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Biochemistry

Specific oligosaccharide form of the *Rhizobium meliloti* exopolysaccharide promotes nodule invasion in alfalfa

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ABSTRACT **_Rhizobium meliloti_ strain SU47 produces both high molecular weight (HMW) and low molecular weight (LMW) forms of an acidic exopolysaccharide, succinoglycan. Genetic studies have shown that succinoglycan is required for alfalfa root nodule invasion. We found that LMW succinoglycan, when applied exogenously to alfalfa roots, restored nodule invasion to _exoA_, _exoB_, _exoF_, and _exoH_ mutants. Nodule initiation signals were not involved, since LMW succinoglycan from _R. meliloti nodD1D2D3_ and _nodA_ mutants and from luteolin-induced wild-type cultures elicited effects similar to LMW succinoglycan from the uninduced wild-type strain. In contrast, LMW fractions from an _exoA_ mutant, nonsuccinylated LMW succinoglycan, and HMW succinoglycan did not promote invasion, nor did LMW exopolysaccharides from _R. leguminosarum_ bv. _trifolii_ and _Rhizobium_ sp. strain NGR234. LMW succinoglycan could be separated by anion-exchange chromatography into several distinct subfractions differing in repeating subunit multiplicities (monomer, trimer, and tetramer) and charge. When tested singly, only the most charged, tetrameric form was active. These results show that a specific oligosaccharide form of succinoglycan promotes nodule invasion in alfalfa. The implications for the mode of action of succinoglycan are discussed.**

The infection of leguminous plants by bacteria of the genus *Rhizobium* leads to the formation of highly differentiated nitrogen-fixing root nodules (1). In response to bacterial signals, a nodule arises from the root cortex, and a tubular infection thread forms in the epidermis that penetrates into the root and carries the bacteria into the nodule. Bacteria are released from the infection thread into the newly formed nodule cells, where they differentiate into nitrogen-fixing bacteroids. To date, two essential signals have been identified in the *Rhizobium meliloti*–alfalfa symbiosis. A plant flavone, luteolin, induces the transcription of the rhizobial nodulation or *nod* genes (2), and an acylated and sulfated *N*-acetylglucosamine oligosaccharide secreted by the bacteria elicits nodule formation (3, 4).

The possibility that the rhizobial exopolysaccharide (EPS) might also be a signal arose when it was shown that transposon Tn*5* mutants of *R. meliloti* that fail to produce any EPS (Exo^-) induce empty non-nitrogen-fixing (Fix^-) nodules on alfalfa roots (5). The normal EPS of *R. meliloti* is succinoglycan, composed of repeating subunits containing seven glucose residues and one galactose residue in particular β-linkages and modified with one acetyl, one succinyl, and one 1-carboxyethylidene (pyruvate) substituent per subunit (6). A high molecular weight (HMW) and a low molecular weight (LMW) form of succinoglycan are produced (7, 8). The strict correlation between the Exo^- and Fix^- phenotypes, plus the fact that most Exo^- mutants are normal with regard to other surface factors (8), provided strong evidence that succinoglycan is required for nodule invasion. Similarly, Exo^- mutants of *Rhizobium* sp. strain NGR234 (9), *Rhizobium leguminosarum* bv. *trifolii* (10), and *R. leguminosarum* bv. *viciae* (11) were defective on their respective hosts. Evidence that nodule invasion relies on structurally specific forms of succinoglycan came from the observation that *R. meliloti exoH* mutants, which produce succinoglycan lacking the normal succinyl substituent (12) and also lacking the LMW fraction (8), also induce empty nodules (12). Furthermore, nodule invasion by Exo^- mutants of *Rhizobium* sp. strain NGR234 or of *R. leguminosarum* bv. *trifolii* could be restored by the exogenous addition of EPS from the parental strain of the same species but not by EPS of different structure from the other species (13).

We report here that invasion of alfalfa by *R. meliloti* Exo^- mutants was restored by the exogenous addition of succinoglycan from *R. meliloti*. A variety of EPSs of different structure all failed to substitute for normal succinoglycan. Furthermore, only a specific subfraction of LMW succinoglycan was active. Preliminary results were presented at the Eighth International Congress on Nitrogen Fixation, Knoxville, TN, May 20–26, 1990, and at the Fifth International Symposium on the Molecular Genetics of Plant–Microbe Interactions, Interlaken, Switzerland, September 9–14, 1990.

