ do not appear to be identical.

NMR analysis. The NMR spectrum of PP618 (*rkpH*) KPS (Fig. 9) is essentially the same as that observed for the KPS from AK631 (26). The PP618 KPS spectrum shows that all of the major KPS components are present; these include the resonances associated with the acetate (2.0 and 2.1 ppm) and β-hydroxybutyrate (1.2 and 2.35 ppm) substituents, as well as those associated with the KDO variant 2-keto-3,5,7,9-tetradeoxy-5,7-diaminononulosonic acid (1.1, 1.7, 2.6, and 4 ppm) (27a). Thus, the NMR spectrum of PP618 KPS strongly suggests that it has the same structure as that of AK631 and that the mutation in PP618 results in ineffective export of KPS and not a structural defect in synthesis. There may be, however, some subtle structural change that is not apparent in the NMR analyses.

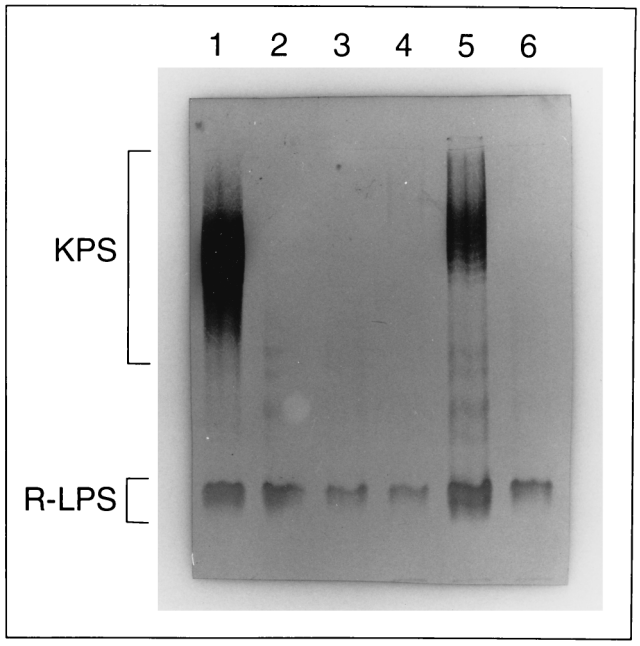

FIG. 6. Immunoblot analysis of crude extracts. The PAGE lanes contained the same samples as in Fig. 5. After transfer, the membrane was probed with polyclonal antiserum raised against whole cells of *R. meliloti* 41 (anti-Rm41). R-LPS, low-molecular-weight form of LPS (22).

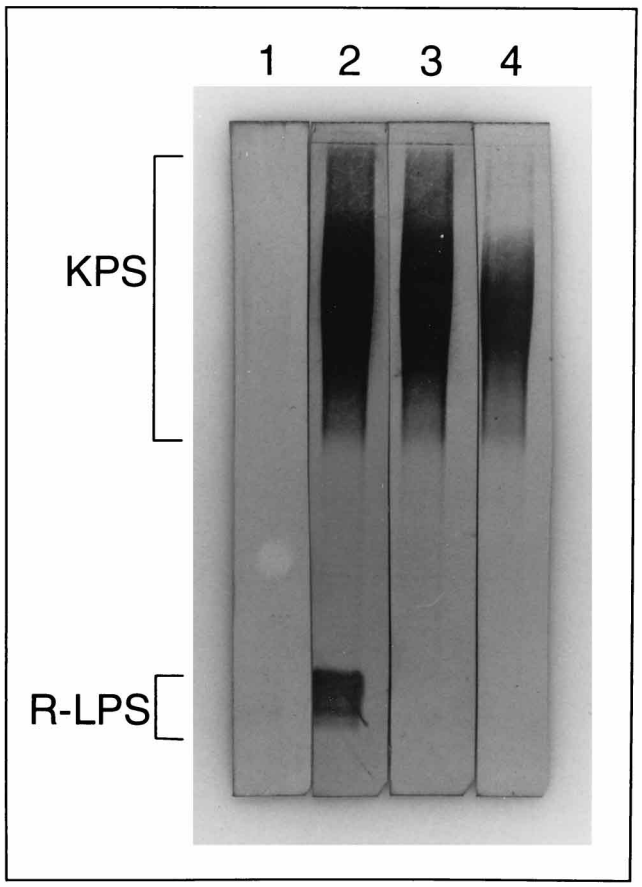

FIG. 7. Immunoblot analysis of the crude extract of strain AK631 (all four lanes) with anti-Rm41 serum preadsorbed with an equal amount of whole cells of cultured bacteria. The antiserum was preadsorbed with AK631 (lane 1), no bacteria (lane 2), PP674 (lane 3), and PP618 (lane 4). R-LPS, see the legend to Fig. 6.

DISCUSSION

Chromosomal loci necessary for the production of different KPS molecules in well-studied (entero)bacteria are clustered in a tight arrangement (29). Two of the functional regions responsible for postpolymerization modification, lipid substitution, and export of the polysaccharides appear to be common, showing strong sequence conservation and similar organization in different species, including *N. meningitidis*, *H. influenzae*, and *E. coli* (11). The central region flanked by these common gene clusters is unique for a given serotype and is involved in the synthesis and polymerization of the specific KPS (5).

