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Rhizobium meliloti Chromosomal Loci Required for Suppression of Exopolysaccharide Mutations by Lipopolysaccharide

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Mutants of alfalfa symbiont *Rhizobium meliloti* SU47 that fail to make extracellular polysaccharide (*exo* mutants) induce the formation of nodules that are devoid of bacteria and consequently do not fix nitrogen. This Fix$^-$ phenotype can be suppressed by an *R. meliloti* Rm41 gene that affects lipopolysaccharide structure. Here we describe mutations preventing suppression that map at two new chromosomal loci, *lpsY* and *lpsX*, present in both strains. Two other *lps* mutations isolated previously from SU47 also prevented suppression.

Studies with mutants indicate that specific surface molecules are necessary for successful bacterial invasion of legume root nodules by *Rhizobium* spp. A few bacterial mutants isolated as defective in symbiosis have obvious cell surface defects (12, 14, 15), and a large number isolated for their surface defects have symbiotic phenotypes (2, 6, 9, 13, 16). In *R. meliloti* the symbiotic Fix$^-$ phenotype of *exo* mutants, which are deficient in succinoglycan exopolysaccharide, can be suppressed by *lpsZ*$^+$, which affects cell surface lipopolysaccharide (LPS) (18). Here we describe two new surface LPS loci, *lpsY* and *lpsX*, that are also involved in *lpsZ* suppression of *exo* mutants.

The suppressing phenotype, called Sxb$^+$, depends on allele *lpsZ*$^+$, originally from megaplasmid pEXO of *R. meliloti* Rm41 (pRmeRm41c); in contrast, pEXO of *R. meliloti* SU47 (pRmeSU47b) has the null allele *lpsZ*^{0} and SU47 is consequently Sxb$^-$ (18). Sxb suppression differs from, and is independent of, suppression by production of a second exopolysaccharide (7, 19). Sxb$^+$ and Sxb$^-$ are correlated with resistance (ϕ^r) and sensitivity (ϕ^s), respectively, to bacteriophages φM1, φM7, and φM12. To identify additional loci required for Sxb$^+$, therefore, we screened for a Tn*5* insert linked to ϕ^s.

A library of random Tn*5* inserts, made in SU47 *str-7* (Rm1021), was transduced with phage φM12h1 (18) into SU47(pEXORm41) *exoB*, which is ϕ^r Sxb$^+$. Six hundred Nm^r colonies were screened for sensitivity to phage φM1, and isolate Ω69::Tn*5* was found to be ϕ^s. The Tn*5* insert was transduced back into the ϕ^r Sxb$^+$ parent by selection for Nm^r and was found to be linked completely to ϕ^s (400 of 400 colonies tested), suggesting that the insert is responsible for the phenotype. Next, for mapping purposes, an insert of Tn*5-233* (Gm^r Sp^r; 4) linked to Ω69::Tn*5* was isolated, by transduction of a library of random Tn*5-233* inserts from SU47 with selection for Gm^r Sp^r and screening for loss of Tn*5* Nm^r. Isolate Ω69-23::Tn*5-233*, identified in this way, was then shown to be 76% linked to Ω69::Tn*5* by both phage and drug phenotypes. When Ω69-23::Tn*5-233* (ϕ^s) was transduced into SU47(pEXORm41) *exoB* Ω69::Tn*5* (ϕ^s), all transductants that lost Nm^r became concomitantly ϕ^r, reinforcing the conclusion that Ω69::Tn*5* was responsible for the original ϕ^s phenotype of the recipient.

When Ω69::Tn*5* was transduced into Rm41 or Rm41 *exoB*, the transductants became ϕ^s (Table 1; Rm5832 and Rm5825, respectively). This ϕ^s phenotype resembled that of *lpsZ* mutants (18) in including sensitivity to φM1, φM7, and φM12 but differed in causing no change in sensitivity to φ16-3. In addition, like *lpsZ* mutations, Ω69::Tn*5* gave a Fix$^-$ phenotype in Rm41 *exoB* and in SU47(pEXORm41) *exoB* but a Fix$^+$ phenotype in Rm41, which defines this as an Sxb locus. In transduction (in the SU47 background), however, Ω69-23::Tn*5-233*, which is 76% linked to Ω69::Tn*5*, was not linked to *lpsZ*::Tn*5*. Moreover, plasmids carrying *lpsZ*$^+$ and flanking DNA neither complemented Ω69::Tn*5* to ϕ^r nor suppressed the Fix$^-$ phenotype of *exo* strains carrying Ω69::Tn*5*. These observations indicate that Ω69::Tn*5* marks a second Sxb locus, which we designated *lpsY*.

