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mucS, a Gene Involved in Activation of Galactoglucan (EPS II) Synthesis Gene Expression in *Rhizobium meliloti*

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In addition to the exopolysaccharide succinoglycan, *Rhizobium meliloti* can produce a galactoglucan exopolysaccharide, EPS II. The production of EPS II occurs in certain mutant strains, in strains containing extra copies of EPS II synthesis genes, or in the wild-type strain under phosphate-limiting conditions. We have identified a gene, *mucS*, that is in a locus required for EPS II induction by extra gene copies and by phosphate limitation, and that activates the expression of at least one other EPS II synthesis gene. *mucS* lies within a cluster of EPS II synthesis genes and contains an open reading frame of 190 amino acids. MucS does not show any significant similarity to known genes and may represent a new type of regulatory protein.

The root nodule symbiosis between *Rhizobiaceae* and legumes develops as a result of an exchange of signal molecules between the symbiotic partners (Fisher and Long 1992). The primary signal, a specific lipochitooligosaccharide produced by the bacterial partner, induces the early events of nodule formation (Lerouge et al. 1990). However, additional molecules are needed for subsequent steps. In the case of symbioses that lead to indeterminate (elongate, continuously growing) nodules, such as that between *Rhizobium meliloti* and alfalfa, the bacterial exopolysaccharide is required for the development of the plant-derived infection thread through which the bacteria invade the developing nodule (Leigh and Coplin 1992; Leigh and Walker 1994). Specifically, *R. meliloti* excretes the acidic exopolysaccharide succinoglycan. Mutants with defects in *exo* genes are not able to synthesize succinoglycan, and elicit the formation of nodules but are blocked from invasion at the stage of infection thread development (Leigh et al. 1985, 1994). The addition of specific forms of succinoglycan is able to restore invasion by the mutants (Battisti et al. 1992).

A second exopolysaccharide, EPS II (EPSb or galactoglucan), produced by *R. meliloti* under certain conditions, is able to replace succinoglycan in the invasion step (Glazebrook and Walker 1989; Zhan et al. 1989). EPS II, structurally an entirely different polysaccharide from succinoglycan, is a high molecular weight polymer made up of repeating units of 4,6-*O*-(1-carboxyethylidene)-α-D-Gal*p*1→3(6-O-Ac)-β-D-Glc*p*1→3 (Her et al. 1990; Levery et al. 1991). Like the *exo* genes for succinoglycan synthesis, the genes for EPS II synthesis are located in a distinct cluster on megaplasmid pRmeSU47b (Glazebrook and Walker 1989; Zhan et al. 1989). Glazebrook and Walker (1989) designated these genes *exp*, while we (Zhan et al. 1989) used the designation *muc*. Genetic analysis indicated that EPS II synthesis required the products of at least six contiguous *exp* loci, spanning about 23 kb (Glazebrook and Walker 1989).

The set of conditions under which EPS II is produced suggests that its regulation is complex. Under culture conditions normally used for succinoglycan production—those involving nitrogen limitation for example—EPS II production is not detectable. However, EPS II is produced by wild-type *R. meliloti* (strain Rm1021) under conditions of phosphate limitation (Zhan et al. 1991). In addition, certain mutants produce EPS II even when phosphate is not limiting. Two different mutations in the *R. meliloti* chromosome, *mucR* (Zhan et al. 1989) and *expR* (Glazebrook and Walker 1989), cause EPS II production. *mucR* has been sequenced and further characterized (Keller et al. 1995), and is highly homologous to Ros, which negatively regulates *vir* gene expression and succinoglycan synthesis in *Agrobacterium tumefaciens*. In addition, introducing the *exp* (or *muc*) gene cluster into wild-type *R. meliloti* on a plasmid results in EPS II production. This plasmid-induced EPS II production may be due to multiple copies of genes that are involved in EPS II synthesis. Interestingly, Glazebrook and Walker (1989) identified two loci within the *exp* cluster that were distinguished by the fact that they were necessary for this plasmid effect. One possible distinction between these two loci and the other genes in the cluster may be that they are involved in positive regulation, while the others are biosynthetic genes. Thus, extra copies of positive regulatory genes may be sufficient for EPS II production under conditions where it does not normally occur. In this paper we have identified and sequenced one such regulatory gene. Since the first characterized EPS II regulatory gene is *mucR*, we designate this second one *mucS*.