MATERIALS AND METHODS

Strains and Media. The wild-type parental strain of *R. meliloti* was Rm1021, a streptomycin-resistant derivative of SU47 (14). *R. meliloti* Exo^- mutants were Rm6086 (*exoA32*::Tn*5-233*; ref. 15), Rm6085 (*exoB13*::Tn*5-233*), Rm6087 (*exoF55*::Tn*5-233*), and Rm7164 (*exoH154*::Tn*5-233*). *R. meliloti* strain Rm2012 (*nodD1*::Tn*5 nodD2*::*tm nodD3*::Tn*5-233*; ref. 16), *R. meliloti* strain Rm5610 (*nodA*::Tn*5*), and *R. leguminosarum* bv. *viciae* R1PRE (wild type) were obtained from F. Ausubel. *Agrobacterium tumefaciens* strain A348 (wild type) was obtained from E. Nester; *R. leguminosarum* bv. *trifolii* ANU794 (wild type), from A. Hirsch; and *Rhizobium* sp. strain NGR234 (wild type), from B. Rolfe. Liquid cultures of *R. meliloti* for plant nodulation tests were routinely grown in Luria–Bertani (LB) medium (17) supplemented with 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$. L agar containing Calcofluor (a fluorescent stain for succinoglycan) was made as described (5).

Isolation and Analysis of EPS. Salts/glutamate/mannitol (SGM) medium (7) was used for *R. meliloti* succinoglycan production as described (8). *R. leguminosarum* bv. *trifolii* and *R. leguminosarum* bv. *viciae* were grown in SGM medium supplemented with 0.1 mg of thiamine, 0.1 mg of calcium pantothenate, and 50 mg of NaCl per liter. *A. tumefaciens* was grown in SGM medium containing 0.1 mg of thiamine per liter, and *Rhizobium* sp. strain NGR234 was grown in yeast/mannitol (YM) medium (18). All strains were grown (50 ml in 250-ml Erlenmeyer flasks) at 30°C on a rotary shaker (200 rpm) for 5 days. Cultures were centrifuged, and

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Abbreviations: EPS, exopolysaccharide; HMW, high molecular weight; LMW, low molecular weight; cfu, colony forming units.

then the EPS in the supernatants was fractionated into HMW or LMW forms by Bio-Gel A-5m chromatography followed by desalting by Bio-Gel P-2 chromatography as described (8). In cases where 20 mg or more of LMW succinoglycan was needed, pooled *R. meliloti* culture supernatants (200–500 ml) were fractionated into LMW and HMW forms by ultrafiltration (Amicon PM30 membranes). Occasionally a crude EPS preparation was made by dialyzing culture supernatant. Nonsuccinylated HMW and LMW succinoglycans were obtained by ultrafiltration of *R. meliloti* strain Rm7164 culture supernatants. Quantitation of EPS by the anthrone–sulfuric acid method and proton NMR spectroscopy were performed as described (8). The neocuproine assay (19) was used to quantitate reducing sugar content of EPS samples.

Fractionation of LMW Succinoglycan. LMW succinoglycan (5–20 mg) was loaded onto a column (1.6 × 30 cm) of QAE Sephadex (Sigma Q-50-120) that was equilibrated with 5 mM Mops [3-(*N*-morpholino)propanesulfonic acid; Calbiochem]/5 mM KCl, pH 7.0. The column was then rinsed with 150 ml of the equilibration buffer, and material was eluted with 400 ml of a linear gradient (5–500 mM KCl) in 5 mM Mops (pH 7.0), followed by 150 ml of 5 mM Mops/500 mM KCl, pH 7.0. Fractions (5 ml) were collected throughout the entire run, and then aliquots (0.3 ml) were assayed for carbohydrate. Fractions were pooled, lyophilized, and desalted (8). An aliquot from each peak fraction was then subjected to Bio-Gel P-4 chromatography and proton NMR spectroscopy to confirm its size and chemical identity. For P-4 chromatography, samples (1 mg unless otherwise noted) were run on a column (1.6 × 100 cm) of Bio-Gel P-4 (200–400 mesh) with a pyridine acetate buffer (pH 5) at 55°C as described (20). Fractions (1 ml) were collected and assayed for carbohydrate. The void volume was determined with blue dextran or HMW succinoglycan, and the salt volume was determined with $CoCl_2$.

