Suppression of the Fix<sup>−</sup> Phenotype of *Rhizobium meliloti* 
*exoB* Mutants by *lpsZ* Is Correlated to a Modified Expression of the K Polysaccharide

BRADLEY L. REUHS,* MYRON N. V. WILLIAMS, JOHN S. KIM, RUSSELL W. CARLSON, AND FRANÇOIS CÔTÉ

Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia 30602

Received 18 January 1995/Accepted 16 May 1995

The rhizobial production of extracellular polysaccharide (EPS) is generally required for the symbiotic infection of host plants that form nodules with an apical meristem (indeterminate nodules). One exception is *Rhizobium meliloti* AK631, an *exoB* mutant of Rm41, which is deficient in EPS production yet infects and fixes nitrogen (i.e., is Fix<sup>−</sup>) on alfalfa, an indeterminate nodule-forming plant. A mutation of *lpsZ* in AK631 results in a Fix<sup>−</sup> strain with altered phage sensitivity, suggesting that a cell surface factor may substitute for EPS in the alfalfa-AK631 symbiosis. Biochemical analyses of the cell-associated polysaccharides of AK631 and Rm5830 (AK631 *lpsZ*) demonstrated that the *lpsZ* mutation affected the expression of a surface polysaccharide that is analogous to the group II K polysaccharides of *Escherichia coli*; the polysaccharide contains 3-deoxy-β-manno-2-octulosonic acid or a derivative thereof in each repeating unit. Rm5830 produced a polysaccharide with altered chromatographic and electrophoretic properties, indicating a difference in the molecular weight range. Similar results were obtained in a study of Rm1021, a wild-type isolate that lacks the *lpsZ* gene: the introduction of *lpsZ* into Rm1021 *exoB* (Rm6903) both suppresses the Fix<sup>−</sup> phenotype and results in a modified expression of the K polysaccharide. Chromatography and electrophoresis analysis showed that the polysaccharide extracted from Rm6903 *lpsZ*<sup>+</sup> differed from that of Rm6903 in molecular weight range. Importantly, the effect of *LpsZ* is not structurally specific, as the introduction of *lpsZ* into *Rhizobium fredii* USDA257 also resulted in a molecular weight range change in the structurally distinct K polysaccharide produced by that strain. This evidence suggests that *LpsZ* has a general effect on the size-specific expression of rhizobial K polysaccharides.

*Bradyrhizobium* and *Rhizobium* spp. (rhizobia) are gram-negative bacteria that participate in a mutualistic association with leguminous plants. The establishment of this association involves a molecular interaction between the host plant and the microsymbiont: flavonoid molecules that are secreted by the host plant (i.e., are Fix<sup>−</sup>) and elicit the production of only those of the 17 nodulins elicited in wild-type infection (13, 15). This deficiency may be partially overcome by the addition of size-specific EPS, isolated from the parent strain, to the inocula of Rm1021 *exo* mutants (2). Mutants that produce an EPS I that lacks the succinyl groups are also defective in nodulation (12), although this may not be strictly a structural effect (i.e., the size range of the EPS may also be affected). Spontaneous mutants of strain Rm1021 that produce a second type of EPS (EPS II) were identified by a highly mucoid colony morphology. EPS II, which comprises acetylated, pyruvylated glucose-galactose disaccharide repeating units, can substitute for EPS I in the infection of alfalfa (9, 29).

Rm41 is a separate isolate of *R. meliloti* that is able to produce both EPS I and EPS II and exhibits a normal host range (19, 27). *R. meliloti* AK631, which is a nonmucoid derivative of Rm41, carries a mutation in *exoB* and is deficient in UDP-galactose synthesis. As a consequence of the mutation, AK631 cannot produce EPS I or EPS II, yet it is fully infective (Fix<sup>+</sup>) on alfalfa (20); this is the only reported exception to the EPS requirement in indeterminate nodulation. Subsequent studies showed that mutations in either the *lpsZ* gene or the *fix-23* gene region of strain AK631 resulted in a Fix<sup>−</sup> phenotype and altered surface properties (19, 20, 26, 27), indicating that the gene functions are related to infectivity. Rm1021 does not carry the *lpsZ* gene, and in contrast to Rm41, an *exoB* mutant of Rm1021 is noninfective on alfalfa; however, this Fix<sup>−</sup> phenotype can be suppressed by the introduction of the *lpsZ* gene from AK631 (27), an observation that is fundamental to this report.

