Exogenous Suppression of the Symbiotic Deficiencies of *Rhizobium meliloti* *exo* Mutants

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The acidic exopolysaccharide (EPS I) produced by *Rhizobium meliloti* during symbiosis with *Medicago sativa* has been shown to be required for the proper development of nitrogen-fixing nodules. Cloned DNA from the *exo* region of *R. meliloti* is shown to stimulate production of the low-molecular-weight form of this exopolysaccharide, and in this report we show that the symbiotic deficiencies of two *exo* mutants of *R. meliloti*, the *exoA* and *exoH* mutants, can be rescued by the addition of this low-molecular-weight material at the time of inoculation. For *exoA* and *exoH* mutants, rescue with a preparation containing low-molecular-weight exopolysaccharide induces the formation of nitrogen-fixing nodules which appear somewhat later and at a reduced efficiency compared with wild-type-induced nodules; however, microscopic analysis of these nodules reveals similar nodule morphology and the presence of large numbers of bacteroids in each.

The symbiotic relationship between *Rhizobium* spp. and leguminous plants is a complex one involving multiple signals from both partners. For *Rhizobium meliloti* and its host, *Medicago sativa* (alfalfa), this interaction results in the formation of a specialized root nodule, in which the bacteria differentiate into nitrogen-fixing bacteroids. A distinct bacterially derived signal that has been identified is the NodRm-1 factor, a sulfated and acylated glucosamine oligosaccharide (20). This molecule has been shown to induce cortical cell proliferation and differentiation into nodule structures in a dose- and structure-dependent fashion when applied to the roots of axenically grown alfalfa seedlings (20).

Another molecule that might serve as a signal in *R. meliloti*-alfalfa symbiosis is the acidic exopolysaccharide succinoglycan (EPS I). The oligosaccharide subunit of EPS I consists of one galactose and seven glucose residues joined in specific linkages and modified with pyruvate, acetate, and succinate substituents in a 1:1:1 ratio (1, 9). Two size classes of EPS I, high and low molecular weight, are distinguishable on the basis of their abilities to be precipitated out of culture supernatants with 3 and 10 volumes of ethanol, respectively (9, 21).

exo mutants can be identified easily because of the fact that Calcofluor, a laundry whitener, fluoresces brightly under long-wave UV light when bound to EPS I (6, 13). In the presence of Calcofluor, wild-type strains fluoresce with a characteristic halo around the colonies. Using this as a screen, we have isolated a considerable number of mutants that affect EPS I production and have classified them into at least 16 complementation groups so far (3, 5, 6, 10, 12, 15, 16, 19). It has been shown previously that mutants which do not produce EPS I are unable to form nitrogen-fixing nodules (6, 13, 15). These mutants can, however, elicit the formation of small, round, white nodules which do not contain bacteria.

In order to explore the role of EPS I in nodule invasion

and development and to characterize the important features of this molecule, we examined whether *R. meliloti* *exo* mutants could be rescued in *trans* by the addition of EPS I. Djordjevic et al. (4) have previously reported that mutants of *Rhizobium* sp. strain NGR234 and *Rhizobium trifolii* which are defective in exopolysaccharide production could be rescued to form nitrogen-fixing nodules on their respective hosts if homologous exopolysaccharide or oligosaccharide was added (4). In contrast, our attempts to rescue *R. meliloti* *exo* mutants' abilities to induce Fix⁺ nodules on alfalfa by the addition of the high-molecular-weight fraction of EPS I were unsuccessful. We found instead that the symbiotic deficiencies of *R. meliloti* *exo* mutants could be partially suppressed if a preparation containing low-molecular-weight EPS I was added to the bacterial suspension prior to inoculating the plant. Since we had observed that parental strain Rm1021 grown in M9-glutamate-mannitol medium (11) produces only a small amount of low-molecular-weight EPS I, we decided to introduce plasmid pEX115 (see Fig. 2) into Rm1021 in the hopes of increasing the amount of low-molecular-weight EPS I produced by this strain. This plasmid is a derivative of pLAFR1 (7) that carries a fragment of Rm1021 DNA and was originally identified because its presence in wild-type strain Rm1021 results in a large fluorescent halo when the strain is plated on Calcofluor-containing medium. It seemed possible that this large halo was due to diffusible low-molecular-weight material, and in fact, we found that strain Rm1021(pEX115) produces 54% of its total exopolysaccharide as the low-molecular-weight fraction (i.e., precipitated by 10 volumes of ethanol from the culture supernatant) whereas Rm1021 produces only 20%. This suggests that the plasmid causes the larger halo by increasing the amount of EPS I that is low molecular weight and presumably diffusible. Using low-molecular-weight material derived from the supernatant of an Rm1021(pEX115) culture by precipitation with 10 volumes of ethanol, we attempted to rescue two *exo* mutants of *R. meliloti*: the *exoA* mutant (15), which produces no detectable EPS I, and the *exoH* mutant (12, 15), which produces EPS I missing its succinate modification. Neither mutant is capable of inducing Fix⁺ nodules on alfalfa plants.