Protease-Treated LMW Succinoglycan. A 6-mg sample of LMW succinoglycan (prepared by Bio-Gel A-5m chromatography) in 0.01 M Tris·HCl, pH 7.8/0.005 M EDTA was treated with proteinase K (50 mg per ml) for 3 hr at 37°C. A control sample (no proteinase K present) was also run in parallel. The samples were then refractionated by Bio-Gel A-5m chromatography and desalted (to remove enzyme and EDTA), and aliquots (0.5 mg) from each treatment were analyzed by protein NMR spectroscopy.

Nodulation Response of Exo⁻ Mutants to EPS. All EPS samples used in nodulation experiments were desalted (8), filter-sterilized (Millipore HA 0.45-μm filters), and streaked (0.1 ml) on L agar plates containing Calcofluor to ensure sterility before use. The chemical identity of all succinoglycan samples was routinely confirmed by proton NMR spectroscopy. Alfalfa (*Medicago sativa* cv. Iroquois) seedlings were grown on Jensen's agar (40 ml per plate) as described (5).

EPS (0.1 mg, also more if noted) was applied along the root 20–24 hr prior to inoculation. Three or four plants were used for each treatment. Four to six weeks after inoculation, plant growth, size, coloration, and nodule appearance were determined, and nodule occupancy [colony-forming units (cfu)] was measured for 6–12 nodules from at least two plants. To determine nodule occupancy, nodules were surface-sterilized as described (5) in 50% (vol/vol) household bleach for 5 min and crushed. The contents were plated on L agar containing Calcofluor and scored for cfu and Calcofluor staining after 3–4 days at 30°C. In some experiments, nodules or whole plants were tested for nitrogen fixation by the acetylene reduction assay before crushing.

Results were scored positive if, for three out of four or all plants, shoots were 4–6 cm high, leaves were light green, at least three nodules per plant were elongate and slightly pink, and more than half of the nodules crushed contained 10^2–10^3 cfu. Results were negative if for all plants shoots were 1–2 cm high, leaves were yellow or brown, all nodules were round and white, and all crushed nodules contained <10 cfu.

Microscopy of Nodules. Six-week-old nodules were fixed in 3.5% gluteraldehyde/1% paraformaldehyde in 0.05 M cacodylate buffer (pH 7.4) for ≈17 hr at room temperature. Nodules were rinsed in cacodylate buffer, fixed in 1% aqueous OsO_4 at 4°C for 3 hr, and dehydrated through a graded series of ethanol. Nodules were embedded in Spurr low-viscosity embedding medium (Polysciences) by using acetone as a transition solvent. Sections (1–2 μm) were cut and stained with 1% toluidine.

RESULTS

Nodule Invasion by Exo⁻ Mutants in the Presence of LMW Succinoglycan. Wild-type *R. meliloti* forms nodules on alfalfa that are elongate, pink because of leghemoglobin, and filled with bacteroids. Nitrogen fixation results in vigorous plant growth on nitrogen-free medium. In contrast, Exo⁻ mutants fail to invade, forming round white nodules on small plants with leaves that are yellow or brown. We found that LMW succinoglycan from Rm1021 applied exogenously to alfalfa roots corrected the nodule invasion defect of Exo⁻ mutants. Table 1 and Fig. 1 show the results of a typical experiment. All plants that were inoculated with the mutant and treated with LMW succinoglycan grew markedly better than untreated plants. Roughly half of the nodules tested on three different plants responded to LMW succinoglycan, showing some elongation or pink color, reducing acetylene, and releasing anywhere from 19 to 14,000 cfu upon crushing. In contrast, on untreated plants, only one nodule had these characteristics but was not sufficient to promote plant growth. Colonies that arose from nodule crushes had the same antibiotic resistances (indicating the presence of the transposon that caused the Exo⁻ mutation) and Calcofluor