Williams et al. (26, 27) proposed that a modification of the *R. meliloti* AK631 EPS effects the suppression the Fix<sup>−</sup> phenotype normally associated with *exoB* (EPS<sup>−</sup>) mutants and that
lpzZ participates in this modification. LPS involvement was suggested by alterations in the cell surface properties of an lpzZ mutant, as determined by phase binding, and a clear correlation of the Fix phenotypes of AK631 and AK631 lpzZ to the dissimilar chromatographic profiles of 3-deoxy-d-manno-2-octulosonic acid (Kdo)-containing polysaccharides extracted from these strains. The presence of the Kdo and LPS-specific fatty acids in the eluted material was evidence that it was the LPS that had been affected by the mutation.

Putnoky et al. (19, 20) demonstrated that the Fix phenotypes of AK631 and AK631 fix-23 mutants were also correlated to phase sensitivity. On the basis of differences in the chromatographic elution patterns of Kdo-containing material from extracts of AK631 and AK631 fix-23, they also concluded that the LPS was responsible for the suppression of the Fixphenotype. The presence of Kdo in the bacterial extracts was assumed to be diagnostic for LPS, as this sugar has been found in all rhizobia LPSs studied to date and had not been reported as a constituent of other rhizobial polysaccharides.

A recent report has shown that both Rhizobium fredii USDA205 and R. meliloti AK631 produce non-LPS, acidic polysaccharides that are analogous to the group II K antigens (capsular polysaccharides or K polysaccharides [KPSs]) of Escherichia coli (21). The major KPS isolated from R. fredii USDA205 comprised disaccharide repeating units of β-D-galp-(1→5)-β-D-kdap-(2→)n. Although the complete structure of the R. melliloti AK631 KPS has not been determined, present data indicate that it consists of disaccharide subunits of Kdo, or a Kdo derivative, and a deoxyaminohexose (24). The characterization of these polysaccharides opened the conclusions of the fix-23 and lpzZ studies to question, and it has since been shown that fix-23 is involved in the production of the KPS (18). Polycarboxylate gel electrophoresis (PAGE), immunochromatographic, and chromatographic data presented in this report demonstrate that an lpzZ mutation in AK631 results in the distinct expression of the KPS, particularly in the molecular weight range of the homologous KPS; finally, the introduction of a plasmid carrying the R. melliloti lpzZ gene into R. fredii USDA257, which produces a structurally distinct KPS, also results in a similar modification in size range, providing evidence for a general effect of the gene product.

**TABLE 1. R. meliloti strains used in this study**

| Strain           | Chromosomal background | pSYM-b | Relevant genotype | Specific gene(s) | Phenotypea | Source or reference |
|------------------|------------------------|--------|-------------------|------------------|------------|---------------------|
| Rm41             | Rm41                   | Rm41   | Wild type (lpzZx) |                  | EPSb Fixc | A. Kondorosi        |
| AK631            | Rm41                   | Rm41   | exoB631 (lpzZx)  |                  | EPSb Fixc | A. Kondorosi        |
| Rm5830           | Rm41                   | Rm41   | exoB631 lpzZ88-Tn5 (lpzZx) | EPSb Fixc | 27       |
| Rm1021c          | SU47                   | SU47   | Wild type (lpZ)   |                  | EPSb Fixc | F. Ausubel          |
| Rm5903           | SU47                   | SU47   | Multicopy lpZ    |                  | EPSb Fixc | 27       |
| Rm1021/pMW23     | SU47                   | SU47   | exoB631 (lpZx)   |                  | EPSb Fixc | 27       |
| Rm5903/pMW23     | SU47                   | SU47   | exoB631, multicopy lpZx | EPSb Fixc | 27       |
| Rm5816           | SU47                   | SU47   |                    |                  | EPSb Fixc | 27       |

a The Fix phenotype is based on a lack of acetylene reduction; however, the actual defect is in the infection process.
b Origin of exo gene-bearing, 1,500-kb megaplasmid; the exo genes control EPS synthesis.
c The wild-type pSYM-b of Rm41 contains the lpzZ gene.

d The exoB gene product is a UDP-glucose-4'-epimerase; a mutation in this gene results in a deficiency in EPS production.

