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The *Sinorhizobium meliloti* Lon Protease Is Involved in Regulating Exopolysaccharide Synthesis and Is Required for Nodulation of Alfalfa

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While screening for *Sinorhizobium meliloti* Pho regulatory mutants, a transposon mutant was isolated that constitutively expressed higher levels of acid and alkaline phosphatase enzymes. This mutant was also found to form pseudonodules on alfalfa that were delayed in appearance relative to those formed by the wild-type strain, it contained few bacteroids, and it did not fix nitrogen. Sequence analysis of the transposon insertion site revealed the affected gene to have high homology to Lon proteases from a number of organisms. In minimal succinate medium, the mutant strain was found to grow more slowly, reach lower maximal optical density, and produce more extracellular polysaccharide (EPS) than the wild-type strain. The mutant fluoresced brightly on minimal succinate agar containing calcofluor (which binds to EPSI, a constitutively expressed succinoglycan), and gas chromotographic analysis of purified total EPS showed that the glucose-to-galactose ratio in the *lon* mutant total EPS was 5.0 ± 0.2 (mean ± standard error), whereas the glucose-to-galactose ratio in the wild-type strain was 7.1 ± 0.5. These data suggested that in addition to EPSI, the *lon* mutant also constitutively synthesized EPSII, a galactoglucan which is the second major EPS known to be produced by *S. meliloti*, but typically is expressed only under conditions of phosphate limitation. ^{13}C nuclear magnetic resonance analysis showed no major differences between EPS purified from the mutant and wild-type strains. Normal growth, EPS production, and the symbiotic phenotype were restored in the mutant strain when the wild-type *lon* gene was present in *trans*. The results of this study suggest that the *S. meliloti* Lon protease is important for controlling turnover of a constitutively expressed protein(s) that, when unregulated, disrupts normal nodule formation and normal growth.

The Lon protease is a ubiquitous and conserved protein throughout the prokaryotes. It is an ATP-dependent tetrameric enzyme displaying complex allosteric activation by the binding of ATP, DNA, and its target protein(s). The target signal in substrate proteins is obscure but seems to involve the tertiary structure of the protein and not a primary amino acid sequence. Most proteins do not display this target structure unless mutated or denatured, but some proteins always display this signal and are subject to constant degradation by Lon (reference 36 and references therein). Most known targets of Lon degradation are regulatory proteins whose physiological function(s) is moderated by a shifting balance between transient increases in expression and turnover by protease action. Lon protease has been implicated as an important regulator in differentiation processes including sporulation in *Bacillus subtilis* (46), fruiting body formation in *Myxococcus xanthus* (20), or switching from swarmer to planktonic cell type in *Vibrio parahaemolyticus* (50). In general, *lon* mutations in nondifferentiating bacteria result in minor decreases in degradation of aberrant proteins (34), accompanied by hypersensitivity to UV irradiation (36).

Lon protease has also been shown to be involved in capsule (22) and EPS (18) production in bacteria. In *Escherichia coli*, overexpression of colanic acid is due to the excess amounts of RcsA, a positive transcriptional accessory factor that enhances the activity of RcsB. The latter is the response regulator member of the RcsC-RcsB two-component regulatory pair (22). Increased activity of RcsB results in transcriptional activation of capsular biosynthesis genes and excess capsule production. Because RcsA is the normal substrate for the Lon protease, mutation of the *lon* gene dramatically increases the production of capsular polysaccharides in these strains (25). In the wild-type strain, the balance between RcsA production and proteolysis is one mechanism that normally controls capsule production. Rcs homologs have also been implicated in controlling cell surface polysaccharide production in *Klebsiella* and *Erwinia* (reviewed in reference 42).

The regulation of cell surface polysaccharides is highly relevant to the *Rhizobium*-legume symbiosis. Polysaccharides closely associated with the bacterial cell surface have been shown to be important in early infection events for rhizobia, such as *Rhizobium leguminosarum* (8). Polysaccharides released from the bacteria as extracellular polysaccharides (EPS) appear to fulfill a similar role for other rhizobia, such as *Sinorhizobium meliloti* (17). Current hypotheses propose that these polysaccharides may act as a suppressor of plant defense systems, which would otherwise inhibit the rhizobia from infecting the plant (8, 39). *S. meliloti* produces two major EPS molecules, EPSI and EPSII (5). Regulation of both has been extensively studied (reviewed in references 5 and 33). EPSI (a succinoglycan) is constitutively expressed under normal laboratory growth conditions, and its presence can be conveniently detected by the binding of the fluorescent compound calcofluor. When grown on agar media containing calcofluor, EPSI-producing strains fluoresce brightly (17). EPSII (a galactoglucan)

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is not normally expressed in growing cultures, but the genes coding for EPSII synthesis are upregulated in response to phosphate starvation (43, 51, 58).

As part of our studies of phosphate assimilation and regulation in *S. meliloti*, we conducted transposon mutagenesis experiments in which we were searching for a negative regulator of the *S. meliloti* Pho regulon (i.e., a homolog to the *E. coli phoR*). During screening of Tn*5*B22 transposon mutants, we encountered a transconjugate that expressed elevated phosphatase activity on high-phosphate medium. An analysis of the transposon insertion site showed that the affected gene was a homolog to *lon* cloned from a variety of bacteria. Further studies with this mutant found that it was affected in growth rate, EPS synthesis, and nodule formation. The data summarizing the free-living and symbiotic phenotype of this organism are presented in this paper.