Table 1. Nodule invasion response to exogenous LMW succinoglycan

	Plant observations			Nodule observations				
Rhizobium root treatment	No. Obs.	Shoot height, cm	Leaf color	No. Obs.	No. e or p*	No. Fix+†	No. with bact.‡	Avg. cfu × 10^{-3} of nodules with bact.§
Rm1021 (wild type)	2	18, 12	Green	12	12	12	12	62
exoA mutant (no EPS)	2	1, 1	Brown	12	1	1	1	0.34
exoA mutant + LMW succinoglycan	3	6, 3.5, 2	Green	30	21	15	16	3.8

LMW succinoglycan (50 μg dissolved in 0.1 ml of sterile H_2O) was applied along the root 24 hr prior to inoculation with an *exoA* mutant, and plants were observed 6 weeks later. No. Obs., number observed.
*Number elongate or pink.
†Number reducing acetylene.
‡Number containing >10 cfu.
§Average number of cfu from nodules containing >10 cfu.

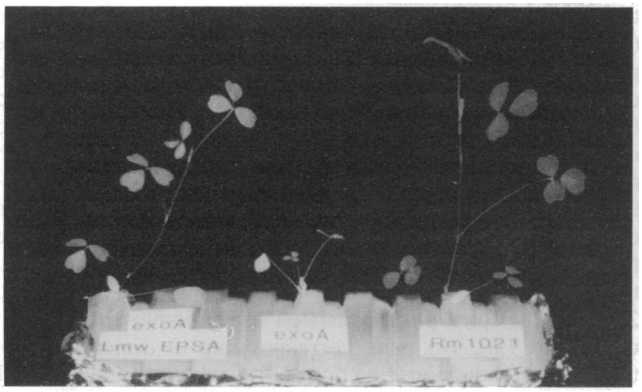

FIG. 1. Four-week-old alfalfa plants inoculated with (left to right) Rm6086 (*exoA*) after a 24-hr pretreatment with LMW succinoglycan (100 μg), Rm6086 without EPS, and Rm1021 (wild type).

staining as the original inoculum, indicating that the nodules had not been invaded by an Exo$^+$ contaminant. Furthermore, *exoA* mutants isolated from nodules of treated plants retained their nodule invasion defect when reinoculated on alfalfa seedlings in the absence of EPS, so the bacteria had not become invasion competent from some genetic alteration. Plants treated with LMW succinoglycan but not inoculated grew poorly and did not nodulate.

LMW succinoglycan promoted invasion by other *exo* mutants as well and did so under several treatment methods. Alfalfa seedlings were inoculated and grown under the following conditions: (*i*) no LMW succinoglycan present (control); (*ii*) LMW succinoglycan (100 μg) incorporated into the molten agar just before pouring, and roots inoculated 24 hr after planting; (*iii*) LMW succinoglycan (1 mg dissolved in 0.1 ml of sterile H_2O) applied along the length of the root 24 hr prior to inoculation; and (*iv*) the same as *iii* applied immediately prior to inoculation. Three plants from each treatment were inoculated with four different Exo$^-$ mutants (*exoA*, *exoB*, *exoF*, and *exoH*) and the parental strain Rm1021 (Exo$^+$). Whereas untreated plants grew poorly and formed only round white nodules, treated plants always grew better and contained a portion of elongate slightly pink nodules that contained bacteria and reduced acetylene. No discernible effect of LMW succinoglycan on Rm1021-inoculated plants was observed.

Ultrastructure of Nodules Invaded by Exo$^-$ Mutants in the Presence of Succinoglycan. Light micrographs of nodule sections (Fig. 2) showed that nodules formed by the *exoA* mutant in the presence of LMW succinoglycan contained typical invasion and symbiotic zones. Infection threads were plentiful and appeared to be distended. Bacteroids were present in the symbiotic zone. None of these structures were found in nodules formed by the *exoA* mutant in the absence of EPS.