RESULTS

mucS::Tn5, an insertion mutation that eliminates multicopy induction of EPS II synthesis.

Previous work in our lab showed that pMuc, a cosmid containing the EPS II synthesis loci identified by Glazebrook

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The sequence is assigned GenBank accession number U51475.

and Walker (1989), elicited EPS II synthesis in the wild-type Rm1021 background (Zhan et al. 1989). One manifestation of this plasmid-induced EPS II production, as distinct from normal succinoglycan production, was the development of mucoidy on LB agar plates. This made it possible to identify Tn*5* insertions in pMuc that eliminated its ability to induce EPS II synthesis in an Rm1021 background (H. Zhan and J. Leigh, unpublished). One such insertion mutation, designated *mucS*::Tn5, is studied here. By restriction mapping, *mucS*::Tn5 lay near the *expG-expD* junction of Glazebrook and Walker (1989), and did not correspond to either of the loci determined by them to be required for plasmid-induced EPS II production.

Another situation in which *R. meliloti* produces EPS II is in the wild-type strain Rm1021 under phosphate-limiting conditions. We showed that the *mucS*::Tn*5* mutation in the genome eliminated EPS II production in this situation as well. To make sure that our assessment of EPS II production was not confounded by succinoglycan production, we constructed the *mucS*::Tn5 mutation in the *exoA* background Rm6086 (see Materials and Methods). We then grew the strain on phosphate-limiting MOPS-KOH agar medium. The absence of colony mucoidy in Rm6086 *mucS*::Tn5, as contrasted with the positive control Rm6086, showed that EPS II synthesis did not occur.

mucE::Tn3HoKm, a *lacZ* fusion in a gene required for EPS II synthesis.

To determine the regulatory effect of *mucS*::Tn5, we constructed a *lacZ* reporter gene fusion to an EPS II synthesis gene using the transposon Tn3HoKm. Tn3HoKm insertions in pMuc were made in *E. coli*, then pMuc with insertions was transferred into Rm1021, where blue colonies on yeast mannitol (YM) plates with X-Gal were sought. Several blue colonies were found and screened by cosmid isolation and restriction digestion. One fusion, *mucE*::Tn3HoKm, mapped in the *expE* locus according to the restriction map of Glazebrook and Walker (1989), and was used for further studies. To demonstrate that the *mucE*::Tn3HoKm insertion was in a locus required for EPS II production, we constructed this mutation in the genome of the *exoA* mutant Rm6086 as for *mucS*::Tn5 above. Under phosphate limiting conditions, the *mucE*::Tn3HoKm mutation eliminated EPS II production.

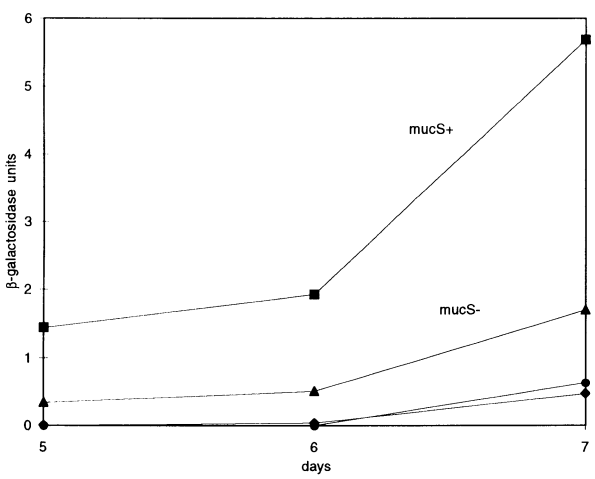

Fig. 1. Effect of *mucS*::Tn5 on *mucE* expression. β-Galactosidase activities for cultures at various times after inoculation are shown. The values shown are averages of two cultures each. The experiment was repeated and similar trends were observed. Only results with no phosphate added are shown. Diamonds, Rm8501; circles, Rm8501 *mucS*::Tn5-233; triangles, Rm8501 *mucS*::Tn5-233 *mucE*::Tn3HoKm; squares, Rm8501 *mucE*::Tn3HoKm.