MATERIALS AND METHODS

Bacterial strains, growth media, and transposon mutagenesis. The *S. meliloti* wild-type strain 104A14 used in this work has been described previously (49). Unless otherwise noted, the basic medium used for culturing all *S. meliloti* strains was a minimal mannitol medium described previously by Summers et al. (51) but which was modified such that succinate replaced mannitol as the sole carbon source. The basal medium contains no added inorganic phosphate (P_i) salts but is buffered by 10 mM morpholinepropanesulfonic acid (MOPS; pH 7.0) and amended with P_i as required. In some experiments, phosphatase activity was compared in cells incubated in the minimal succinate medium that contained 50 mM KH_2PO_4 ($+P_i$) or zero P_i ($-P_i$). *S. meliloti* bacteroids were isolated on yeast-extract mannitol agar (YMB agar [47]). *E. coli* DH5α (45) was used as a host strain for plasmid constructions, and *E. coli* S17-1 (47) was employed for conjugal transfer of plasmids to *S. meliloti*; both *E. coli* strains were cultured on Luria-Bertani medium (45). Ampicillin (100 $\mu g \cdot ml^{-1}$) and gentamicin (25 $\mu g \cdot ml^{-1}$ for agar media, 15 $\mu g \cdot ml^{-1}$ in broth cultures) were included as required.

Transposon mutagenesis was used to screen for a Pho regulatory mutant that was overexpressing alkaline phosphatase on $+P_i$ minimal succinate agar; growth on high-phosphate medium represses the synthesis of alkaline phosphatase (1). Previously described methodology (1, 51) was used for transposon mutagenesis of strain 104A14 with transposon Tn*5*B22 (48). Transconjugates were spread onto minimal succinate agar supplemented with gentamicin and 5-bromo-4-chloro-3-indolylphosphate (XP), the latter being a chromogenic phosphatase substrate. A single blue colony was twice streak purified to obtain a pure culture and is referred to as RmMSU9. Phosphatase activity in periplasmic extracts of RmMSU9 was measured by using methods described previously (13) except that cell suspensions of all strains were adjusted to an optical density (OD) of 0.6 (absorbance at 595 nm [A_{595}] was measured) prior to periplasm protein extraction, and that activity was assayed with the pH adjusted to 5.3, 6.3, and 8.3.

Nucleic acid manipulations and plasmids. The protocols of Sambrook et al. (45) were used for routine manipulations of plasmid and chromosomal DNA. The Tn*5*B22 insertion site in the mutant was characterized by subcloning Tn*5*B22 along with flanking chromosomal DNA. The transposon-chromosome junction was then sequenced, with the resulting nucleotide sequence data used to conduct searches of public databases. Briefly, total chromosomal DNA was harvested from the mutant, digested with *Xma*I, and then ligated into pBluescript KS(+) (Stratagene, La Jolla, Calif.). The ligation mix was transformed into *E. coli* DH5α cells (45), and plasmids from transformants resistant to ampicillin and gentamicin were analyzed by restriction analysis to verify that each contained a single cloned fragment. Southern blotting was then used to verify that the cloned fragment was identical to that in the genome of the mutant strain. A plasmid containing the entire Tn*5*B22 transposon and flanking DNA was recovered and is referred to as pMLS148. The primers 5′-AACGACGGGATCCATAAT-3′ and 5′-CCATGTTAGGAGGTCACATGGAAGTCAG-3′ were used to initiate sequencing from the *lacZ* and transposase termini, respectively (48).

A *Bam*HI-*Xma*I fragment of pMLS148 containing the 5′ portion of the *lon* gene flanking the transposon was used to probe an *S. meliloti* 104A14 cosmid library; the construction of this library has been described previously (1). One hybridizing cosmid, c11E8, was chosen for further study and subcloning. A 3.4-kb *Xma*I fragment bearing the *lon* gene was subcloned from c11E8 and into the multiple cloning site of pUCP19 (55), and the *lon* gene alone was removed as a 2.7-kb *Bam*HI fragment and cloned into pRK311 (16) to form pMLS150. This plasmid, c11E8, and pRK311 were transferred into the mutant strain and the wild-type *S. meliloti* strain via conjugation with *E. coli* S17-1 (47).

Sequencing of the insert subcloned in pMLS150, as well as DNA flanking Tn*5*B22 (described above), was accomplished with an ABI 377 DNA sequencer (Perkin-Elmer, Norwalk, Conn.) by using synthetic primers complementary to the transposon termini (described above) and to nucleotide sequences determined within the cloned fragments. Sequence homology searches were conducted by the BLAST network service (2), and sequence alignments were done with the GAP program (14). Both strands of pMLS148 were sequenced.

Polysaccharide isolation and characterization. Cultures were grown to early stationary phase (A_{595} was ~1.0 for wild-type cultures and ~0.75 for the *lon* mutant) in minimal succinate broth, and then exopolysaccharides were precipitated from the culture supernatant and purified by using the methods described by Doherty et al. (17). The final EPS material was dialyzed against six changes (4 liters each) of distilled water over a 48-h period. EPS was quantitated by using the anthrone assay (56) with glucose as the standard and then normalized to total protein by using the Bio-Rad microassay (Richmond, Calif.) with bovine serum albumin as the standard. For gas chromatographic (GC) analysis of EPS, precipitated and purified EPS samples were subject to acid hydrolysis (6), reduced with sodium borohydride (19), and derivatized to thrimethylsilyl ethers (52) prior to being analyzed in a Varian 3300 gas chromatograph, using a flame ionization detector and separation on an HP-1701 column.

Total EPS was also analyzed by ^{13}C nuclear magnetic resonance (NMR) spectrometry. Samples were prepared by dissolving 8.8 mg of freeze-dried EPS from the *lon* mutant and 13.8 mg of EPS from the wild-type strain in 0.7 ml of D_2O (Cambridge Isotope Laboratories) and sonicating the samples for approximately 24 h. The spectra were collected with a Bruker DRX 500 spectrometer at 60°C at a carbon frequency of 125.77 MHz with WALTZ-16 decoupling of the protons. For each, 60,000 transients of 32k complex points were collected with a total recycle delay of 1.54 s. The data were processed by using an exponential window function with a line broadening factor of 2 Hz and then zero filled to a final size of 32k real points.