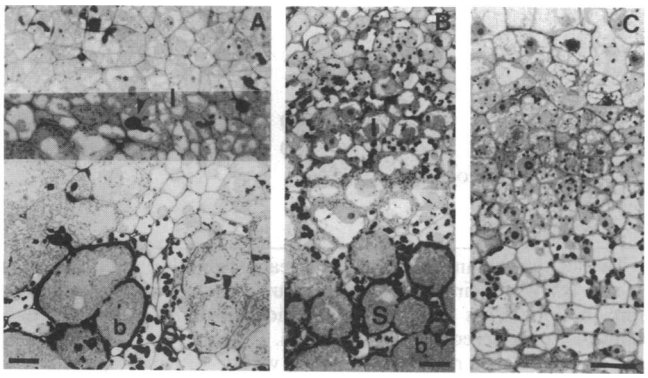

FIG. 2. Light micrographs of nodules formed by Rm6086 (*exoA*) after a 24-hr pretreatment with LMW succinoglycan (100 μg) (*A*), Rm1021 (wild type) (*B*), and Rm6086 without EPS (*C*). Portions of the invasion zones (I) and symbiotic zones (S) are shown. Infection threads (arrowheads), bacteroid-filled cells (b), and individual bacteroids (arrows) are indicated. Darkly staining granules are starch. (Bars = 100 μm.)

Invasion-Promoting Activities of Various EPSs. To establish the specificity of the EPS requirement for nodule invasion, we surveyed various forms of EPS for their abilities to correct the nodule invasion defect of the *exoA* mutant. Only the LMW form of succinoglycan was active; neither HMW succinoglycan nor dialyzed cell-free culture supernatants of Rm1021 containing both HMW and LMW succinoglycan promoted nodule invasion. (The inability of dialyzed supernatant to promote invasion may reflect an inhibitory effect of exogenously added HMW succinoglycan. We have observed such an effect with Rm1021.) Sonication of HMW succinoglycan decreases the viscosity in solution because of strand breakage; however, efforts to generate active succinoglycan by sonication of HMW succinoglycan failed. The invasion-promoting activity was not a non-EPS factor that comigrated with LMW succinoglycan, since LMW fractions (equivalent to 0.1 mg EPS) obtained from the *exoA* mutant by Bio-Gel A-5m chromatography and desalted as usual did not promote invasion. [*exoA* mutants have been shown to secrete no detectable succinoglycan but to be normal with regard to other surface factors (8).] In addition, unfractionated dialyzed supernatants from *exoA*, *exoB*, *exoF*, and *exoH* mutants did not promote nodule invasion (they had no effect on the nodulation ability of Rm1021). Heat (80°C for 30 min) or protease treatment had no effect on the activity of LMW succinoglycan, suggesting that activity was not due to some contaminating proteinaceous or heat-labile factor. In agreement with genetic evidence (12), succinylation appeared to be important; neither HMW nor LMW succinoglycan from a nonsuccinylating *exoH* mutant (8, 12) promoted invasion. *nod* genes that control nodule formation were not involved in production of active succinoglycan, since LMW succinoglycan samples from *nodD1D2D3* and *nodA* mutants and from Rm1021 cultured in the presence of the *nod* gene-inducer luteolin elicited the same response as did LMW succinoglycan from Rm1021 cultured without luteolin; these LMW succinoglycan samples also contained the normal subfractions as determined by Bio-Gel P-4 chromatography (below).

HMW and LMW EPSs from *R. leguminosarum* bv. *trifolii* and *Rhizobium* sp. strain NGR234 and HMW EPSs from *R. leguminosarum* bv. *viciae* and *A. tumefaciens* were also tested at both 0.1- and 0.5-mg doses; none were active.

Fractionation of LMW Succinoglycan and Biological Activity of Subfractions. We found that LMW succinoglycan could be further fractionated into several oligomeric subfractions

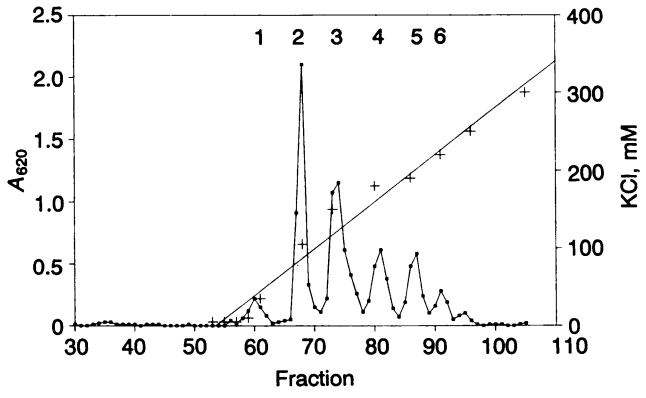

FIG. 3. QAE Sephadex chromatography of LMW succinoglycan (13 mg loaded). Boxes represent absorbance at 620 nm in the anthrone–sulfuric acid assay for carbohydrate. +, KCl concentration (mM).