### MATERIALS AND METHODS

**Strains, media, growth conditions, and phage assays.** The *R. melliloti* strains used are described in Table 1. *R. melliloti* and *R. fredii* cells were grown in TY and YEM, respectively, at 27°C to late log phase (optical density at 600 nm [OD600] of ~0.9). The phage and assay conditions have been previously described (26). Drops of phage suspension (10 to 10²) were spotted on lawns of *R. melliloti* cells in lambda top agar.

**Polysaccharide extraction and chromatography.** The extraction and initial purification protocols have been previously described (18, 21). Briefly, the cells were pelleted by centrifugation, washed, repelleted, and extracted with hot phenol-water. Initial purification of the crude cell extract was performed on Sepharose 4B (30 mM triethylamine, 10 mM EDTA [pH 7]); the eluted fractions were chemically assayed for Kdo by the thiobarbituric acid (TBA) assay and for hexasaccharides with phenol-sulfuric acid and then assayed for LPS by PAGE. This procedure was followed by gel filtration chromatography over Sephadex G-150 superfine (0.2 M NaCl, 1 mM EDTA, 50 mM Tris base, 0.25% deoxycholic acid [pH 9.25]). Further purification was performed on DEAE-Sephadex. The column was washed with H₂O (3 × bed volume), and the retained polysaccharides were eluted by using a linear gradient of 0.2 to 0.8 M ammonium formate.

**SDS whole cell disruption.** Whole cell disruptions of 2-ml culture pellets were performed using a French press. The cultures were grown to late log phase, pelleted, washed with 1 ml of Tris buffer (0.05 M Tris base, pH 6.85), and repelleted. The pellets were disrupted in 0.1 ml of sodium dodecyl sulfate (SDS) buffer (50 mM Tris base, 10% glycerol, 1% SDS, 1 mM EDTA, 0.001% bromophenol blue) at 100°C for 3 min prior to PAGE.

**PAGE and immunoblotting.** PAGE, immunoblotting, and antibody development protocols have been previously described (18, 21). PAGE was performed on the phenol-water extracts or SDS-disrupted cells, using 18% acrylamide gels with deoxycholic acid as a detergent; the gels were either silver stained or Alcian blue/silver stained. For immunoreaction analyses, 18% polyacrylamide gels were transferred to nitrocellulose or Nytran+ (Schleicher & Schuell, Keene, NH), using a Trans-Blot SD apparatus (Bio-Rad, Richmond, Calif.), incubated with anti-Rm41, and developed with goat anti-rabbit alkaline phosphatase conjugate and substrate. Rabbit anti-Rm41 was donated by Dale Noel (Marquette University, Milwaukee, Wis.).

**Effects of plant root extract.** The effects of plant-derived compounds on polysaccharide production were tested on 2-ml cultures of *R. melliloti* AK631 and Rm5830. The growth conditions and protocol, as well as the root extraction procedure, have been previously reported (22). For PAGE analysis, control cultures and cultures containing various root extracts (500 μg/ml) of flavonoids (3 μg/ml) were grown to late log phase, adjusted to identical OD₆₀₀ values, pelleted, and analyzed by PAGE following SDS whole cell disruption. For TBA assays, duplicate cultures were adjusted to identical OD₆₀₀ values, duplicate aliquots were removed from each, and the TBA assay was performed on the cell pellets. The correlation of root extract concentration to polysaccharide production was established by PAGE studies (as described above), in which the cell pellets from cultures containing various concentrations of alfalfa root extract (0, 12.5, 25, 50, and 100 μg/ml) were examined by PAGE; in these experiments, the gels were very lightly stained to avoid background effects. The gels were then scanned with a laser densitometer, and the data were analyzed by ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.). The degree of staining was established by summation of the OD values at each pixel (50 by 50 μm) present in a fixed area in the central (most uniform) portion of the high-molecular-weight (HMW) KPS banding region of each lane. The data were then normalized for loading differences (which were slight) by comparison with the values obtained in


**RESULTS**

The *Rm41* background. (i) Strain phenotypes. The strain designations and pertinent descriptions are given in Table 1. Strain construction and Fix phenotypes were reported previously (26, 27). Briefly, *R. meliloti* AK631 is Fix\(^+\) on alfalfa and sensitive to phage \(\phi\) 16-3, whereas strain Rm5830 (AK631 *lpsZ*) is Fix\(^+\) and resistant to phage \(\phi\) 16-3.