Plasmid clones that complement *lpsY69*::Tn*5* for both ϕ^r and Fix, and thus appear to carry *lpsY*$^+$, were isolated from both an Rm41 genomic library (S. Klein, Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, 1987) and an SU47 genomic library (10) and had similar restriction patterns. Mutations in *lpsZ*, in contrast, are complemented only by clones from Rm41 (18). Unlike *lpsZ*$^+$ clones, the *lpsY*$^+$ clones did not by themselves suppress the Fix$^-$ phenotype of SU47 *exoB* mutants. Moreover, when transduced into either SU47 *str-7* or SU47 *exoB str-7*, in contrast to its effect in SU47(pEXORm41) *exoB* described above, *lpsY69*::Tn*5* had no effect on phage, Calcofluor, or Fix phenotypes. Allele *lpsY*$^+$ is therefore phenotypically silent in an *lpsZ*^{0} background.

On M9 mannitol–0.05% Congo red agar, Sxb$^+$ Rm41 derivatives formed salmon-colored colonies (Table 1). The Sxb$^-$ strains Rm41 *lpsZ* and Rm41 *lpsY*, however, both formed dark red colonies. We therefore isolated three dark red colonies after random mutagenesis of Rm41 *exoB* with Tn*5-132* (oxytetracycline resistance [Ot^r]; 1,5). One of these (Rm5841, Table 1) was found to have gained sensitivity to φM1, φM7, and φM12, as well as resistance to φ16-3. This phenotype was not corrected by plasmids carrying *lpsZ*$^+$ (18) or by some of the *lpsY*$^+$ plasmids described above, but it was corrected by the other *lpsY*$^+$ plasmids. Thus, this mutation, designated *lpsX3*::Tn*5-132*, appears to be in a gene linked to but separate from *lpsY*. That conclusion was confirmed by tight linkage between *lpsY69*::Tn*5* and *lpsX3*::Tn*5-132* (in two separate experiments in which

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TABLE 1. Phenotypes of strains derived from Rm41

Strain	Relevant genotype		Growth of phage		Appearance on Congo Red agar	Fix
	exo	*lps*	φM1, φM7, φM12	φ16.3		
Rm41	+	+	−	+	Salmon, mucoid	+
AK631	*B*	+	−	+	Salmon	+
Rm5831	+	*Z*	+	±	Red, mucoid	+
Rm5830	*B*	*Z*	+	±	Red	−
Rm5832	+	*Y*	+	+	Red, mucoid	+
Rm5825	*B*	*Y*	+	+	Red	−
Rm5843	+	*X*	+	−	Red, mucoid	+
Rm5841	*B*	*X*	+	−	Red	−

lpsY69::Tn*5* was transduced into *lpsX3*::Tn*5-132* with selection for Nm^r and scoring for Ot, a total of 6 Ot^r colonies were found among 1,312 Nm^r colonies, indicating 99.6% linkage). Like other Sxb mutations, *lpsX3*::Tn*5-132* gave a Fix^- phenotype in Rm41 *exoB* but a Fix^+ and ϕ^s phenotype in Rm41. Like $lpsY^+$, $lpsX^+$ was phenotypically silent in an $lpsZ^0$ background.

By transduction, Ω69-23::Tn*5-233* was ~20% linked to *lpsB*::Tn*5* (3), indicating that *lpsY* and *lpsX* map to the chromosome in the region of *cys-11*. To simplify mapping, Tn*5* of *lpsY69*::Tn*5* was replaced with Tn*5-132* (4). Linkage of *lpsY* to *lpsB* was obscured, however, by the finding that the Ot^r phenotype was not expressed in *exoB lpsY lpsB* triple mutants. Nevertheless, *lpsB* mutations were not complemented by any plasmid that complements *lpsY*, nor was *lpsY* complemented by the $lpsB^+$ plasmid p1A (3), consistent with the loose transductional linkage. Linkage to *exo* loci has not been determined.

Alteration in phage sensitivity suggests LPS alterations (11), and in fact purified LPS from *lpsZ* (18), *lpsY*, and *lpsX* mutants of Rm41 all differ in structure from Rm41 lps^+ LPS. In Fig. 1, the low-molecular-weight peak (fractions 40 to 50) corresponds to periplasmic β-2 glucan, while fractions 15 to 35 correspond to various species and aggregates of LPS (18). LPS from *lpsX* mutants appears different from *lpsY* LPS but similar to *lpsZ* LPS.

In light of these results, we have also checked SU47 mutations isolated originally as *lps* (3) for Sxb phenotype, by transduction into the suppressed ($lpsZ^+$) strain SU47 (pEXORm41) *exoB* and scoring for retention (Sxb^+) or loss (Sxb^-) of the Fix^+ phenotype. Most of the *lps* mutations are Sxb^+ (although for some of the strains fixation may be delayed nonreproducibly); these include *lpsB*::Tn*5*-12-1, *lpsB*::Tn*5*-18-1, *lpsC*::Tn*5*-27-1, Ω::Tn*5*-34-3 (class E), Ω::Tn*5*-1F (class F), Ω::Tn*5*-2H (class G), Ω::Tn*5*-4H (class H), Ω::Tn*5*-2G (class J), Ω::Tn*5*-3E (class J), Ω::Tn*5*-8-1 (class K), Ω::Tn*5*-8-2 (class L), and Ω::Tn*5*-15-2 (class M). Two of the *lps* mutations, however, Ω::Tn*5*-4P (the single class I insert) and Ω::Tn*5*-1H (class J), are Sxb^-. These two Sxb^- mutations strongly support the implication of LPS in Sxb suppression.