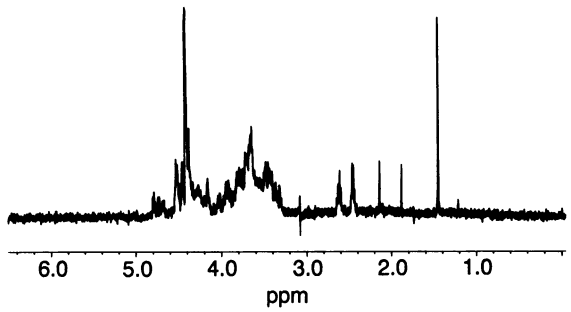

FIG. 4. Proton NMR spectrum of QAE fraction 6, showing close similarity to authentic succinoglycan (8). Peak assignments are as follows: 1.45 ppm, pyruvate methyl protons; 2.15 ppm, acetate methyl protons; 2.45 and 2.6 ppm, succinate methylene protons; 3.3–4.8 ppm, carbohydrate region; 4.4 ppm, HDO solvent peak. The peak at 1.9 ppm was present in all of the QAE fractions and has not been identified.

by anion-exchange or gel-filtration chromatography. Fig. 3 shows the elution profile obtained from QAE anion-exchange chromatography. Proton NMR spectroscopy and comparison with authentic spectra (8) showed that QAE chromatographic peaks 2–6 consisted of succinoglycan with all of its discernible features including pyruvate, acetate, and succinate, although quantities could not be determined accurately (see Fig. 4 for NMR of QAE peak 6). A clear spectrum of peak 1 could not be obtained because of insufficient material, but it too appeared to resemble succinoglycan.

Each QAE peak and unfractionated LMW succinoglycan were chromatographed by Bio-Gel P-4 gel-filtration chromatography to determine the succinoglycan size distribution (Fig. 5). Unfractionated LMW succinoglycan yielded three peaks that were eluted within the fractionation range of the column. The molecular weight of each peak was estimated from the molar ratio of the observed total carbohydrate to observed reducing sugar, based on the assumption that each repeating subunit of succinoglycan contains eight sugar residues and each molecule of succinoglycan regardless of size contains a single reducing end. The ratios showed that the first peak consisted of tetramer (four repeat units, ratio 31), the second peak consisted of trimer (three repeat units, ratio 25), and the third peak consisted of monomer (one repeat unit, ratio 7.8). No peak corresponding to dimer was detected. Based on migration on the P-4 column, QAE peaks 1 and 2 both consisted of monomeric subfractions. QAE peak 3 consisted of a mixture of all three oligomeric sizes, while QAE peak 4 contained a mixture of trimer and tetramer. QAE peaks 5 and 6 consisted of the tetrameric form. Since the tetramers in QAE peaks 3, 4, 5, and 6 each eluted differently in ion-exchange chromatography, they may differ in their degrees of modification by the carboxylated 1-carboxyethylidene (pyruvate) and succinyl groups. Total carbohydrate-to-reducing sugar measurements of each QAE peak supported the conclusions drawn from P-4 chromatography. Thus, the calculated ratios were as follows: peak 1, 8; peak 2, 9; peak 3, 17; peak 4, 25; peak 5, 34; peak 6, 31.

To determine if one or all of the oligomeric subfractions were biologically active, we tested each QAE peak individually for nodule invasion-promoting activity. Only peak 6 was active (Table 2).

