The Acetyl Substituent of Succinoglycan Is Not Necessary for Alfalfa Nodule Invasion by *Rhizobium meliloti* Rm1021

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***Rhizobium meliloti* Rm1021 requires a Calcofluor-binding exopolysaccharide, termed succinoglycan or EPS I, to invade alfalfa nodules. We have determined that a strain carrying a mutation in the *exoZ* locus produces succinoglycan that lacks the acetyl substituent. The *exoZ* mutant nodulates alfalfa normally.**

The soil bacterium *Rhizobium meliloti* fixes nitrogen in a symbiotic relationship with alfalfa. The bacteria induce nodules on the plant root through the action of a novel signal molecule, the Nod factor, and invade these nodules through special structures called infection threads (reviewed in references 4 and 7). Mutants of *R. meliloti* Rm1021 that do not produce a Calcofluor-binding exopolysaccharide, termed succinoglycan or EPS I, fail to invade alfalfa nodules. This exopolysaccharide is a polymer of octasaccharide repeat units, with acetyl, succinyl, and pyruvyl substituents (1, 15), as shown in Fig. 1. Recent work suggests that a low-molecular-weight form of this polysaccharide may have a function in nodulation (3, 19).

R. meliloti colonies producing succinoglycan fluoresce under UV light on medium containing the dye Calcofluor, and this phenotype has been used to screen for mutants altered in succinoglycan production. By these means, a cluster of *exo* genes necessary for succinoglycan synthesis, which is located on the second symbiotic megaplasmid present in *R. meliloti* (6, 8), has been defined (9, 13, 14). At least 16 loci in this cluster that affect succinoglycan production have been defined to date. Mutations in the *exoA*, *exoB*, *exoF*, *exoL*, *exoM*, *exoP*, *exoQ*, *exoT*, and *exoY* genes completely abolish production of succinoglycan and nodule invasion (9, 13, 14, 16, 20). Strains carrying mutations in the *exoH* gene produce a derivative of succinoglycan that lacks the succinyl modification (11), and these strains are also symbiotically ineffective (Fix[−]).

The strain Rm8341 was isolated in a Tn5 mutagenesis of the *exo* region of strain Rm1021 (13). This strain was originally characterized as fluorescent on Calcofluor medium and symbiotically effective (Fix⁺) on alfalfa. It carries a Tn5 insertion in a 1.6-kb *EcoRI* fragment near the *exoB* gene. Recently, an open reading frame, termed *exoZ*, was described in this region (5). A strain carrying a deletion mutation in this open reading frame shows a delay in the appearance of UV fluorescence on Calcofluor plates but is Fix⁺ on alfalfa (5). By restriction mapping, we found that the Tn5 insertion in Rm8341 is located within this open reading frame and truncates approximately the last 10 to 15% of the coding region (data not shown). Upon reexamination, the strain Rm8341 was found to exhibit a delay in the appearance of fluorescence on Calcofluor medium, as was described for

the earlier *exoZ* deletion mutant. Therefore, we have renamed this Tn5 insertion *exoZ341*.

Proton nuclear magnetic resonance spectroscopy of exopolysaccharide from Rm8341. Exopolysaccharide was prepared from Rm1021 and Rm8341 culture supernatants by precipitation with 3 volumes of ethanol as described previously (12). Exopolysaccharide samples (1 to 5 mg) were exchanged several times with D₂O and resuspended in D₂O, and one-dimensional proton spectra were obtained at 60°C with the 500-MHz instrument operated by the Francis Bitter National Magnet Laboratory at Massachusetts Institute of Technology. The spectra, shown in Fig. 2, are extremely similar except that the spectrum of Rm1021 succinoglycan shows a peak at 2.15 ppm representing the methyl protons of the acetyl group and the spectrum of succinoglycan from Rm8341 does not. Therefore, the *exoZ341* mutation blocks the acetylation of succinoglycan.

Deesterification of succinoglycan subunits from wild-type, *exoZ341*, and *exoH154* strains. To obtain further evidence that the sole alteration in the polysaccharide from the *exoZ341* strain was the lack of the acetyl substituent, octasaccharide subunits derived from the polysaccharide were deesterified and examined by thin-layer chromatography. Octasaccharide subunits were generated from polysaccharides from Rm1021, *exoZ341*, and *exoH154* strains by cleav-

FIG. 1. Structure of the octasaccharide repeat unit of *R. meliloti* succinoglycan (EPS I) (1, 15). The positions of the acetyl and succinyl substituents have been determined recently by neutral permethylation and collision mass spectroscopy (15).