Effect of *mucS*::Tn5 on *mucE*::Tn3HoKm expression.

To study the effect of the Tn*5* insertion in *mucS* on the expression of *mucE*::Tn3HoKm, we placed these mutations, individually and together, in the Lac$^-$ background Rm8501 (see Materials and Methods). Thus, we had strains Rm8501, Rm8501 *mucE*::Tn3HoKm, Rm8501 *mucE*::Tn3HoKm *mucS*::Tn5-233, and Rm8501 *mucS*::Tn5-233. The strains were grown for 7 days in MOPS-KOH liquid medium with no phosphate added and with 0.1 mM phosphate added. Samples were taken for measurement of β-galactosidase activity at 5, 6, and 7 days. Only basal levels of β-galactosidase activity (0.5 units or less) were detected in strains lacking the *mucE*::Tn3HoKm insertion (Fig. 1), as well as in all strains grown in medium with phosphate added (not shown). Therefore, phosphate limitation is required for *mucE* expression as well as for EPS II production. In the absence of added phosphate, Rm8501 *mucE*::Tn3HoKm expressed about fourfold higher levels of β-galactosidase activity than Rm8501 *mucE*::Tn3HoKm *mucS*::Tn5-233 (Fig. 1). The effect of the *mucS*::Tn5-233 insertion on *mucE* expression suggested that a gene, *mucS*, was a positive regulator.

Subcloning the *mucS* locus.

To identify the *mucS* gene, we sought to subclone the smallest fragment of pMuc that could complement the regulatory phenotype of the *mucS*::Tn5-233 mutation. Plasmids were mated into Rm7504 (Rm8501 *mucS*::Tn5-233

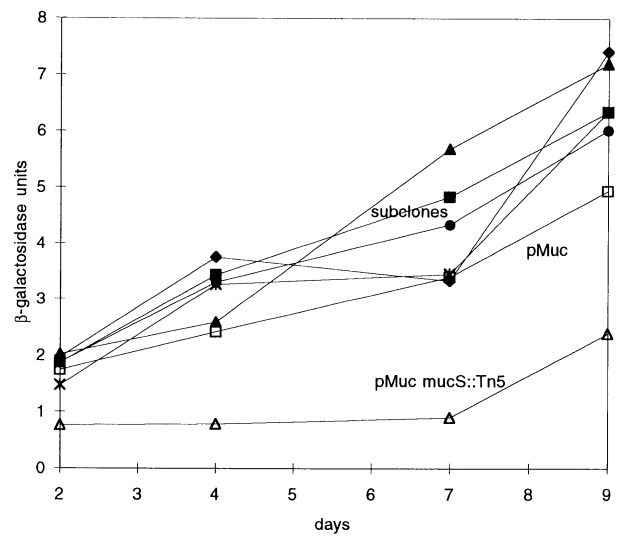

Fig. 2. Complementation of *mucS*::Tn5-233 in an Rm7504 background. β-Galactosidase activities for cultures at various times after inoculation are shown. Open square, pMuc; open triangle, pMuc *mucS*::Tn5; closed square, p*mucS*EE; closed diamond and closed triangle, p*mucS*BB in two orientations; closed circle, p*mucS*BE; crossed symbol, p*mucS*BgE. Results with vectors pLAFR1 and pSP329 (not shown) differed from pMuc *mucS*::Tn5 by no more than 0.3 units. The values shown are averages of two cultures each. The experiment was repeated and similar trends were observed.

mucE::Tn3HoKm) and complementation of the *mucS* mutation was judged by measuring the β-galactosidase activity arising from *mucE*::Tn3HoKm. All cultures were incubated in MOPS-KOH liquid medium with no phosphate added. As expected, pMuc complemented the mutation while pMuc *mucS*::Tn5 did not (Fig. 2). Subclones from the region of pMuc that contained the site of insertion of *mucS*::Tn5 (see Fig. 3) were made in the broad host range vector pSP329 and mated into Rm7504. Subclones constructed with the *Eco*RI fragment, the *Bam*HI fragment in both orientations, the *Bam*HI-*Eco*RI fragment, and the *Bgl*II-*Eco*RI fragment, were all positive (Fig. 2), as was the *Kpn*I-*Cla*I fragment (not shown). The smallest of these, containing the *Bgl*II-*Eco*RI and the *Kpn*I-*Cla*I fragments, were used to make *Exo*III deletions from the right and left, respectively (see Materials and Methods). On the right, the region between the ends of the smallest positive deletion (R4, Fig. 3) and the negative deletion (R3) represented an apparent border of the region required for complementation. On the left, all five deletions tested positive. Therefore it was concluded that the region between the right border of deletion R4 and the left border of deletion L5 (Fig. 3) contained the *mucS* gene.