For electrophoretic analysis of cell surface-associated polysaccharides, cells were washed and extracted by using the methods described by Carlson et al. (9), followed by dialysis against distilled water as described above. Samples were electrophoretically separated and stained as described by Reuhs et al. (41). Briefly, samples were mixed with an equal volume of sample loading solution that contained 10% (vol/vol) glycerol, 0.25% (wt/vol) sodium deoxycholate (DOC), 0.125 M Tris (pH 6.8), and 0.002% bromphenol blue. They were then electrophoresed through acrylamide gels which were comprised of a stacking phase that was 4% acrylamide polymerized in a buffer comprised of 0.5% (wt/vol) DOC and 0.125 M Tris-Cl (pH 6.8) and a resolving phase that was 18% acrylamide polymerized in a buffer containing 0.5% (wt/vol) DOC and 0.375 M Tris base (pH 8.8). The running buffer contained 0.290 M glycine, 0.037 M Tris base, and 0.25% (wt/vol) DOC. The gels were then stained for either lipopolysaccharides (LPS) alone or for both LPS and capsular polysaccharides (referred to as KdoPS [41]), again using the methods described by Reuhs et al. (41).

Plant growth and inoculation. For nodulation kinetic studies, alfalfa seeds were surface sterilized and aseptically germinated on YMB agar as described previously (35). Axenic seedlings were transferred to test tubes (2.5-cm diameter and 20 cm long), incubated in the dark for 24 h at 25°C, and then inoculated with 100 μl of a washed *S. meliloti* cell suspension, delivering approximately 1.0×10^5 cells directly to the distal 2 cm of the root of each seedling. The inoculant dose in each case was based on prior experiments in which the OD_{595}s of washed cell suspensions of each strain were calibrated to viable counts obtained on minimal succinate agar plates. After inoculation, the plants were transferred to a growth chamber and incubated at 25°C with a light intensity of approximately 400 $\mu mol \cdot m^2 \cdot s^{-1}$ and a photoperiod consisting of 16 h of light and 8 h of darkness. Plants were scored daily for the presence of nodules.

To assess the symbiotic competence of the Lon^- mutant, axenic alfalfa seedlings were cultured in Magenta growth boxes (Sigma, St. Louis, Mo.) as described previously (35) and grown under temperature and light conditions described above for the nodulation kinetic studies. For each strain, the stability of the transposon and/or plasmids during symbiosis was assessed. Nodules were surface sterilized as described previously (1) and then crushed in sterile 0.85% (wt/vol) saline solution and serially diluted, and aliquots were spread onto YMB agar. One hundred isolated colonies of each strain were subcultured onto minimal succinate agar and minimal succinate plus antibiotic agar to determine retention of antibiotic resistance. Total DNA was extracted from a random sample of 10 nodule isolates of the mutant strain and used in Southern blot analysis (pMLS148 used as probe) to verify that the transposon was retained in its original position.

Nucleotide sequence accession number. The sequence of the cloned *lon* gene is available under accession no. AF167159.

RESULTS

Mutant identification and growth characteristics. The *S. meliloti lon* mutant, RmMSU9, was originally identified as overexpressing phosphatase activity on $+P_i$ agar medium. A single blue colony arising on minimal succinate agar supplemented with gentamicin and XP was subcultured to purity, and then the phosphatase activity was quantified in periplasmic extracts of cells grown in minimal succinate broth cultures. Periplasmic phosphatase activity in RmMSU9 and 104A14 cells was compared at pH levels corresponding to the pH optima of the two

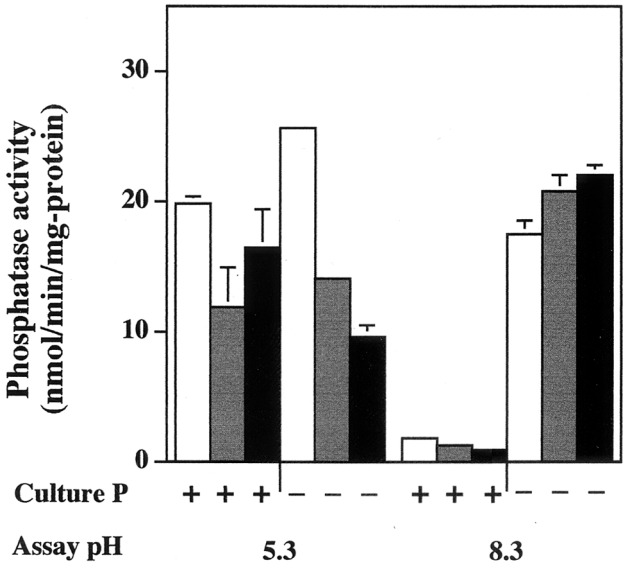

FIG. 1. Acid and alkaline phosphatase activities in periplasmic extracts of *S. meliloti*. The wild-type strain 104A14 is shown by black bars, the *lon* mutant RmMSU9 with white bars, and RmMSU9 (c11E8) (cosmid providing the wild-type *lon* gene in *trans*) as gray bars. Data are the average ± standard error of the means of at least two independent experiments in which each experimental mean was derived from periplasmic extracts of three separate early-stationary-phase cultures. Cells were incubated 16 h in minimal succinate broth with (+) or without (−) phosphorus.

acid phosphatases (pHs 5.3 and 6.3) and the alkaline phosphatase (pH 8.3) that are known to occur in *S. meliloti* (1, 13). Phosphatase activity in RmMSU9 was significantly and consistently higher than that observed in the wild-type strain 104A14 at only pHs 5.3 and 8.3 (Fig. 1) and verified the XP phenotype observed on agar media. Acid phosphatase activity in RmMSU9 was greater than in 104A14 under both $+P_i$ and $-P_i$ incubation conditions, and it increased nominally in P_i-stressed RmMSU9 cells (Fig. 1). Alkaline phosphatase activity in RmMSU9 grown with high levels of phosphate was low relative to phosphate-stressed mutant or wild-type cells, but it nevertheless was consistently at least twofold greater than in 104A14 under the same $+P_i$ incubation conditions (Fig. 1). Alkaline phosphatase levels in RmMSU9 increased from about 2 nmol/min/mg of protein in $+P_i$ cells to approximately 17.5 nmol/min/mg of protein after phosphate starvation. 104A14 responded as documented previously (1), with alkaline phosphatase being present at low levels in phosphate-replete cells (~0.9 nmol/min/mg of protein) and then increasing roughly 20-fold in response to P_i starvation. RmMSU9 carrying cosmid c11E8 (see below), which contains the wild-type *lon* gene, demonstrated acid and alkaline phosphatase activity more similar to that of the wild-type strain. From these experiments, it was concluded that the increased alkaline phosphatase activity in RmMSU9 represented a small but reproducible deviation from the normal Pho regulatory pattern of alkaline phosphatase expression, which is repressed by high phosphate levels (1).