Figure 1 shows the time course of the appearance of pink

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FIG. 1. Rescue of *exo* mutants of *R. meliloti* by the addition of a preparation containing low-molecular-weight EPS I. *R. meliloti* strains were grown in M9-glutamate-mannitol medium (11) for 7 days at 30°C and prepared for inoculation experiments as described previously (13). Low-molecular-weight material from strain Rm1021 was precipitated from culture supernatants with 10 volumes of ethanol as described elsewhere (11, 21), and the amount of hexose present was quantitated by the anthrone method (14). Plants were inoculated with bacteria concomitantly with this low-molecular-weight preparation in petri dishes containing Jensen's medium as described previously (13) and were examined weekly for nodule formation. Each inoculum was tested on five plants. The points represent averages from several experiments using the same preparation of low-molecular-weight material. Closed circles, Rm1021, no added polysaccharide; open circles, the *exoH* mutant plus low-molecular-weight material at a concentration of 5 μ g/ml of hexose; squares, the *exoA* mutant plus low-molecular-weight material at a concentration of 5 μ g/ml of hexose; triangles, either the *exoA* or *exoH* mutant with no added polysaccharide.

Fix⁺ nodules on alfalfa plants inoculated with Rm1021, the *exoA* mutant, or the *exoH* mutant plus or minus this low-molecular-weight preparation at a concentration of 5 μ g/ml of hexose, as quantitated by the anthrone method (14). One week after inoculation with Rm1021, pink nodules began to appear whether or not low-molecular-weight material was added, and by 2 weeks every plant had pink nodules (Fig. 1). When plants were inoculated with the *exoH* mutant plus low-molecular-weight material at a concentration of 5 μ g/ml of hexose, pink nodules were observed after 3 weeks; after 6 weeks, about 80% of plants had one or more pink nodules. When the *exoA* mutant plus low-molecular-weight material at a concentration of 5 μ g/ml of hexose was used as the inoculum, the appearance of pink nodules was somewhat delayed, but after 6 weeks, about 60% of the plants had one or more pink nodules. Different concentrations (5 to 160 μ g/ml of hexose) of the high-molecular-weight exopolysaccharide preparation were also used in rescue experiments, but no rescue of either the *exoA* mutant or the *exoH* mutant was observed; nor did the addition of high-molecular-weight EPS I appear to increase the efficiency of the rescue observed with the low-molecular-weight material (data not shown).

To verify that the observed rescue was not due to mutation or reversion, bacteria from several representative nodules were examined for their antibiotic resistance markers and also their Calcofluor and Fix phenotypes. It was found that in each case, the bacteria recovered from nodules had

FIG. 2. Genetic maps of *exo* region on pRmSU47b (top line) and *exo* region-containing plasmids (15). *exo* genes are indicated by heavy lines. Sites for restriction endonuclease *Eco*RI are shown (R). Shown below are plasmids pEX115 (this work) and pD34, pEX154, and pEX41 (described in reference 14).

the same markers, phenotypes on Calcofluor plates, and phenotypes on plants as did the original *exoA* and *exoH* mutants, indicating that the added low-molecular-weight preparation alone was responsible for their abilities to form Fix⁺ nodules.