(ii) PAGE and immunoblot analyses. SDS whole cell disruption was used for PAGE analysis of LPS because the phenol-water extraction procedure results in an inconsistent fractionation of the different LPS forms into the water phase. Two different stains were used in this study: silver stain, which stains only the LPS, and Alcian blue/silver stain, which also stains the KPS (21). The PAGE (silver stain) banding pattern of the AK631 LPS, which consisted of low- and high-mobility banding regions (with and without O antigen, respectively), was essentially the same as the LPS banding pattern of Rm5830, the *lpsZ* mutant (Fig. 1A). Slight differences in mobility result from differences in loading and well location on the gel. This LPS PAGE pattern is common in rhizobial studies (4).

Phenol-water extracts of AK631 and Rm5830 were used for PAGE (Alcian blue/silver stain) analyses of the KPS (Fig. 1B). The Alcian blue-specific banding regions result from various size ranges of the KPSs (21). In contrast to the LPSs, the respective KPSs showed significant differences in electrophoretic mobility of the KPS: (i) the major banding region of the KPS from the *lpsZ* mutant displayed a lower mobility than that of AK631 (upper arrows), suggesting a difference in molecular weight distribution or net charge; and (ii) a high-mobility, Alcian blue-specific banding region (lower arrow) was virtually absent in the Rm5830 lane. The ladder pattern of the AK631 KPS actually continues further down the lane, but it is obscured by the LPS I. Although the banding patterns of the respective KPSs show a wide range in the degree of polymerization, a major proportion of the extracted polysaccharide falls into the size ranges indicated by the arrows, as shown by the protuberance of the bands in those regions. The relative mobilities of the two major forms of the KPS from AK631 indicate average molecular weights of ~5,000 to 7,000 (high mobility) and ~20,000 to 25,000 (low mobility) compared with standards (not shown). The vast majority of the KPS from Rm5830 appears to have an average molecular weight of ~25,000 to 35,000 (uppermost arrow), although there are minor amounts of LMW polysaccharide (down to the 8,000 to 12,000 range). Ion-exchange chromatography of the respective KPSs showed no significant differences in the net charge of the polysaccharides (discussed below), indicating that the PAGE differences are due to size variation.

In immunoblot analyses, the KPS from AK631 was efficiently electrophoretically transferred to a nitrocellulose membrane (from a polycrylamide gel) and detected by anti-*Rm41*, whereas the Rm5830 KPS failed to bind the membrane (Fig. 1C). The fact that the difference in staining was due to affinity for the membrane, and not the antiserum, was established by the use of a different antiserum, which failed to bind the membrane (Fig. 1D). The difference in KPS size range is also visible here. The LPSs of the two different strains showed the same affinity for the membranes and antiserum.

(iii) Chromatographic analysis and purification. Initial separation of the extracted polysaccharides was performed by Sepharose 4B gel filtration chromatography (Fig. 2). The profiles show that HMW, Kdo-rich KPSs were present in the extracts of both *R. meliloti* AK631 and *R. meliloti* Rm5830 (peak 4B-1). LPS represents less than 5% of the TBA-positive material in this profile (21); therefore, the Kdo elution profile is essentially that of the KPS. The earlier elution of the 4B-1 component from Rm5830 indicates a higher average molecular weight for the KPS from that strain, which supports the PAGE data discussed above. In addition to peak 4B-1, an LMW Kdo-rich peak is present in the profile of AK631. Subsequent NMR analyses showed that this polysaccharide was similar to the HMW KPS (discussed below) and that it was responsible for the high-mobility ladder pattern found only in the PAGE analysis of AK631 (discussed above). This LMW KPS peak is absent in the Rm5830 profile; the small peak in that profile is from LPS, which was apparent in the PAGE profile of the fractions (not shown). PAGE analysis also showed that LPS eluted into many of the column fractions (represented by inset bars), including those of peak 4B-1, which is a result of aggregation; this makes interpretation of differences in LPS by this method difficult. Subsequent carbohydrate analyses of the
LPSs has failed to show any difference in the Rm5830 LPS (24).