Following cloning of the transposon and flanking DNA, the sequence flanking the Tn*5*B22 insertion site was determined. Homology searches using the partial sequence of the interrupted gene suggested the gene encoding the Lon protease had been affected. Following isolation of the cosmid clone c11E8 that contained DNA homologous to the interrupted gene, subcloning and Southern blot analysis identified a 3.4-kb *Xma*I fragment that contained the *lon* gene. The nucleotide sequence of this subclone showed an open reading frame (ORF) capable of encoding an 806-amino-acid protein having a molecular mass of 89.5 kDa. This ORF was preceded by a potential ribosome binding site and followed by a strong transcriptional terminator. The inferred protein was 62% identical and 78% similar to the Lon protease from *E. coli* (11) and was 82% identical and 90% similar to Lon from *Brucella abortus* (accession no. AF042348). Located 221 bp downstream from the *lon* gene, a second ORF was identified in the same orientation having 100% inferred amino acid identity to HupB (results not shown), a histone-like protein HU subunit previously sequenced from *S. meliloti* (29). This close association of *lon* and *hupB* has also been noted in other organisms (7, 50).

Growth of the *lon* mutant was inferior to that of the wild-type strain, with growth ceasing when culture OD (A_{595}) reached approximately 0.75 (Fig. 2). We also examined UV sensitivity of the *S. meliloti* mutant because *E. coli lon* mutants have been shown to be sensitive to UV light (26). After exposing 104A14 and RmMSU9 to UV light ranging from 1 to 25 mJ/cm^2 (Stratalinker 1800 UV Crosslinker; Stratagene), no differences between strains were observed (78 to 0.025% survival). In addition, microscopic examination did not reveal cell elongation in the *lon* mutant strain under either normal growth conditions or following UV irradiation (results not shown), a trait that is also associated with the *E. coli lon* mutant (24).

To verify that the growth phenotype was associated with the *lon* mutation, the *lon* gene was isolated and separated from the downstream *hupB* gene. The resulting construct, pMLS150, contained 350 bp upstream of the *lon* gene but lacked a downstream sequence, including that which encodes the last 23 predicted amino acids of *lon*. Nevertheless, pMLS150 restored the mutant to normal growth patterns in minimal succinate broth (Fig. 2).

Symbiotic phenotype. Because *lon* mutations have been implicated in differentiation processes of other bacteria, we ex-

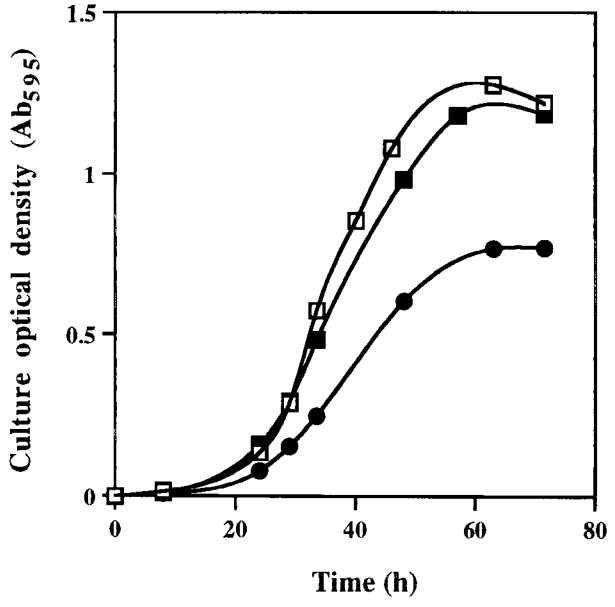

FIG. 2. Growth of *S. meliloti* wild-type and *lon* mutant strains in minimal succinate medium. Culture ODs were determined at the given times. Data points are from a single culture for each strain and are representative of one of three experiments showing a growth phenotype of the mutant, but with wild-type growth restored by the *lon* gene supplied in *trans*. □, wild-type strain 104A14; ●, *lon* mutant RmMSU9; ■, RmMSU9(pMLS150).

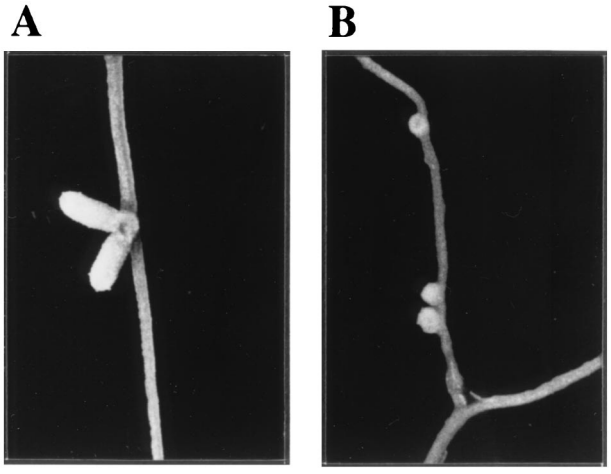

FIG. 3. Photographs of nodules formed by the wild-type and *lon* mutant strains. Shown are nodules formed by the wild-type strain 104A14 (A) and the *lon* mutant RmMSU9 (B). Roots were harvested 6 weeks after inoculation.

plored the possibility that the *lon* mutant would be defective in some stage of symbiosis formation with alfalfa. Inoculation of sterile alfalfa seedlings with RmMSU9 resulted in the formation of callus-like nodules (Fig. 3B), which were in contrast to the pink elongated nodules formed by the wild-type strain (Fig. 3A). Quantification of symbiotic parameters indicated that the nodules formed by the *lon* mutant could not support plant growth beyond that of uninoculated controls due to the inability to fix nitrogen (Table 1). Further, in studies examining nodulation kinetics, nodule formation by the *lon* mutant was significantly delayed and a significant number of plants remained nodule-free during the scoring period in which all plants inoculated with 104A14 were nodulated (Fig. 4). Upon extended incubation in the growth chamber (typically 20 to 25 days), all plants inoculated with the *lon* mutant eventually became nodulated (results not shown). As with the culture growth profile shown above in Fig. 2, the presence of the wild-type *lon* allele restored the mutant to a normal nodulation and symbiotic phenotype (Table 1 and Fig. 4); complementation was not due to the presence of the control plasmid pRK311.