R. meliloti and *R. fredii* also produce cell surface polysaccharides that are structurally analogous to group II K antigens found in *E. coli* (25); however, the organization of the genetic determinants required for KPS production is different from that of the *kps* (*cps*, *cap*, and *bex*) regions of enterobacteria. In *R. meliloti* 41, two loci affecting KPS production have been described. The *rkpZ* gene which is not present in the genome of *R. meliloti* 1021 is located on the pRm41c megaplasmid, while the *fix-23* region is on the chromosome (24, 31). Moreover, further gene clusters not linked to the *fix-23* region have been found to affect KPS production in *R. meliloti* 41 (15a). Previously, we have identified six *rkp* genes, termed *rkpABCDEF*, in the first complementation unit of the *fix-23* region; mutations in these genes resulted in deficient KPS expression (22). The similarity of the predicted gene products to fatty acid synthase suggested their involvement in the synthesis of a lipid molecule which might serve as a lipid carrier or anchor for KPS. In this work, nucleotide sequence determination of the DNA region contiguous with the first six genes revealed the presence of four additional genes, termed *rkpGHIJ*, whose predicted protein products exhibited similarity to different proteins involved in the modification and transfer of lipid molecules. Biochemical and immunological analyses of mutants carrying Tn*5* insertions in these genes demonstrated a complete or partial lack of CPS on the cell surface. Two of the 11 *R. meliloti rkp* genes sequenced have shown similarity to genes required for KPS production in enterobacteria. High sequence similarity between RkpZ of *R. meliloti* and KpsC of *E. coli* has been reported (20). Despite this similarity, the respective phenotypes of the mutants were not identical: the *E. coli kpsC* mutant produced only a reduced amount of intracellular KPS (6), whereas mutation of *rkpZ* of *R. meliloti* resulted in the same amount of exported, higher-molecular-weight KPS as in the wild type (26). The *rkpJ* gene exhibits a high level of sequence identity to *lipB* of *N. meningitidis* and *kpsS* of *E. coli*, which is located in the same gene cluster as *kpsC*. The protein products of these genes are thought to be involved in lipid substitution of CPS (6, 12, 20). This lipid modification is a requirement for polysaccharide translocation to the cell surface in these species, since KPS of *kpsS* and *lipB* mutants remain in the cytoplasm. The lack of attachment of the lipid anchor in *E. coli* also results in overpolymerization of the polysaccharide (6). In contrast, the *rkpJ* mutants (PP671 and PP633) underpolymerize the polysaccharide compared to the wild-type strain. Thus, a direct analogy to the KPS expression mechanism in *E. coli* (and other gram-negative enterobacteria) is not adequate to explain KPS synthesis and transport in *R. meliloti*. This is supported by the fact that the *fix-23* region is present in other rhizobia (including *Agrobacterium* spp.) which produce very distinct KPS (27a), yet no similar region in enterobacteria has been reported.

Recently, Reuhs et al. proposed a model for KPS expression in rhizobia (26). According to this model, an oligosaccharide containing 10 to 15 disaccharide repeat units is synthesized first. These LMW subunits are then polymerized to HMW KPS and exported, possibly in conjunction with a lipid molecule produced by a fatty acid synthase complex encoded in the first complementation unit of the *fix-23* region (26). Involvement of

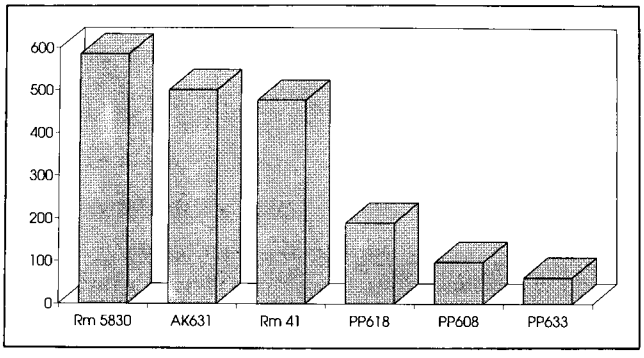

FIG. 8. ELISA response of mutant cells to anti-Rm41 serum preadsorbed with PP674 cells. The *y*-axis values are OD_{492} (10^3) and are averages of six readings, i.e., three ELISAs of duplicate experiments. The standard deviation from the average value was less than 2%. When anti-Rm41 serum was used without preadsorption with PP674 cells, the ELISA responses of the mutants were similar to that of the parent.