Sequence analysis of *mucS*.

We sequenced from the left border of deletion L3 to the right border of deletion R4 (Fig. 3). The sequence (GenBank accession number U51475) is shown in Figure 4. We found one open reading frame (ORF) of 190 amino acids (predicted molecular weight: 22,590 daltons). Sequencing revealed that the noncomplementing deletion R3 and the *mucS*::Tn5 insertion both interrupted the ORF (Fig. 4). The ORF showed no significant similarity to any known gene, either at the nucleotide or the amino acid level. A putative ribosome binding site preceded the ORF, as did two upstream direct repeats. Hydropathy analysis of the ORF (not shown) revealed a potential transmembrane region from amino acid 78 (nucleotide 926) through 95 (nucleotide 979).

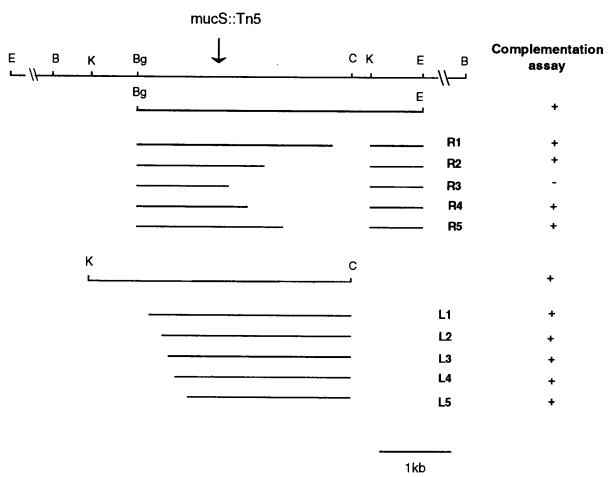

Fig. 3. Restriction map of the *mucS* region, deletion subclones, and complementation results. Orientation is opposite that of Glazebrook and Walker (1989), and the *Eco*RI fragment indicated corresponds to their *Eco*RI fragment reaching from *expA* to *expE*. The arrow shows the position of the *mucS*::Tn5 insertion. The position of the *mucE*::Tn3HoKm insertion lies off the map to the left. Fragments subcloned into pSP329 and tested for complementation of *mucS*::Tn5-233 are shown as horizontal lines, representing, in the case of deletions, DNA remaining after deletion. Restriction enzyme sites are as follows: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; K, *Kpn*I.

Complementation assay of smaller *mucS* subclones.

To eliminate the possibility that genes upstream from the *mucS* ORF were required for function, smaller subclones were obtained through PCR. As before, these were cloned into the broad host range vector pSP329, transferred to Rm7504 through conjugation, and checked for complementation (Fig. 5). Subclones comprising nucleotides 101 through