For subsequent NMR analysis, the 4B-1 pool from strain AK631 was chromatographed over Sephadex G-150, using an eluent that included deoxycholic acid to prevent LPS aggregation; this resulted in the separation of the HMW KPS, which eluted in the column void (G-150-1), from the LPS (presented in reference 21). The Rm5830 KPS also eluted into the Sephadex G-150 void volume (G-150-1; not shown). The respective KPSs also exhibited similar elution properties on DEAE-Sephadex, indicating that there was not a significant difference in the net charge (not shown), although this step did result in the separation of the KPSs (DEAE-2) from HMW neutral polysaccharides (DEAE-1), composed of glucose, mannose, and xylose, which are produced by both strains. Prior to this purification step, these sugars were presumed to be components of the R. meliloti KPS (21). The complete structure of the polysaccharide from AK631 has not yet been determined; however, present data indicate that it consists of acetyl and 3-OH-butanoyl-substituted repeating units of Kdo, or a Kdo derivative, and deoxyaminohexose (24). Importantly, fatty acids were not detected in the analysis of purified KPS.

(iv) \(^{1}H\) NMR analysis. Proton NMR was performed on the DEAE-2 pools of each KPS. The only significant difference between the \(^{1}H\) NMR spectra of the KPSs from AK631 and Rm5830 (Fig. 3) is found at δ 2 to 2.15, in the region of acetyl resonances, suggesting a different substitution pattern. Both spectra contain the resonances associated with β-linked Kdo. The small, sharp resonances are due to trace contaminants
Yet, as with fix-23 mutations (18), it is this component that is affected by the lpsZ mutation.

(v) Regulation of polysaccharide expression. The expression of *R. fredii* LPS, KPS (22), and EPS (7) has been shown to be affected by host plant-derived compounds; therefore, the possibility of similar effects with *R. meliloti* KPS expression was tested. A PAGE comparison of SDS-disrupted cells (Fig. 4A) shows that alfalfa root extract specifically elicited an elevated expression of HMW KPS by AK631. Soybean root extract had no apparent effect. The polyacrylamide gels were very lightly but evenly stained with Alcian blue prior to silver staining to minimize background and highlight any contrast (hence the difference between Fig. 4A and 1B). The silver staining of the high-mobility LPS shows that the sample loadings were virtually identical. Several flavonoids (apigenin, leuteolin, naringenin, and genistein) were also tested, with no effect (not shown). The same set of experiments was performed on Rm5830, and neither the root extracts nor the flavonoids had any effect on Rm5830 (not shown). Chemical analysis (TBA assay) of total Kdo content also showed that growth with the alfalfa root extract resulted in elevated levels of TBA-positive material in the cell pellets of AK631 (Fig. 4B). The difference can be attributed to the KPS, as it is far more abundant than LPS and contains a greater proportion of Kdo (21). The root extract had no effect on Rm5830. A clear correlation between alfalfa root extract concentration and KPS expression was shown by PAGE (Fig. 4C); the effects were quantified by densitometry analysis of the HMW KPS banding region and normalized to the high-mobility LPS banding region. The gels were lightly silver stained with Alcian blue prior to silver staining to minimize background and highlight any contrast.

from chromatography eluents and handling; e.g., the doublet at δ 1.35 is due to lactate (a common problem in NMR analysis). The contaminant resonances which are distinguished by narrow line width, in comparison with the polysaccharide (this is related to relaxation time) are more significant in the Rm5830 spectrum because the sample was much less concentrated. The 1H NMR spectrum of the LMW KPS from AK631 revealed no obvious differences from that of the HMW form (not shown). The most important aspect of these NMR spectra, which are of intact polysaccharides, not hydrolysis products (as in 27), is that they do not have any resonances associated with fatty acids and therefore, are not the spectra of LPSs (see reference 21).