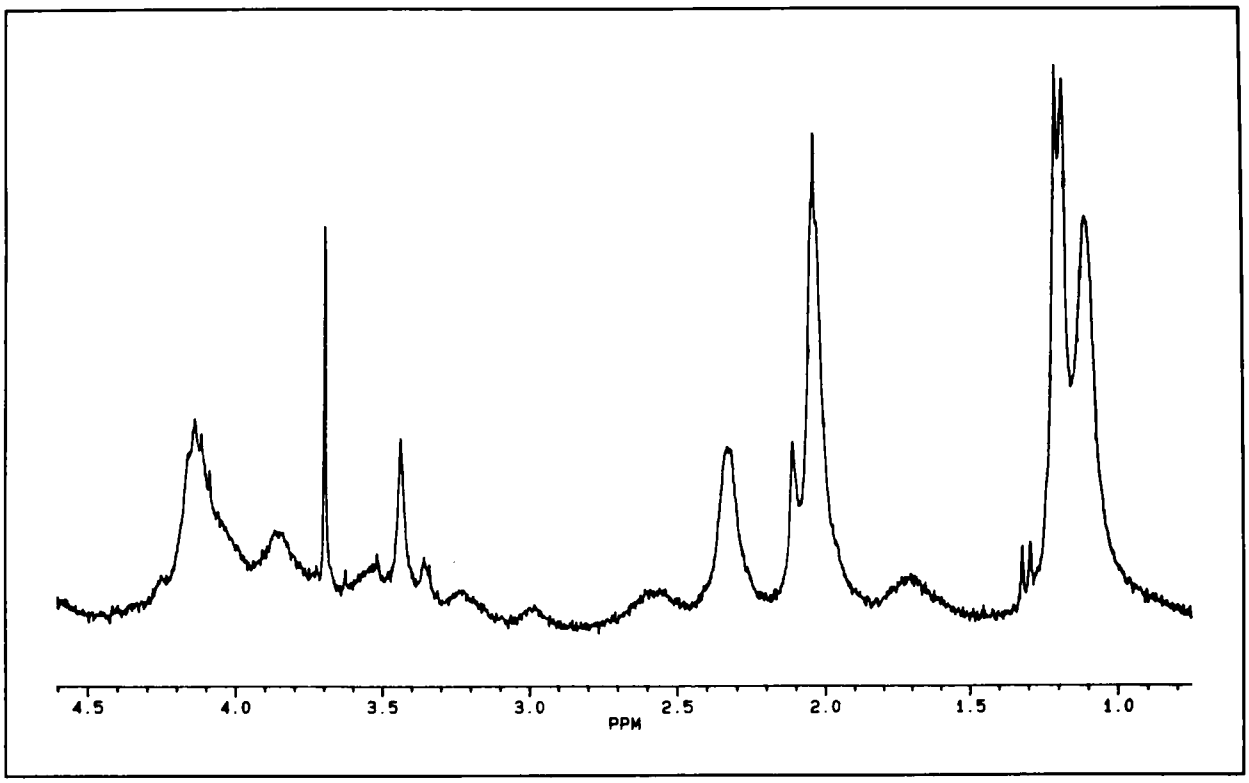

FIG. 9. ^{1}H NMR spectrum of purified KPS from PP618.

the *rkp* genes in these processes is supported by our findings that (i) complementation unit I mutants, such as PP674, produced only the LMW form of KPS; (ii) immunological studies indicate that these LMW molecules are harbored intracellularly, and (iii) mutation of the *rkpGHI* genes resulted in limited release of HMW KPS, while the *rkpJ* mutants were the most deficient in KPS polymerization and export, producing only small amounts of LMW KPS that was not exported to the cell surface. The sequence homology of these genes does not offer a clear explanation for the specific functions. The fact that fatty acids were not detected in purified KPS suggests that (i) the lipid molecules are removed during export to the surface or (ii) there is a novel lipophilic molecule that does not contain the usual fatty acids. Genetic data seem to support the assumption that the lipid part is removed from the polysaccharide and then recycled, since no induction of the *rkp* genes has been found by using *lacZ* reporter gene fusions (22), even though alfalfa root extracts specifically elicited increased expression of HMW KPS (26).

The surface polysaccharides may have an essential role in symbiotic nodule development, presumably acting as signals for the plant host in some strains (10). In fact, a recent report shows that the K antigen of *R. meliloti* AK631 elicits specific mRNA accumulation in alfalfa (4). Another report shows that the production of K antigens by rhizobia is widespread and that the K antigens are strain-specific antigens (27); i.e., unlike EPS, the K antigens differ in structure from strain to strain within the same species. These facts, along with the apparent ubiquity of the *rkp* region in rhizobia (including *Agrobacterium* spp.), suggest a somewhat sophisticated mechanism for modifying the bacterial capsule in response to environmental and/or host plant factors.

ACKNOWLEDGMENTS

We are grateful to Z. Györgypal for help with computing. We also thank S. Jenei and Z. Sárai for skillful technical assistance.

This work was supported by grants OTKA TO16674, TO20340, and TO13008; by PHARE Accord Program H9112-0233; by grant PECO CIPA-CT93-0156; by grant NSF IBN-9305022; by U.S. DOE grant DE-FG09-93ER20097; and by U.S.-Hungarian Science and Technology Joint Fund grant JF No. 513. E. Kiss and A. Kereszt were recipients of a short-term fellowship of the joint Hungarian Academy of Sciences-CNRS research program to the two participating institutes (Institut of Genetics and Institut des Sciences Végétales).

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