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   1  ACCAGGCGGCGTCGAGGGGGGCGCTCACCGCCGAAGAGGTGAGGAAGCCG
  51  CGCAGCTCGGTGAGGTCGCGCAGCGACTGCGTCGCGCGCGCAAGTCCCTG
 101  ATCGGCCGATGCCTGGACGGCGGCGGTCAGGACCGGCAGGTTCAGCCAGC
 151  GGACCACGGCGCTGCCCATGGCCTGAAAGGTGAGGGCGCGAATGTAGTCG
 201  AGCACGCCCAGAACGATCAGCGCGCCCAGGGCGAGAATCGTCAGCATGAC
 251  GAGCGTATCCATCGTCTGGCTGTTCAACACCCTGTCATGCACCTGCATCA
 301  TATAGAGGGGCATGGTGAGCTGCAGGAGGTTGATGCAGGCGCTGAGCAGG
 351  GCTGCATAAAGAAGCGTCACGACGAACACACGCCGGGCCCGCAGAACCAG
 401  CAGCTTCGGCGCGATTGTTCCGTTTTGCGCCGGTGTTGCCCGGTTGGGGC
 451 [CCTTGCTAATCAAAGGATTTCGAACAGTATCACGCATGGGGCGATCTTCT
 501  AATTAGGCATTCGCGGGTTTCATGGGGCGATCTGGTCCAGATTCACTCAA
 551  ATCACAGTATTGTTGCGATAAGTGTGACACAATAAACATCGCCAGCTCAA
 601  GAGCACGCAATTTCGGGGCAGGGGTGTTATGAAATTACTTCAAGTTTTGA
                                                  M  N
 651  AGTAATTTTCCGGAATTGGAAGTACGTTCCCAATGGAGAGAGGGATGAAC
                          ↓
      H  R  I  L  Y  P  F  A  D  F  G  D  T  V  A  I  L
 701  CACAGGATACTCTATCCGTTCGCAGACTTTGGAGACACTGTTGCAATCCT
       P  A  N  E  T  Q  R  K  G  L  D  T  P  V  D  D  R
 851  TCCCGCGAATGAAACGCAGCGGAAAGGCCTCGATACCCCTGTAGACGATC
       D  G  D  D  S  L  V  T  Y  F  E  L  A  R  V  M
 801  GTGACGGAGATGATTCGCTCGTCACCTATTTCGAGCTGGCCCGGGTCATG
      E  R  A  S  R  R  F  S  G  L  L  R  A  E  L  T  K
 851  GAGCGTGCAAGCCGCCGCTTTTCCGGCCTGTTGCGTGCGGAACTGACGAA
       L  G  V  E  D  I  G  P  A  Q  A  M  V  L  L  A  I
 901  GCTCGGGGTCGAAGATATCGGTCCCGCGCAGGCGATGGTTCTTCTGGCCA
        G  E  A  E  L  S  V  G  E  L  L  D  R  G  H  Y
 951  TCGGCGAGGCGGAACTCTCGGTGGGAGAGCTTCTGGACCGGGGCCACTAT
      V  G  S  N  I  S  Y  Y  L  K  Q  L  A  D  G  D  Y
1001  GTGGGTTCGAACATCTCCTATTATCTGAAGCAGCTTGCCGACGGCGACTA
       I  D  R  I  A  S  Q  R  D  K  R  S  A  R  I  R  L
1051  CATCGACCGGATCGCTTCGCAGCGTGACAAGCGTTCGGCCCGCATCCGGC
        S  E  K  G  R  Q  L  C  A  G  L  R  Q  A  A  K
1101  TTTCAGAGAAAGGCAGGCAGCTTTGCGCCGGCCTGCGACAGGCGGCTAAG
                                              Δ
      G  Y  E  R  A  L  S  H  G  D  Q  D  R  R  N  L  E
1151  GGCTACGAACGCGCCCTCAGCCATGGCGATCAGGACCGGCGGAATCTGGA
       T  A  F  Q  T  L  H  R  L  R  L  V  W  G  N  A  A
1201  GACCGCCTTCCAGACACTGCACCGCCTCGAACTCGTCTGGGGGAATGCCG
        R  Y  G  I  *
1251  CACGCTACGGCATCTGATGCTCCATCTGCATGTTCCTCAAAGCGTAACCG
1301  AGCAAGGGCGGTAACATGCA]
```

Fig. 4. Sequence of *mucS*. A putative ribosome binding site is underlined, and 13-base direct repeats are overlined. Downward arrow indicates the position of the *mucS*::Tn5 insertion. Upward Δ symbol shows the right end of deletion R3 (Fig. 3). Brackets indicate the smallest PCR subclone that complemented *mucS*::Tn5-233.

1320 and 451 through 1320 were positive. A subclone from nucleotide 451 through 1175 was negative. Therefore, nucleotides 1 to 451 are dispensable, while nucleotides starting from 1175 within the ORF are necessary. These results confirm that the ORF, *mucS*, is responsible for regulation of *mucE* expression.

DISCUSSION

We have identified a gene, *mucS*, that is involved in activation of at least one other gene required for EPS II synthesis. That the *mucS* ORF is responsible for regulation was demonstrated in complementation assays in which deletions or a transposon insertion that interrupted the coding sequence, but not deletions that ended outside the gene, restored regulation of *mucE* expression to a *mucS*::Tn5-233 mutant. The deduced amino acid sequence of *mucS* did not show any significant similarity to other known proteins and appears to represent a new type of regulatory protein.