Our analyses of the *R. meliloti* DNA present in plasmid pEX115 showed that it encompasses the *exoP* to *exoH* loci (Fig. 2). To localize the region responsible for the increased production of low-molecular-weight exopolysaccharide material, several plasmids were introduced into Rm1021 and the fraction of low-molecular-weight EPS I produced was quantitated. It was found that Rm1021(pD34) produces the same percentage of low-molecular-weight EPS I as Rm1021, whereas Rm1021(pEX154) produces an amount of low-molecular-weight material comparable to that produced by Rm1021(pEX115). Introduction of plasmid pEX41, which carries only the *exoL*, *exoK*, and *exoH* loci, also increased the fraction of low-molecular-weight polysaccharide produced to approximately 60%. When derivatives of pEX154 containing Tn5 insertions in these loci were introduced, it was found that disruptions in either *exoH* or *exoK* resulted in no increase in the amount of low-molecular-weight material produced compared with that from the wild type, but an insertion in *exoL* allowed increased low-molecular-weight EPS I production. The results indicate that overexpression of *exoK*⁺ can affect the ratio of high- to low-molecular-weight EPS I. It is less clear whether the overexpression of *exoH*⁺ is also required or whether Tn5 insertions in *exoH* have a polar effect on the expression of *exoK*. Our observations are consistent with those of Leigh and Lee (11), who showed that *exoH* mutants do not produce a detectable amount of low-molecular-weight EPS I. Examination of the low-molecular-weight material obtained by precipitation with 10 volumes of ethanol by proton magnetic resonance indicated that it contained material carrying the acetyl, pyruvyl, and succinyl substituents characteristic of EPS I (data not shown).

Some of the Fix⁺ nodules formed after inoculation with the *exoH* mutant plus low-molecular-weight material as well as some wild-type nodules were fixed, embedded, and sectioned for light microscopy (8). Figure 3 shows a longitudinal section of a pink, nitrogen-fixing nodule induced by *exoH*

FIG. 3. Light microscopy of alfalfa root nodules induced by *R. meliloti* Rm1021 (B and D) or by the *exoH* mutant rescued by the addition of a preparation containing low-molecular-weight EPS I (A, C, and E). Nodules harvested from alfalfa roots 3 to 4 weeks after inoculation were fixed and prepared for microscopy as described elsewhere (8). Samples were processed on a Polaron H1200 vibrating microtome (Bio-Rad Microscience Div., Cambridge, Mass.) into sections 0.5 μ m thick, which were then stained with multiple staining solution (Polysciences, Warrington, Pa.) and examined under a Zeiss light microscope. (A) Longitudinal section of a pink, nitrogen-fixing rescued *exoH* mutant nodule with the meristematic (I), infection (II), nitrogen-fixing (III), and senescent (IV) zones indicated; (B and C) higher magnifications of nitrogen-fixing zones in Rm1021 (B) and rescued *exoH* mutant (C) nodules; (D and E) higher magnifications of infection zones in Rm1021 (D) and rescued *exoH* mutant (E) nodules. Infection threads are marked (I in panels D and E).

rescued by the addition of a preparation containing low-molecular-weight EPS I (panel A). The meristematic (I) and infection (II) zones have the same structures as in nodules induced by the parental RM1021 strain, although infection threads (indicated by "I" and an arrowhead in panels D and E) and infected cells are somewhat larger in the rescued nodule (shown under higher magnification in panels D and E). The nitrogen-fixing zone (III) contains cells filled with bacteroids and amyloplasts and extends along the nodule to the base. Throughout the rescued nodule, as in the wild type, there are many cells containing a large central vacuole. Higher magnification shows that in the nitrogen-fixing zone of the rescued nodule (panel C), the infected cells contain fewer bacteroids than in the wild-type nodule (panel B).

Battisti et al. (2) have independently observed suppression of the symbiotic deficiencies of *R. meliloti* *exo* mutants with low-molecular-weight polysaccharide isolated from strain Rm1021 but not with high-molecular-weight exopolysaccharide. They present evidence that the active species is a highly charged oligomer of succinoglycan that contains four

repeat units of the octasaccharide (2). The results obtained by ourselves and by Battisti et al. (2) for the *R. meliloti*-alfalfa symbiosis are similar to the observations of Djordjevic et al. (4), who showed that low-molecular-weight forms of the homologous exopolysaccharides can rescue the symbiotic deficiencies of certain mutants of NGR234 and *R. trifolii*. However, in contrast to the results obtained with NGR234 and *R. trifolii*, we failed to see rescue in the *R. meliloti* symbiosis when the high-molecular-weight form of the exopolysaccharide was added. Other activities of *R. meliloti* polysaccharides and oligosaccharides have also been reported, including the restoration of symbiosis to poorly infective strains by the addition of exopolysaccharide preparations isolated from highly infective strains (17) and the formation of short infection threads that aborted in the root hair when exopolysaccharide purified by ultrafiltration methods was added to *exo* mutants (18). Further chemical characterization of the active species within the preparation of low-molecular-weight material and investigation into the nature of its interactions with the plant should offer further

insights into the molecular mechanisms involved in the development of nitrogen-fixing nodules.

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