FIG. 4. (A) Alcian blue/silver-stained polyacrylamide gel of SDS-disrupted cell pellets of *R. meliloti* AK631. Lanes: 1, control; 2, cell culture containing 500 μg of alfalfa root extract per ml; 3, cell culture containing 500 μg of soybean root extract per ml. The late-log-phase cultures were brought to identical OD₆₀₀ values with additional growth medium, and 2 ml of each was removed and pelleted by centrifugation prior to PAGE preparation. (B) Total Kdo determination of cell pellets from cultures of AK631 (1) and Rm5830 (2) grown with 0 μg (solid bars) and 500 μg (striped bars) of alfalfa root extract per ml. The late-log-phase cultures were brought to identical OD₆₀₀ values with additional growth medium prior to the removal of aliquots. Each datum point represents the average assay response, as calculated from duplicate assays of duplicate cultures. Kdo assay response is the response to the TBA assay (A₅₅₀). (C) Plot of the relative ODs of HMW KPS bands from a lightly stained polyacrylamide gel. The cell cultures contained the following concentrations of alfalfa root extract: 0, 12.5, 125, 250, and 500 μg/ml. The ODs were determined by densitometry analysis, and the values were normalized to the high-mobility LPS values to account for any loading variation between wells. See Materials and Methods for details.

FIG. 5. Spot tests of designated bacteriophages on lawns of Rm1021 (lpsZ⁺), Rm1021/pMW23 (lpsZ⁻), Rm6903 (exoB⁺), and Rm5816 (exoB lpsZ⁻). Clearing indicates sensitivity to the phage.
stained to avoid background interference; in fact, the differences were more pronounced, if less reliable, with more darkly stained gels.

The \textit{Rm1021} background. (i) \textbf{Strains and relevant descriptions.} The strains used in this portion of the study are also described in Table 1. The wild-type strain (\textit{Rm1021}) lacks the \textit{lpsZ} gene (\textit{lpsZ}'), and \textit{exoB} (\textit{EPS}') derivatives of \textit{Rm1021} are \textit{Fix}− on alfalfa. \textit{EPS}− (\textit{Rm1021}) and \textit{EPS}− (\textit{Rm6903}) strains were used in the phage studies. Two strains were used for PAGE and immunochromatological analyses of \textit{lpsZ}′: \textit{Rm6903}/pMW23, which carries \textit{lpsZ}′ on a 4-kb fragment of \textit{Rm41} DNA in medium-copy-number vector pRK404, and \textit{Rm5816}, which carries the entire 1,700-kb \textit{pSYM-bRm41} megaplasmid, including \textit{lpsZ}. Strain construction and phenotypic analysis have been reported previously (26, 27). All \textit{exoB} \textit{lpsZ}′ strains were shown to be \textit{Fix}− on alfalfa, despite the lack of \textit{EPS} production; however, \textit{Rm5816} showed much higher levels of effective nodulation (~90% of plants) than did \textit{Rm6903}/pMW23 (~30% of plants), which indicates that other genes on \textit{pSYM-bRm41} may modulate \textit{lpsZ} activity.

(ii) \textbf{Phage sensitivity of \textit{Rm1021} and derivatives.} Phage spot tests (Fig. 5) showed that either the introduction of \textit{lpsZ}′ (\textit{Rm1021}/pMW23) or a mutation of \textit{exoB} (\textit{Rm6903}) results in resistance to \textit{phi} M7, \textit{phi} M9, and \textit{phi} M10. \textit{Rm1021}/pMW23 shows a resistance to \textit{phi} M6 that is not shared by \textit{Rm6903}, which, conversely, is specifically resistant to \textit{phi} M14. Additional resistances are seen with \textit{lpsZ}′\textit{exoB} strains. Resistance was previously found to be due to loss of phage binding to cells (26, 27). Clearly, both \textit{lpsZ} and \textit{exoB} affect components that are accessible to phage (i.e., cell surface components) and that would be detectable by to the host plant in symbiotic infection.

(iii) \textbf{PAGE and immunoblot analyses.} The PAGE analysis of phenol-water extracts (Fig. 6) showed no Alcian blue-specific bands for \textit{Rm6903} (the upper banding region lane 1 is due to O-antigen-containing \textit{LPS}), indicating that the \textit{KPS} of \textit{Rm6903} does not enter the running gel. In contrast, an Alcian blue-specific banding region of the same relative mobility as the HMW \textit{KPS} from \textit{AK631} is seen with \textit{Rm6903}/pMW23. A parallel gel was silver stained only and confirmed that this material is not \textit{LPS} (not shown). There is no effect on \textit{LPS} migration, although the \textit{LPS} banding pattern of \textit{Rm6903} is different from that of \textit{AK631}. The effect of \textit{lpsZ} is much more pronounced in \textit{Rm5816} (not shown), which carries the entire \textit{pSYM-bRm41}, although there was no indication of a high-mobility banding region, such as that found in \textit{AK631}, in either of the \textit{lpsZ}′ strains. Interestingly, the \textit{KPS} from \textit{Rm5816} binds anti-Rm41 (lane 3), indicating structural identity; this finding was supported by NMR analysis (not shown).