mucS is part of a complex regulatory system that controls EPS II synthesis. Other factors that may regulate EPS II synthesis include other positively acting loci in the EPS II synthesis cluster; the cellular response to phosphate limitation; and the negatively acting genes in the chromosome, *mucR* and *expR*. There is little information to indicate whether *mucS* interacts directly with any of these other factors. Glazebrook and Walker (1989) identified two loci that were required for the "plasmid effect," in which multiple copies of the EPS II synthesis gene cluster resulted in stimulation of EPS II synthesis. Mapping indicates that neither of these loci corresponds with the position of *mucS*, also required for the plasmid effect. If the other loci represent positive regulators as does *mucS*, there may be several positively acting regulators in the cluster. These regulators could work independently, but the fact that disrupting any one of them eliminates plasmid-induced EPS II production suggests that they may each be an essential part of the same regulatory pathway. Their presence in multiple copies is, of course, an artificial situation, in which they may exert their regulatory effect without the necessity for whatever signal they may transduce.

Phosphate limitation is another factor that controls EPS II synthesis. The expression of genes for EPS II synthesis is stimulated when phosphate is limiting, and this stimulation appears to rely on a regulatory locus that also stimulates alkaline phosphatase activity, a common cellular response to phosphate starvation (Zhan et al. 1991). Although the presence of a plasmid with extra copies of EPS II synthesis genes is not required for stimulation by phosphate limitation, we have shown here that *mucS* is necessary. It is possible that *mucS*, and perhaps the other possible regulatory genes in the EPS II cluster, participate directly in transducing the phosphate limitation signal to regulate gene expression. However, it is equally likely that *mucS* and the phosphate limitation signal work independently.

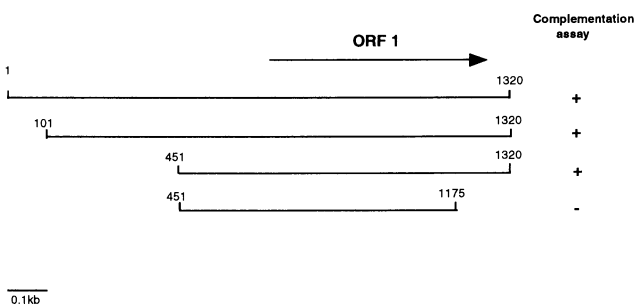

Fig. 5. Complementation assay of PCR subclones around *mucS*. ORF 1 refers to the *mucS* open reading frame. Nucleotide numbers refer to Fig. 4.

The regulatory picture is further enriched by the existence of two negative factors, *mucR* (Zhan et al. 1989) and *expR* (Glazebrook and Walker 1989). The only available information concerning where these genes fit in is that neither excess phosphate, nor a mutation in the apparent phosphate limitation response regulator mentioned above, eliminates EPS II synthesis in a *mucR*::Tn5 mutant (Zhan et al. 1991). Therefore *mucR* either acts downstream of the initial phosphate limitation sensing mechanism (and therefore a *mucR* mutation is epistatic), or *mucR* acts independently of phosphate limitation. Other work on *mucR* (Keller et al. 1995, Zhan et al. 1989) indicates that it is a positive regulator of succinoglycan synthesis as well as a negative regulator of EPS II production, and so may regulate the balance between the synthesis of the two exopolysaccharides. Its homology to *ros* in *A. tumefaciens* and the effect there on virulence as well as succinoglycan synthesis suggests that it is a gene of widespread importance. The functional interaction of *mucR* with *mucS* or other genes has not been investigated; for example, a *mucR mucS* double mutant has not been tested. *mucR* and *mucS* could act by independent regulatory pathways, or *mucR* and *mucS* could act together, one controlling the other's expression or affecting its activity.