Over the course of three different symbiotic competency experiments, few *lon* mutant *S. meliloti* cells could be recovered from the callus-like nodules. Typically, about 50 to 100 colonies were obtained from undiluted nodule homogenates sampled from surface-sterilized nodules. By contrast, roughly 10^5 isolates were routinely recovered from wild-type nodules (data not shown). These results indicated that an early step in nodule infection is blocked as a consequence of the *lon* mutation. All isolates obtained from the mutant nodules retained the Gent^r marker, and Southern blot analysis of total DNA extracted from randomly sampled isolates showed no evidence of transposon instability in this mutant during symbiosis. As determined by tetracycline resistance, retention of c11E8 in RmMSU9 was only approximately 55% and perhaps explains the slightly reduced plant dry matter accumulation by plants inoculated with RmMSU9(c11E8) relative to that of plants inoculated with RmMSU9(pMLS150).

EPS phenotype and partial characterization. The morphology of nodules formed by the mutant was strikingly similar to *S. meliloti* nodulation mutants that were found to be altered with respect to their extracellular polysaccharide composition (e.g., see references 17, 30, and 37). This observation, along with the fact that cell surface polysaccharide synthesis is affected in *E. coli lon* mutants (53), suggested that synthesis of capsular polysaccharides and/or EPS may be altered in RmMSU9. As assessed with hot phenol extracts separated on polyacrylamide gel electrophoresis (PAGE) gels and alcian blue plus silver staining, we found no evidence that capsular polysaccharide (KdoPS) synthesis in the *lon* mutant was altered (Fig. 5). Various paired volume samples from equivalent cell biomass of both 104A14 and RmMSU9 (as measured by total protein), as well as paired samples diluted in a twofold series (results not shown), failed to reveal any differences in KdoPS staining (alcian blue plus silver stain) between the Lon mutant and the wild-type strain. However, when stained for only LPS (silver staining alone), one major component was missing in the RmMSU9 extracts and was also not observed in extracts from RmMSU9(pMLS150) or RmMSU9(c11E8) (Fig. 5).

The *lon* mutant also produced and released significantly more EPS than the wild-type strain. Total EPS recovered from supernatants of five separate early-stationary-phase cultures for each strain showed the mutant synthesized 0.100 ± 0.010 (mean ± standard error) mg of glucose equivalents per mg of protein, in contrast to 0.035 ± 0.004 and 0.022 ± 0.004 mg of glucose equivalents per mg of protein for 104A14 and RmMSU8(pMLS150), respectively. As in the symbiotic competence studies, pMLS150 again contained DNA that returned the mutant to wild-type status with respect to EPS synthesis.

In an attempt to determine which EPS was being overproduced (EPSI or EPSII), we examined total EPS by using ^{13}C NMR and GC. ^{13}C NMR analysis showed that total EPS obtained from RmMSU9 appeared similar to that from 104A14 (Fig. 6) and both were similar to that previously reported for *S. meliloti* (28, 37), displaying signals corresponding to the acetyl, pyruvyl, and succinyl substitutions. The lone exception was an additional signal at 38 ppm, which was found to arise from free succinate derived from the medium (determined with scan of sterile medium) and was surprising given the extent that the samples were dialyzed. The GC analysis showed that the glucose-to-galactose ratio for RmMSU9 EPS was 5.0 ± 0.2 (mean ± standard error; two independent cultures, three subsamples each), in contrast to 7.1 ± 0.5 for the wild-type strain. Both strains fluoresced equally brightly on minimal succinate agar media containing calcofluor (results not shown), indicating that EPSI was being synthesized in both strains.

DISCUSSION

While attempting to identify and isolate an *S. meliloti phoR* mutant, we isolated a mutant that had higher than normal lev-

TABLE 1. Symbiotic phenotypes of the *S. meliloti* wild-type strain 104A14, the *lon* mutant RmMSU9, and the RmMSU9 complemented with cosmid c11E8 or pMLS150[a]

Strain	Symbiotic parameter		
	Shoot dry wt (mg per plant)	No. of nodules per plant	Nodule fresh wt (mg per plant)
104A14(pRK311)	13.5 ± 0.8	7.1 ± 1.0	5.6 ± 0.4
RmMSU9(pRK311)	5.3 ± 0.5	8.2 ± 0.8	5.9 ± 0.5
RmMSU9(c11E8)	11.2 ± 0.5	8.6 ± 1.0	8.3 ± 1.2
RmMSU9(pMLS150)	13.9 ± 1.4	6.7 ± 0.6	7.0 ± 0.8
Uninoculated control	5.8 ± 0.3		

[a] Data are from one of two experiments showing the symbiotic defect of the *lon* mutant and represent the means ± standard errors of five replicates for each treatment. Wild-type and control strains contained the c11E8 and pMLS150 parent plasmid pRK311.