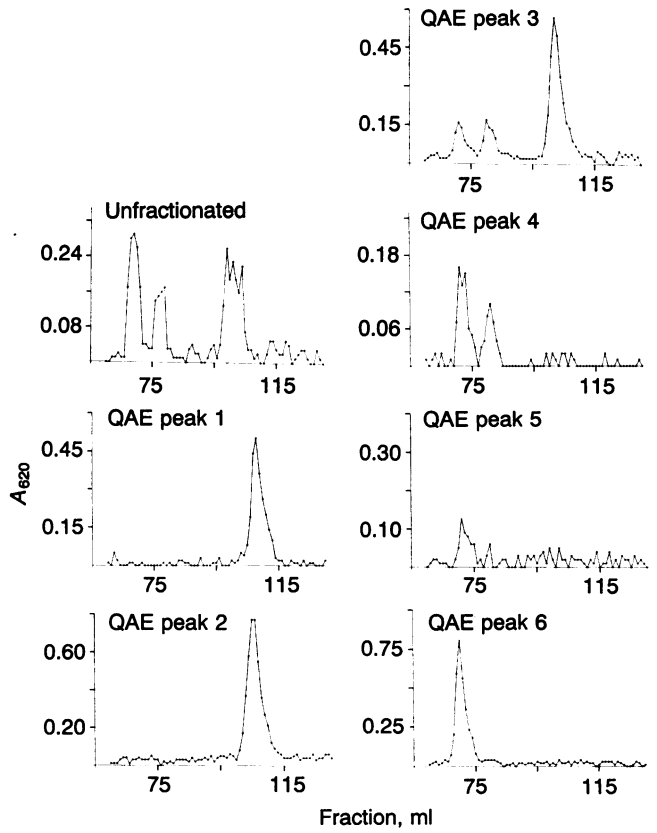

FIG. 5. Bio-Gel P-4 chromatography of LMW succinoglycan fractions. EPS sample and amount (mg) loaded: unfractionated LMW succinoglycan, 0.5; QAE peak 1, 0.4; QAE peak 2, 0.5; QAE peak 3, 0.5; QAE peak 4, 0.2; QAE peak 5, 0.1; QAE peak 6, 0.6. A_{620} values are readings obtained in the anthrone–sulfuric acid assay. Void and salt volumes were at fractions 60 and 146, respectively.

DISCUSSION

R. meliloti exo mutants fail to invade nodules because of a block in infection thread penetration (12, 21). Here we have demonstrated that succinoglycan when added exogenously can overcome the block in invasion. Nodules formed by *exo* mutants in the presence of succinoglycan resembled normal nodules in appearance, contained bacteroids, and fixed nitrogen. Infection threads were present in the interior of the nodules. While the invasion response to exogenous succino-

Table 2. Nodule invasion response to subfractions of LMW succinoglycan

Succinoglycan fraction	Nodule invasion Exp. 1	Exp. 2	Exp. 3
QAE 1	ND	–	–
QAE 2	–	–	–
QAE 3	–	–	–
QAE 4	–	–	–
QAE 5	–	–	–*
QAE 6†	ND	+	+‡
QAE 1-6	ND	+‡	+
LMW succinoglycan	+	ND	+
None	–	–	–
Rm1021§	++	++	++

Two or three plants were used for each condition in each experiment. LMW succinoglycan (0.1 mg unless otherwise noted) was applied along roots 20–24 hr before inoculation with Rm6086, and plants were observed after 4–4.5 weeks. –, Plant tops 1–3 cm, leaves pale or brown, and nodules round and white; +, plant tops 4–6 cm, leaves light green, and some nodules elongate and white or pink; ++, plant tops 6–8 cm, leaves dark green, and nodules elongate and pink. ND, not determined.

*A few nodules were elongate and white on both of two plants.

†0.05 mg of succinoglycan was added.

‡Plant growth was uniformly +, and one of two plants contained visibly elongate nodules.

§Plants were inoculated with Rm1021, and no EPS was added.

glycan was quite marked, it was not as pronounced as the invasion that follows inoculation with Exo$^+$ *R. meliloti*. This may simply be due to the fact that optimal conditions have not been achieved or that succinoglycan is needed internally during nodule invasion and, when added exogenously, does not easily penetrate to its site of action. Alternatively, some additional form of succinoglycan (such as HMW succinoglycan) that is not added in our assay or does not penetrate the root surface may be necessary for optimal efficiency. The distended appearance of the infection threads in nodules formed by the *exo* mutant in the presence of LMW succinoglycan might also be related to an internal function of EPS that is not supplied by exogenous addition.