MATERIALS AND METHODS

Media and supplements.

E. coli strains were grown in LB medium (Table 1). *R. meliloti* was grown in yeast mannitol (YM, Vincent 1970) or in LB supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$. Antibiotic concentrations were (μg/ml) streptomycin sulfate (Sm) 500, neomycin sulfate (Nm) 200, gentamycin sulfate (Gm) 15, spectinomycin sulfate (Sp) 100, tetracycline-HCl (Tc) 10, chloramphenicol (Cm) 25, kanamycin sulfate (Km) 50, and Ampicillin (Amp) 100. To assess EPS II synthesis or EPS II gene expression, phosphate-free morpholinopropane sulfonate buffered medium (MOPS-KOH) was prepared as described (Zhan et al. 1991). Solid media contained 1.5% Bacto Agar. For *R. meliloti*, solid media were supplemented with 0.02% Calcofluor to determine the presence of succinoglycan (Leigh et al. 1985).

Genetic and molecular techniques.

Plasmids were moved into *R. meliloti* and *E. coli* in triparental matings with the helper plasmid pRK600 (Ruvkun et al. 1982). Tn*5* insertions into pMuc were obtained by mating the plasmid first into the Tn*5*-containing *E. coli* strain MT614, then from this strain into *E. coli* C2110 selecting for cotransfer of tetracycline resistance and kanamycin resistance. Tn3HoKm insertions into pMuc were obtained using pTn3HoKm (Glazebrook and Walker 1989) and the method of Stachel et al. (1985).

mucS::Tn5 and *mucE*::Tn3HoKm insertions were transferred from pMuc into the Rm1021 genome by homogeniti-

zation (forced double homologous recombination with incompatible plasmid pPH1JI, Ditta 1986). Then, after we checked for tetracycline sensitivity (indicating loss of pMuc), *mucS*::Tn5 and *mucE*::Tn3HoKm were transduced into Rm1021 where Gm and Sp sensitivity confirmed the absence of pPH1JI. Southern hybridization confirmed the presence of the appropriate transposon insertions. Both mutations were also moved from the Rm1021 background into Rm6086 (Rm1021 *exoA*::Tn5-233) by transduction (Finan et al. 1986a).

Rm 7501 (Rm8501 *mucS*::Tn5) was constructed in the Lac$^-$ background Rm8501 by homogenitization. Rm7502 (Rm8501 *mucS*::Tn5-233) was made by replacing Tn*5* with Tn5-233 (encoding Gm and Sp resistance). This was done by conjugating in pRK607 and selecting for Gm and Sp resistance and Nm sensitivity (De Vos et al. 1986). We then transduced in *mucE*::Tn3HoKm to give Rm7504 (Rm8501 *mucS*::Tn5-233 *mucE*::Tn3HoKm). *mucE*::Tn3HoKm was also independently transduced into Rm8501 to give Rm7503 (Rm8501 *mucE*::Tn3HoKm).

Recombinant DNA techniques were as described (Ausubel et al. 1990; Maniatis et al. 1982). Fragments of pMuc were cloned first in pUC18, then transferred into the broad host range vector pSP329 for introduction into *R. meliloti*. pSP329, made by S. Porter in the lab of E. Nester, is a 7.5-kb plasmid of the IncP1 incompatibility group encoding tetracycline resistance and containing the pUC18 polylinker. ExoIII deletions on both ends of the *mucS* region were performed using the EraseABase kit (Promega Biotec., Madison, WI), following manufacturer's instructions except that the plasmids were isolated either by CsCl gradient centrifugation or using the Qiagen Midi prep isolation kit and protocol. Deletions were performed on fragments cloned into pSP329. For deletion from right to left, the *Bgl*II-*Eco*RI subclone was used after digestion with *Cla*I and *Kpn*I to create an ExoIII-susceptible end to the left only. Similarly, for deletion from left to right, the *Kpn*I-*Cla*I fragment was used after digestion with *Kpn*I and *Bgl*II.