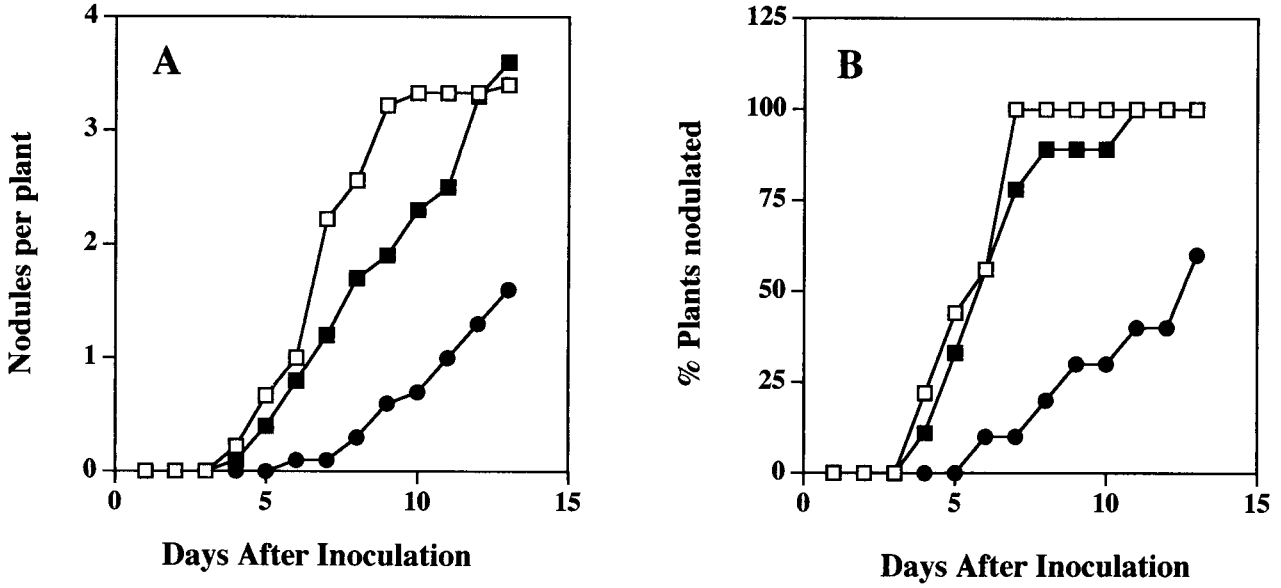

FIG. 4. Nodulation kinetics of the wild-type and *lon* mutant strains used in this study. Shown are the number of nodules formed per plant (A) and percentage of plants nodulated (B) following inoculation with the wild-type strain (□), the *lon* mutant RmMSU9 (●), or RmMSU9 complemented with the wild-type *lon* allele contained in pMLS150 (■). Results are from one of three experiments documenting the delayed and reduced nodulation of the *S. meliloti lon* mutant RmMSU9.

els of phosphatase activity while growing on an agar medium that contained high levels of phosphorus (Fig. 1). This phenotype is consistent with the PhoR$^-$ phenotype in *E. coli* (54), but an analysis of the transposon insertion site and sequencing of the complementing gene determined that the interrupted gene was *lon*. In addition to displaying phosphatase, growth, and EPS phenotypes in a minimal succinate medium (Fig. 2), the *lon* mutant also exhibited abnormal symbiotic behavior, being much slower than the wild-type strain in nodule formation, and the abnormal nodules formed by this mutant failed to fix nitrogen (Fig. 3 and 4; Table 1). The various defects of this mutant were reversed to normal when the wild-type *lon* allele (missing the C-terminal 23 amino acids) was provided in *trans* (Fig. 2 and 4; Table 1). Additional DNA appeared to not be required as complementation was achieved by only *lon* and minimal amounts of upstream flanking DNA; computer analysis failed to identify a complete ORF in the approximate 300 bp of DNA upstream of *lon* cloned in pMLS150 (results not shown). The complementing fragment in pMLS150 also lacked the 3′ region of the gene that codes for the terminal 23 amino acids. The apparent nonessential nature of this part of the C terminus might be explained by the complete lack of conservation in the terminal 29 inferred amino acids of various Lon proteins (alignments not shown).

The nodulation phenotype of RmMSU9 (Fig. 3 and 4) was found to be very similar to that described for other *S. meliloti* mutants affected in EPS synthesis (e.g., see references 17, 30, and 37), and previously published information regarding the role of Lon in other bacteria also suggested that production of surface polysaccharide may be somehow affected. Qualitative assessment of *S. meliloti* capsular polysaccharides (structurally distinct from LPS and referred to as KdoPS [41]), by staining in polyacrylamide gels failed to show any difference between the *lon* mutant and the wild-type strain. This was the case regardless of quantity of material loaded in the gel or time allowed for staining.

When staining for LPS alone, however, the *lon* mutant was found to lack a single major band. This alteration in LPS was

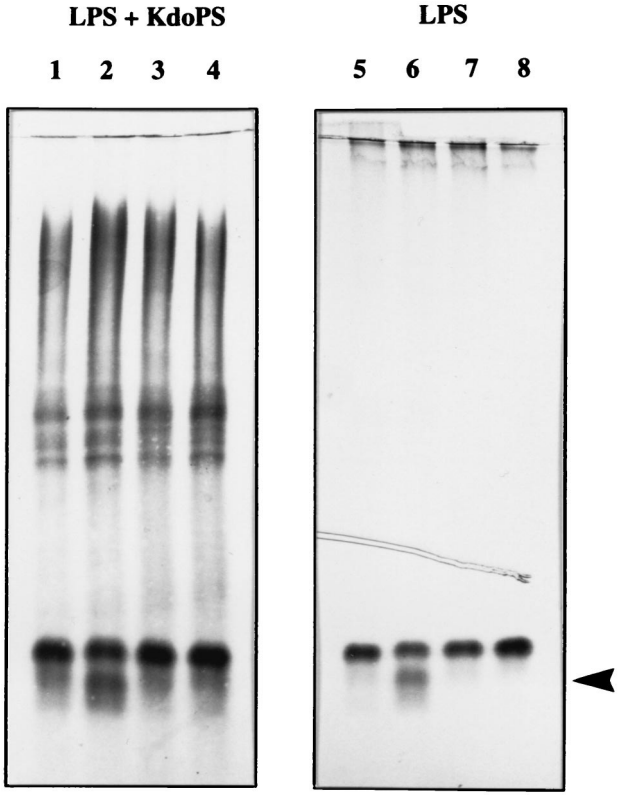

FIG. 5. Analysis of cell surface-associated polysaccharides of the wild-type and *lon* mutant strains used in this study. Shown are capsular polysaccharides and lipopolysaccharides (left) and silver strains of only the LPS (right) of the *lon* mutant RmMSU9 (lanes 1 and 5), wild-type strain 104A14 (lanes 2 and 6), RmMSU9(pMLS150) (lanes 3 and 7), and RmMSU9(c11E8) (lanes 4 and 8) as visualized by alcian blue plus silver staining. The arrow denotes the LPS band that is missing in the *lon* mutant.