(iv) \textbf{Chromatography.} The Sepharose 4B profiles of the crude cell extracts from \textit{Rm6903} and \textit{Rm6903}/pMW23 (Fig. 2C and 2D) show that \textit{Rm6903} produced an HMW \textit{KPS} that eluted primarily in the column void (peak 4B-1), which is similar to the elution of the 4B-1 fraction from \textit{Rm5830}. In contrast, the \textit{KPS} from \textit{Rm6903}/pMW23 was retained on the column. Clearly, the presence of \textit{lpsZ} affects the molecular weight distribution of the \textit{KPS}s from both the \textit{Rm1021} and the \textit{Rm41} \textit{EPS}− derivatives, and this effect is correlated to the suppression of the \textit{Fix}− phenotype.

\textbf{PAGE analysis of \textit{R. fredii} USDA257 and \textit{R. fredii} USDA257/pMW23 (\textit{lpsZ}′).} To determine if the biochemical effects were structurally specific, the \textit{lpsZ}′-carrying pMW23 was mobilized into \textit{R. fredii} USDA257, which produces a structurally distinct \textit{KPS} (23), and the crude cell extracts were examined by PAGE (Fig. 7). The dark-staining regions are due to the \textit{KPS}s of \textit{R. fredii} USDA257 and \textit{R. fredii} USDA257/pMW23; the distinct bands below (and beneath) the \textit{KPS} region are from the O-antigen-containing \textit{LPS}s. The arrows indicate the median mobility of the respective \textit{KPS}s and show that \textit{lpsZ}′ effects a size range modification in \textit{R. fredii} KPS expression. The \textit{KPS} from strain USDA257 is similar to that of \textit{R. mелилити} \textit{AK631} in that it contains Kdo in the repeating units; however, it differs in the hexosyl component and is unsubstituted; importantly, it does not bind anti-Rm41 (23, 24). The \textit{LPS} of strain USDA257 was unaffected by the introduction of the plasmid.

\section*{DISCUSSION}

Numerous studies have focused on the significance of rhizobial \textit{LPS} and \textit{EPS} in rhizobium-legume symbiosis. The results presented here and in a previous report (18) demonstrate that the specific expression of the Kdo-rich \textit{KPS} by \textit{R. mелилити} \textit{AK631} (\textit{Rm41} \textit{exoB}) is correlated to the suppression of the Fix− phenotype and the megaplasmid (\textit{pSYM-b})-borne \textit{lpsZ} gene effects a modification in polysaccharide production. A mutation of \textit{lpsZ} is correlated to five observed phenomena. (i) The HMW \textit{KPS} elutes earlier in gel filtration chromatography and has a lower mobility in PAGE, indicating
a size range modification, (ii) the HMW KPS displays a diminished affinity for nitrocellulose membrane in immunoblot analyses; and (iii) the LMW KPS is virtually absent (this report). In addition, (iv) the lpsZ strain is Fix− on alfalfa, and (v) it shows a different pattern of phage sensitivity (26, 27).

In contrast to Rm41, Rm1021 does not carry lpsZ, and the exoB mutant of that strain is unable to infect the host plant. The introduction of medium-copy-number pMW23 into Rm1021 exoB (Rm6903) was sufficient to restore a Fix+ phenotype, albeit at a low level; however, when the entire Rm1021 pSYM-b megaplasmid was replaced with pSYM-bRm41, the level of effective nodulation approximated that of the wild-type strain. Rm6903/pMW23 (lpsZ−) was also affected in the size-specific expression of the KPS, and as in the nodulation studies, the presence of pSYM-bRm41 results in a more conspicuous effect. The introduction of lpsZ+ into R. fredii USDA257, which produces a structurally distinct KPS (23, 24), also results in a modification in KPS size range, showing that the gene ultimately affects KPS expression and that the effect may not be structurally dependent.