Primers for PCR were obtained from DNA International and BioLabs. Primers were designed to contain a *Bam*HI site and a *Xba*I site near their 5′ ends to facilitate cloning into the multiple cloning sites of the vectors. PCR was performed basically as described (Ausubel et al. 1990) in a Mini Cycler from MJ Research. The 100-µl reaction volume contained a final 2.5 mM $MgCl_2$ concentration. All reaction steps were carried out for 1 min at 94°C for denaturation, 50°C for annealing, and 72°C for polymerization for a total of 30 cycles.

β-Galactosidase assays were performed as described by Miller (1972). The A_{550nm} coefficient of 1.345 was used for *R. meliloti* as determined empirically by J. Jelesko.

DNA sequencing and analysis.

DNA sequencing was performed using the Sequenase kit (United States Biochemical Corp., Cleveland, OH) on single-

Table 1. Bacterial strains, plasmids, and transducing phage used in this study

Strain, plasmid, or phage	Genotype	Source or reference
E. coli		
HB101	*rps*L20 *pro*A2 *rec*A13 *hsd*S20(r_B^- m_B^-) *supE*44	G.C.Walker
MM294A	*pro-82 thi-1 endA1 hsdR17 supE44*	G.C.Walker
MT616	MM294(pRK600)	Finan et al. 1986b
MT614	MM294A *recA56* Ω6::Tn*5*	T. Finan
DH5αF′	F′ φ*80d lacZ* Δ*M15* Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17* (r_K^-, m_K^-) *supE44*λ$^-$*thi-1 gyrA relA1*	Gibco-BRL
C2110 Nal^r Rif^r	*polA1 rha his*	E. Nester
R. meliloti		
SU47	Wild type	Vincent 1941
Rm1021	SU47 Sm^R	F. Ausubel
Rm6086	Rm1021 *exoA32*::Tn5-233	G. Walker
Rm8501	Lac$^-$ derivative of Rm1021	G. Walker
Rm7501	Rm8501 *mucS*::Tn5	This work
Rm7502	Rm8501 *mucS*::Tn5-233	This work
Rm7503	Rm8501 *mucE*::Tn3HoKm	This work
Rm7504	Rm8501 *mucS*::Tn5-233 *mucE*::Tn3HoKm	This work
Rm7505	Rm6086 *mucS*::Tn5	This work
Rm7506	Rm6086 *mucE*::Tn3HoKn	This work
Plasmids		
pRK2013	Nm^R, ColE1 replicon with RK2 *tra* genes	Figurski and Helinski 1979
pRK600	Cm^{R}Nm^S, pRK2013 Nm^s::Tn*9*	Finan et al. 1986b
pRK607	pRK2013::Tn5-233	De Vos et al. 1986
pPH1JI	IncP Gm^R Sp^R tra$^+$	Ruvkun et al. 1982
pLAFR1	Tc^R, *R. meliloti* clone bank vector	F. Ausubel
pMuc	*R. meliloti* cosmid clone of EPS II synthesis genes	Zhan et al. 1989
pSP329	Tc^R, broad host range vector	S. Porter
pTn3HoKn	pHoHo1 Km^R	Glazebrook and Walker 1989
p*mucS*EE	8.7-kb *Eco*RI-*Eco*RI fragment cloned into pSP329	This work
p*mucS*BB	7.3-kb *Bam*HI-*Bam*HI fragment cloned into pSP329	This work
p*mucS*BE	5.7-kb *Bam*HI-*Eco*RI fragment cloned into pSP329	This work
p*mucS*BgE	4.4-kb *Bg*II-*Eco*RI fragment cloned into pSP329	This work
p*mucS*KC	4.0-kb *Kpn*I-*Cla*I fragment cloned into pSP329	This work
p*mucS*BgC	3.3-kb *Bgl*II-*Cla*I fragment cloned into pSP329	This work
Phage		
φM12	Transducing phage	Finan et al. 1986a

stranded fragments cloned into M13mp18 and M13mp19 or on double-stranded pUC18 derived subclones. The *mucS*::Tn5 insertion junction was determined by sequencing from a primer complementary to a small region of the Tn5 inverted repeats.

The nucleotide and amino acid sequences were analyzed using the University of Wisconsin Genetics Computer Group (GCG) programs. The predicted open reading frame (ORF) was obtained with the CodonPreference program employing the codon usage table RHIZO.COD determined by J. M. Cherry with the CodonFrequency program. Homologous DNA searches from the EMBL and GenBank databanks were done using the FASTA software. Predicted hydrophobicity was analyzed by the Goldman-Kyte-Doolittle method by means of the PEPPLOT program.

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