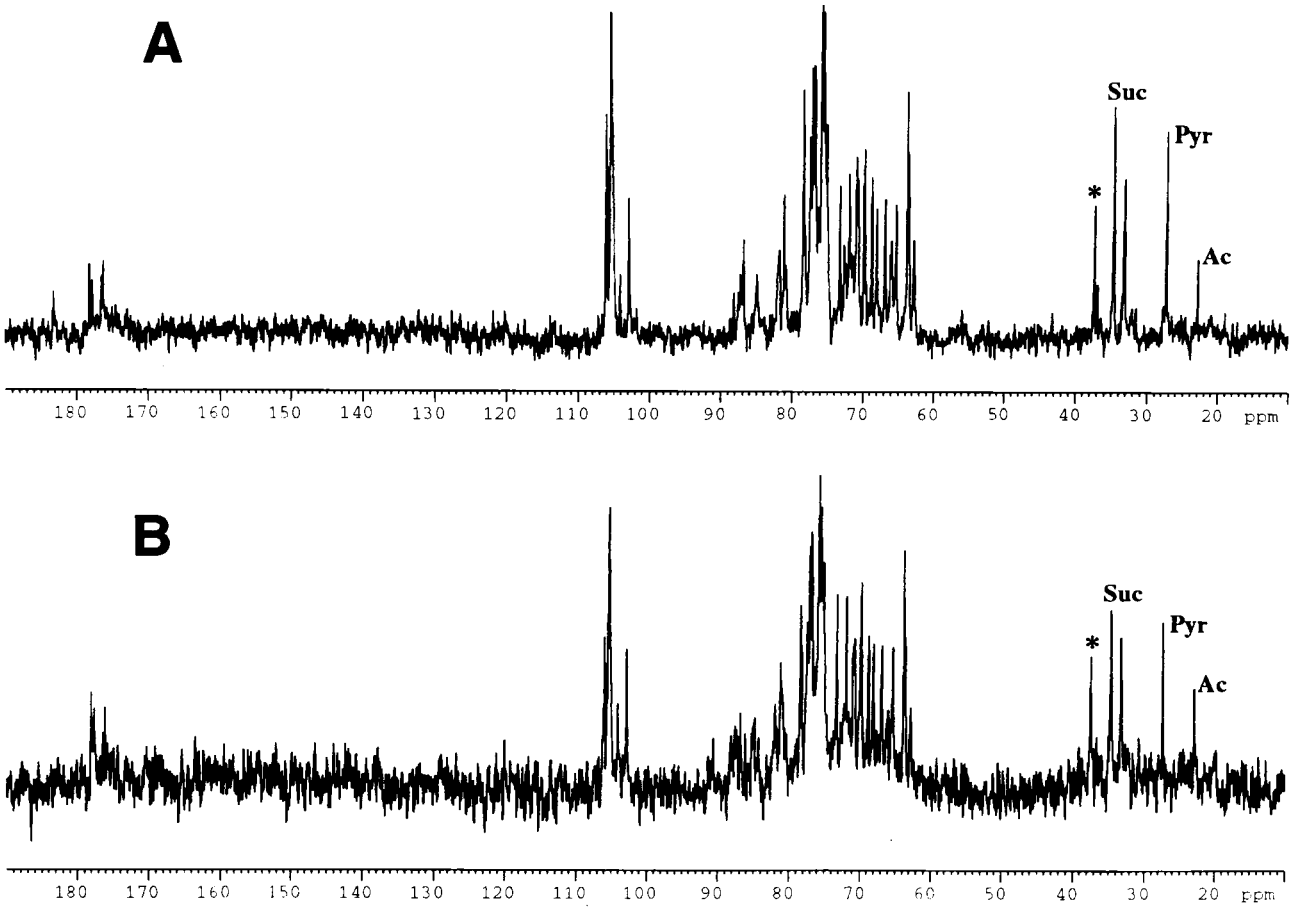

FIG. 6. ^{13}C NMR spectra of EPS derived from *S. meliloti* wild-type strain 104A14 (A) and the *lon* mutant RmMSU9 (B). The acetyl (Ac), pyruvyl (Pyr), and succinyl (Suc) signals were assigned based on previous identifications (28, 37). The signal at 38 ppm (*) was found to arise from free succinate.

likely not the basis for the symbiotic phenotype as the plasmid and cosmid clones that rescued the phosphatase (Fig. 1), growth (Fig. 2), and symbiotic phenotypes (Table 1; Fig. 4), and EPS regulation failed to replace the missing LPS component (Fig. 5). The absence of a wild-type LPS profile for RmMSU9(pMLS150) or RmMSU9(c11E8) also implies the possibility of an operon arrangement whereby the insertion of the Tn*5*B22 transposon had polar effects on expression of genes downstream from *lon* that were not included in the cloned DNA that rescued the symbiotic phenotype. Interestingly, all strains failed to exhibit staining of what might be interpreted as an LPS I species (see reference 27 for review). Prolonged silver staining and varying amounts of LPS extract did not result in bands or staining activity in the PAGE experiments that were obviously distinct from that observed in alcian blue plus silver stains (results not shown). Weak staining in the LPS I region of silver stain-PAGE experiments has been noted previously for *S. meliloti* (44), and the lack of a symbiotic phenotype associated with altered LPS in the Lon^- mutant is consistent with previous reports regarding the symbiotic performance of *S. meliloti* LPS mutants (12, 32).