In transduction among the three class J mutations, with Tn*5* replaced by Tn*5-233* as appropriate, Ω::Tn*5*-1H (Sxb^-) was not linked to either Ω::Tn*5*-2G (Sxb^+) or Ω::Tn*5*-3E

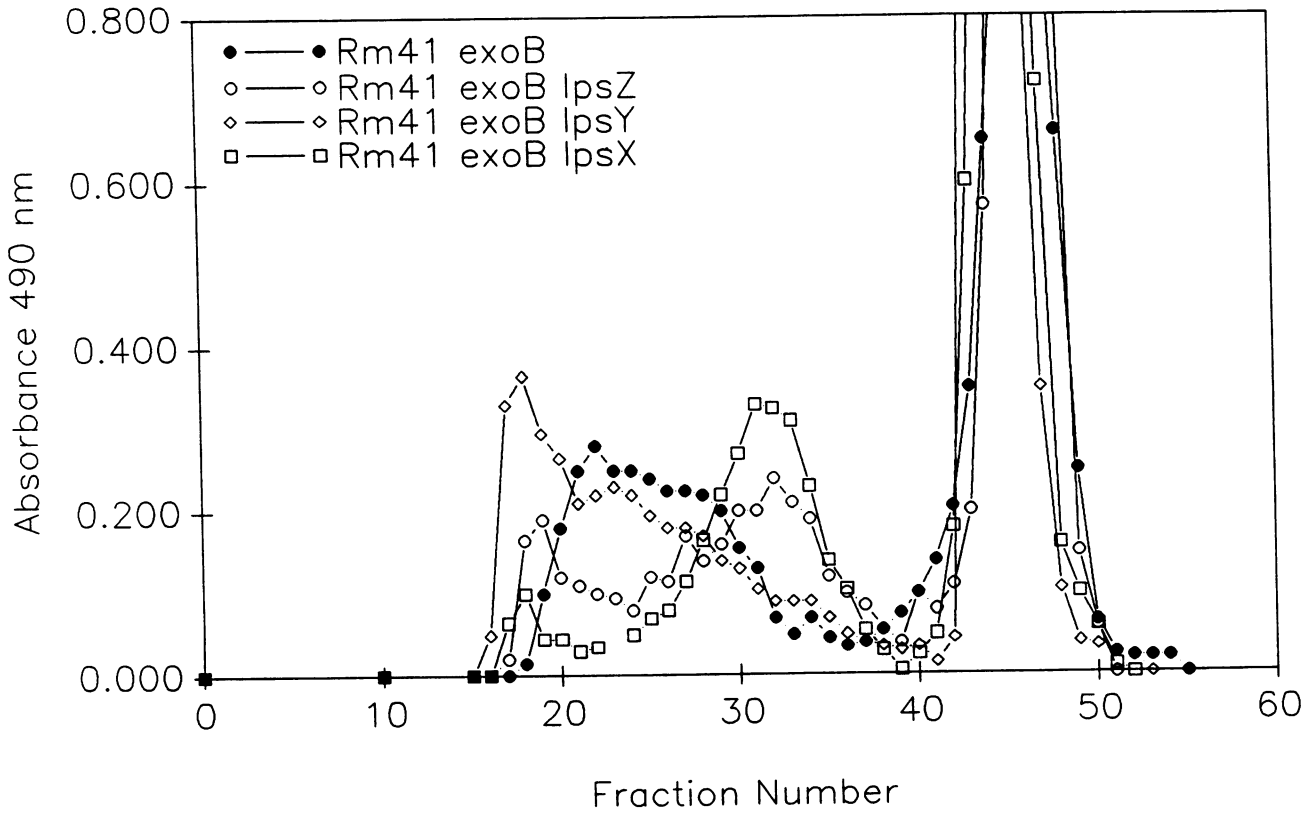

FIG. 1. Gel filtration of LPS. LPS extracted by hot phenol (17) was fractionated through Sepharose 4B in 0.1 M ammonium formate (pH 5.5) elution buffer.

(Sxb^+), which are 100% linked to each other. On this basis Ω::Tn*5*-1H is now reassigned to a new class, class N.

Putnoky et al. (14) have described chromosomal mutations called *fix-23* that are ϕ16-3^r, noninvasive, and Fix^- in Rm41 *exoB* but Fix^+ in Rm41. These mutations are by definition Sxb^- and thus might be related to the *lpsX* mutant described here. Other mutations with similar phenotypes isolated from Rm41 derivatives are not yet reported to have been tested in SU47 (8).

In summary, whereas natural isolate Rm41 is *lpsZ*$^+$ and natural isolate SU47 is *lpsZ*^{0}, both Rm41 and SU47 are *lpsY*$^+$ *lpsX*$^+$. The simplest interpretation is that all three loci, as well as the two *lps* loci defined by the Sxb^- class I and class N mutations, are involved in production of the LPS structure that can substitute in nodule invasion for succinoglycan exopolysaccharide (18).

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