lpsZ is not essential for the effective synthesis of the KPS, as it is produced by AK631 lpsZ and Rm1021 (lpsZ−); instead, it appears to be involved in size range determination. Other effects have not been definitively determined, although the difference in affinity for the nitrocellulose membrane and the possible difference in the acetylation pattern imply that some structural modifications may be associated with lpsZ. Can the size range effect alone explain the capacity of the R. meliloti KPS to substitute for the EPS in nodulation? Battisti et al. (2) found that the addition of a tetramer of exogenous EPS I, isolated from R. meliloti SU47, to the inocula of EPS Fix− derivatives allowed for low levels of infection of alfalfa. If this finding is accurate, this EPS would be polysaccharide with a molecular weight of ~6,000, roughly equivalent to the predicted molecular weight of the LMW KPS, as judged from relative mobility in PAGE analyses. Although the LMW form of the polysaccharide is produced by the fix−23 mutants, which are Fix−, it is most likely harbored intracellularly and released only upon chemical extraction. This observation is supported by both the genetic data for fix−23 and immunochimical studies (18, 24). Alternatively, the LMW form may not be a factor in the infection, as it was the HMW form that was conspicuous in the Rm6903 lpsZ+ strains, and the inclusion of host plant root extract in culture media enhanced the expression of the AK631-specific HMW form of the KPS, not the LMW form.

This latter observation was included as further evidence that the lpsZ gene ultimately affects the expression of the KPS; however, this apparent modification in KPS expression may also have some significance in symbiosis. In addition, previous studies have shown a similar effect in R. fredii USDA205 (22). The possibility exists that bacterial factors, other than the nod factor, have a positive or negative function in the infection process and that there is plant-specific regulation of these factors. The observations presented here have stimulated further investigations into biotic and abiotic regulation of cell surface expression.

A substantial similarity between the predicted amino acid sequences of lpsZ and kpsC was recently reported (17); kpsC is one of several genes involved in the expression of the group II capsules (K antigens) of E. coli. A companion report suggests that a mutation in kpsC affects the acylation, size range, and export of the K5 polysaccharide (3). Despite the homology, the phenotypes of the lpsZ and kpsC mutants are not identical: AK631 lpsZ produces an abundance of the KPS, and the cellular distribution is roughly similar to that in AK631 (this report and references 24 and 27), whereas a mutation of the kpsC gene of E. coli resulted in the intracellular accumulation of greatly reduced amounts of the K5 PS. The similarity of KpsC and LpsZ does, however, substantiate the biochemical data, which show that lpsZ affects the expression of group II-like K antigens. Many different (group II) K antigens have now been identified in other strains and species of mutualistic (rhizobia) and pathogenic plant symbionts (23), suggesting a widespread importance for these bacterial components in plant-microbe interaction.

A model for KPS expression in rhizobia is suggested by the data gathered thus far from studies of R. meliloti and R. fredii, as well as the homology of LpsZ to KpsC. (i) The polysaccharide repeating units are produced by gene products from an as yet unidentified gene region, and the initial polymerization process in R. meliloti yields polysaccharide subunits of ~4,000 to 7,000 Da (8 to 15 repeating units), which are harbored intracellularly, as shown by the presence of LMW KPS in the fix−23 mutants. (2) A fix−23-related process assembles exportable (lipid-linked?) polysaccharides that are HMW polymers of the subunits, which are then exported to the cell surface. (3) lpsZ, which is not required for the normal expression of the capsule in R. meliloti, modifies the polymerization process, promoting the export of the smaller polysaccharides.

The K antigens of rhizobia are generally found to be of strain-specific size ranges, which indicates that a strain-specific eld (chain length determinant) systems may be common, possibly LpsZ functions in the modification or inhibition of this system.

On the basis of the data presented in this report and in previous reports (18, 21), the present designation of the lpsZ gene should be changed. The gene product clearly affects the KPS(s) but is not completely functionally homologous to the kpsC product; consequently, we suggest that the gene be redesignated rkpZ (rhizobial KPS). This change is important, as the presumed substitution of LPS for EPS in the infection of alfalfa by strain AK631 is frequently cited as an important phenomenon in plant-microbe interactions and biosynthetic regulation.

ACKNOWLEDGMENTS

This work was supported in part by grant IBN-9305022 from the National Science Foundation and by the U.S. Department of Energy-funded Center for Plant and Microbial Complex Carbohydrate Research under extragrant DE-FG09-87ER13810.

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