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FIG. 2. Proton nuclear magnetic resonance spectra of exopolysaccharide from Rm1021 (a) and Rm8341 (*exoZ341::Tn5*) (b). Chemical shift was standardized to the DHO peak (4.372 ppm at 60°C). The singlets at 1.46 and 2.15 ppm represent the methyl protons of the 1-carboxyethylidene (pyruvate) and acetyl groups, respectively. The triplets at 2.47 and 2.63 ppm represent the methylene protons of the succinyl group. The peak at 2.15 ppm is absent in the *exoZ341* mutant.

age of the purified polysaccharides with the succinoglycan depolymerase from the soil bacterium *Cytophaga arvensicola*, which cleaves succinoglycan into the subunits shown in Fig. 1 (2). The octasaccharide subunits were purified by gel filtration chromatography and treated with 0.1 M KOH overnight at room temperature. This treatment removes the acetyl and succinyl esters but not the pyruvate ketal. Untreated and treated octasaccharide subunits were chromatographed on Silica Gel 60 (E. Merck) for three ascents in propanol-nitromethane-water (50:20:20) and visualized with 5% H_2SO_4 in ethanol as described previously (18). The octasaccharide from Rm1021 exopolysaccharide had a mobility between that of maltotetraose and maltopentaose (Fig. 3). The octasaccharide from Rm8341 exopolysaccharide had a lower mobility, consistent with loss of the acetyl substituent, which increases the mobility of oligosaccharides in this thin-layer chromatography system (18). The octasaccharide from the *exoH154* strain had a slightly higher mobility than that from the wild-type strain; therefore, the succinyl substituent appears to decrease the mobility of the octasaccha-

FIG. 3. Thin-layer chromatogram of octasaccharide succinoglycan subunits produced with the succinoglycan depolymerase from *C. arvensicola*. Lanes: 1, maltooligosaccharides; 2, Rm1021 octasaccharide; 3, Rm8341 (*exoZ341*) octasaccharide; 4, Rm7154 (*exoH154*) octasaccharide; 5 to 7, KOH-treated octasaccharide from Rm1021, Rm8341, and Rm7154, respectively. Abbreviations: Glc, glucose; M2 to M9, maltooligosaccharides of two to nine glucose residues.

ride. All of the KOH-treated octasaccharides had the same mobility, suggesting that the only difference among the three octasaccharides is the presence or absence of the ester-linked substituents.

The finding that the *exoZ341* mutant, which fails to acetylate succinoglycan, is Fix^+ on alfalfa demonstrates that the acetyl substituent of succinoglycan is not necessary for its function in nodulation. In contrast, strains carrying mutations in *exoH*, which fail to succinylate succinoglycan, are Fix^- on alfalfa (11). However, the *exoH* mutants also fail to produce a low-molecular-weight form of succinoglycan found in culture supernatants of Rm1021 (10), and it is unclear whether their deficiency in nodulation is due to lack of the succinate modification or lack of low-molecular-weight succinoglycan. It is possible that the succinyl substituent is necessary for generation of the low-molecular-weight material. It should also be noted that removal of the acetyl substituent does not change the net charge of succinoglycan, while removal of the succinyl substituent reduces the negative charge of the polysaccharide. It is possible that a relatively high negative charge is important for the function of EPS I in nodulation.

The sequence of the ExoZ protein has been shown to be homologous to that of NodX of *Rhizobium leguminosarum* bv. *viciae* (5), a host-range nodulation gene that allows this strain to nodulate the legume Afghanistan pea. This homology may be relevant to the biochemical function of NodX. The *nod* gene products synthesize a novel signal molecule termed the Nod factor, which in *R. leguminosarum* is a tetramer or pentamer of *N*-acetyl glucosamine with an unsaturated fatty acid modification and an acetyl substituent on C-6 of the sugar at the nonreducing end (17). This acetyl substituent is necessary for nodulation of the normal hosts of *R. leguminosarum* and is thought to be added by the product of the *nodL* gene (17). Various other chemical substitutions on other Nod factors have been shown to be important determinants of host range (7). NodX may be involved in adding a second acetyl substituent to the *R. leguminosarum*

Nod factor that allows it to be recognized by Afghanistan pea.

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