In contrast to LPS mutants, successful symbiosis in indeterminate nodules such as those formed by *S. meliloti* on alfalfa has been correlated with correct structure (31) and amounts of EPS (17). Overexpression of EPSI in *S. meliloti* 1021 cells is associated with an inability to efficiently colonize callus-like nodules (17), a phenotype similar to that reported here for the *lon* mutant. However, nodulation defects similar to that observed with the *lon* mutant have also been found with *S. meliloti* mutants unable to synthesize EPSI (39). In examining possible *lon* mutation effects on EPS synthesis, RmMSU9 was found to overexpress EPS. Additional experiments were then conducted in an attempt to establish if EPSI was being synthesized or oversynthesized. Both 104A14 and RmMSU9 cells fluoresced brightly on minimal succinate agar medium containing calcofluor (which binds to the succinoglycan EPSI [17]), suggesting that EPSI was being synthesized. Therefore, NMR and GC analyses were employed to obtain evidence regarding overproduction of EPSI and/or synthesis of EPSII.

Previous structural and analytical studies have determined that EPSI and EPSII can be differentiated based upon their glucose and galactose composition. In addition, EPSI and EPSII can be compared based on the presence of succinyl substitutions on the EPSI carbohydrate backbone as opposed to its absence in EPSII (reviewed in references 5 and 33). The glucose-to-galactose ratio is 7:1 in EPSI and 1:1 in EPSII. If EPSI alone was being overproduced in RmMSU9, then the glucose-to-galactose ratio should remain at 7:1. Alternatively, if all of the nearly threefold increase in polysaccharide synthesis was due to EPSII production, then perhaps a glucose-to-galactose ratio closer to roughly 2.0 might be expected. As determined by using GC analysis, a glucose-to-galactose ratio of 5:1 in the *lon* mutant suggests that the additional EPS synthesized was composed of both EPSI and EPSII. Such an outcome should result in the total EPS material being enriched for acetyl and pyruvyl substitutions relative to succinyl groups.

The ^{13}C NMR spectra of total EPS purified from RmMSU9 and 104A14 showed the presence of the succinyl, acetyl, and pyruvyl substitutions thought to be critical for normal nodulation (28, 37) and present in the low-molecular-weight EPSI fraction that promotes nodule invasion (4). While clearly not quantitative, a comparison of the signal intensities of the succinyl, acetyl, and pyruvyl signals for each strain shows subtle differences, perhaps indicating that the Lon$^-$ mutant EPS is enriched for acetyl and pyruvyl groups relative to the succinyl groups. This again would imply that EPSII was being produced along with EPSI. While not convincing evidence by itself, this is consistent with the glucose-to-galactose composition analysis.

Under normal growth conditions, the wild-type *S. meliloti* strain produces only EPSI. However, under conditions of nitrogen limitation, it will overproduce EPSI (17) or can be induced to produce EPSII when phosphate stressed (43, 51, 58). As the above EPS characterization experiments were conducted with cells grown in media that contained nonlimiting amounts of both nitrogen and phosphorus, it appears that regulation of both EPSI and EPSII synthesis is disrupted by the lack of normal Lon protease activity.

As many of the *exp* genes coding for proteins involved in EPSII synthesis are sensitive to phosphorus availability and are controlled by PhoB (43, 51), low-level constitutive expression of alkaline phosphatase in this mutant is not surprising. Alkaline phosphatase is the marker enzyme for the phosphate stress response in bacteria and is controlled by PhoB (see review by Wanner [54]). This is also the case for *S. meliloti* (1). Low-level constitutive expression of alkaline phosphatase in the *lon* mutant is similar to the alkaline phosphatase phenotype observed with the *E. coli phoR* mutant (54). However, these two mutants differ in that alkaline phosphatase induction in phosphate-limited RmMSU9 is essentially at wild-type levels (Fig. 1), as compared to no alkaline phosphatase induction in the *E. coli phoR* mutant. This implies that Lon regulates the relative cellular abundance of a protein that is capable of stimulating *pho* gene expression in *S. meliloti* under high-phosphate-level conditions. Whether PhoB is involved in this scenario is not yet clear, but will be a thrust of future experiments.

As elucidated with *E. coli*, one important function of Lon is related to regulation of cellular processes via degradation of regulatory proteins, such as SulA and RcsA (22, 23). There are a number of regulatory proteins involved in controlling EPS synthesis in *S. meliloti* (reviewed in reference 5), and it would not be unreasonable to expect that some are substrates for the Lon protease. Mutations in ExoR and ExoS result in EPSI overproduction (17), and mutations in MucR and ExpR result in EPSII overproduction (21, 28, 57). However, these are negative-acting regulatory proteins and, as such, are probably not involved in the Lon$^-$ mutant EPS phenotype. Their accumulation due to loss of turnover via protease activity would likely result in reduced EPSI and EPSII production.

Increased levels of positive-acting regulatory proteins would more likely account for increases in EPS production in this mutant, and a number of positive regulatory proteins have been described. Overproduction of SyrM results in increased EPS synthesis (38), and SyrA is apparently involved also (38). Potentially, Lon protease targets might also include ExpG, which has also been shown to have positive regulatory effects on EPSII production (3). In addition, an RcsA-like system may also be involved. RcsA is a positive regulator of capsule synthesis in *E. coli* and is a substrate for Lon (22), and it has been previously hypothesized to be involved in regulating *S. meliloti* EPS synthesis (17). An RcsA homolog has been detected in *R. leguminosarum* by immunological techniques (15), but a direct connection between EPS production and RcsA has not yet been established in the rhizobia. Finally, evidence in the literature also suggests that other possible Lon targets potentially relevant to the *lon* mutant EPS phenotype might include ExoY or ChvI (see references 10 and 40).

While the results of this study likely do not entirely explain the basis for the symbiotic phenotype of the Lon$^-$ mutant, the data do demonstrate an association between Lon function and regulation of EPS production. Given previous reports correlating abnormal alfalfa nodulation with changes in *S. meliloti* EPS structure and content (17, 30, 37), it is likely that at least part of the RmMSU9 symbiotic defect results from abnormal EPS synthesis. It is anticipated that future work aimed at identifying Lon protease substrates will further clarify the basis for the symbiotic defect in RmMSU9 and enhance our understanding of EPS synthesis in *S. meliloti*.

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