The invasion response that we have observed has enabled us to investigate the properties of biologically active succinoglycan. First, only LMW succinoglycan, not HMW succinoglycan, promoted invasion. This contrasts with the results of similar experiments with *Leucaena* and clover in which HMW and LMW EPS from the appropriate species of *Rhizobium* were functional (13). The HMW EPS in the succinoglycan–alfalfa system may differ in the ability to form three-dimensional determinants that resemble the LMW form, to be degraded into LMW forms in the presence of the plant, or to penetrate into an appropriate site of action. LMW succinoglycan was active at a low concentration; experiments where it was incorporated into the agar medium used for plant growth showed that it could elicit a response at 2.5 μg/ml, or in the range of 1 μM. The requirement for a particular form of LMW EPS was quite specific. Only a fraction containing a highly charged (therefore highly modified with pyruvate and succinate substituents) oligomer of succinoglycan that contained four repeat units (tetramer), not fractions containing smaller or less charged forms, was active when tested alone. This was consistent with our finding (unpublished data) that LMW succinoglycan harvested from cultures older than 5 days lacked tetramer and failed to promote invasion. The requirement for a highly charged, LMW form of succinoglycan was also consistent with genetic evidence that succinylation and/or molecular weight are important: *exoH* mutants, which produce nonsuccinylated HMW succinoglycan and very little LMW succinoglycan (8), fail to invade (12). The *nod* genes, which control nodule formation, were not involved in the synthesis of biologically active LMW succinoglycan or the synthesis of normal LMW subfractions.

Neither HMW nor LMW EPS from several heterologous species of *Rhizobium* promoted invasion of alfalfa by the *R. meliloti exo* mutant. This observation is reminiscent of findings in the *Leucaena* and clover systems in which only the EPS from the parental strain of the homologous bacterial species could rescue nodule development and invasion by Exo$^-$ mutants (13). Our results could reflect a specific structural requirement or the failure of the heterologous species to produce EPS of the correct subunit multiplicity and degree of modification.

Interestingly, genetic experiments have indicated that a different *R. meliloti* EPS, EPSb (EPS II), can substitute for succinoglycan in nodule invasion (22, 23). EPSb has been detected only as HMW EPS, and we have not yet obtained oligosaccharide forms to test in our bioassay. EPSb may contain the necessary three-dimensional determinants as a HMW EPS or it may be broken down into LMW forms in the presence of the plant root. Also, experiments in which *Rhizobium* sp. strain NGR234 was engineered to produce succinoglycan have shown that succinoglycan can substitute for the NGR234 EPS during the nodulation of *Leucaena* (24). The exogenous addition of succinoglycan has not been attempted with NGR234 *exo* mutants on *Leucaena*, but the structural identity of succinoglycan with the NGR234 EPS in a large portion of its repeat unit could help to explain the results.

Our results suggest that succinoglycan plays a specific role in alfalfa root nodule development that relies on its particular chemical structure. LMW succinoglycan may function as a specific signal, as does the nodule formation signal nodRm1 (3, 4) or the elicitors that induce plant-defense responses (25). This model would suggest the existence of a specific plant receptor. The requirement for the tetrameric oligosaccharide form of succinoglycan could reflect a requirement to fill a receptor "pocket." The requirement for carboxyl-containing substituents could reflect specific interactions with the receptor or a requirement for correct folding of the EPS molecule. In the latter case, the chelation of divalent cations by the negatively charged groups could result in appropriate folding, as in certain peptide neurotoxins from cone snail venom, in which either disulfide bonds or metal chelation by γ-carboxyglutamate residues provides a rigid conformation (26). LMW succinoglycan could also act as an activator of specific enzymes (e.g., polysaccharases) involved in root hair curling or infection-thread initiation and growth (*exo* mutants are delayed in root hair curling as well as blocked in infection-thread penetration; refs. 12 and 21). In this case, the structural requirement may reflect the need for the presence of acidic residues that would interact with basic amino groups on proteins.

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