Function of Succinoglycan Polysaccharide in Sinorhizobium meliloti
Host Plant Invasion Depends on Succinylation, Not Molecular Weight

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ABSTRACT  The acidic polysaccharide succinoglycan produced by the rhizobial symbiont Sinorhizobium meliloti 1021 is required for this bacterium to invade the host plant Medicago truncatula and establish a nitrogen-fixing symbiosis. S. meliloti mutants that cannot make succinoglycan cannot initiate invasion structures called infection threads in plant root hairs. S. meliloti exoH mutants that cannot succinylate succinoglycan are also unable to form infection threads, despite the fact that they make large quantities of succinoglycan. Succinoglycan produced by exoH mutants is refractory to cleavage by the glycanases encoded by exoK and exsH, and thus succinoglycan produced by exoH mutants is made only in the high-molecular-weight (HMW) form. One interpretation of the symbiotic defect of exoH mutants is that the low-molecular-weight (LMW) form of succinoglycan is required for infection thread formation. However, our data demonstrate that production of the HMW form of succinoglycan by S. meliloti 1021 is sufficient for invasion of the host M. truncatula and that the LMW form is not required. Here, we show that S. meliloti strains deficient in the exoK- and exsH-encoded glycanases invade M. truncatula and form a productive symbiosis, although they do this with somewhat less efficiency than the wild type. We have also characterized the polysaccharides produced by these double glycanase mutants and determined that they consist of only HMW succinoglycan and no detectable LMW succinoglycan. This demonstrates that LMW succinoglycan is not required for host invasion. These results suggest succinoglycan function is not dependent upon the presence of a small, readily diffusible form.

IMPORTANT  Sinorhizobium meliloti is a bacterium that forms a beneficial symbiosis with legume host plants. S. meliloti and other rhizobia convert atmospheric nitrogen to ammonia, a nutrient source for the host plant. To establish the symbiosis, rhizobia must invade plant roots, supplying the proper signals to prevent a plant immune response during invasion. A polysaccharide, succinoglycan, produced by S. meliloti is required for successful invasion. Here, we show that the critical feature of succinoglycan that allows infection to proceed is the attachment of a “succinyl” chemical group and that the chain length of succinoglycan is much less important for its function. We also show that none of the short-chain versions of succinoglycan is produced in the absence of two chain-cleaving enzymes.

S. meliloti 1021 is a soil bacterium and nitrogen-fixing symbiont of the host plants Medicago truncatula cv. “Jemalong A17” and Medicago sativa (alfalfa) (1, 2). Under nitrogen-limiting conditions, S. meliloti induces formation of nodules on host plant roots, invades and colonizes the nodules (1, 2), and then begins to convert or “fix” dinitrogen gas to ammonia, a form that the host can use (2). For successful invasion of host plant roots by rhizobia, the symbiotic partners must exchange multiple signals that promote bacterial entry. Plant flavonoids signal S. meliloti to produce a lipochitooligosaccharide signal called Nod factor (NF) (3). NF induces host plant root hair curling that leads to trapping of microcolonies of S. meliloti within the curl and induces cell division in the root cortex, leading to formation of the nodule primordium (2). Structures called infection threads initiate from these colonized curled root hairs. An infection thread is a progressive ingrowth of root hair cell membrane that leaves behind a tubule filled with S. meliloti and a matrix composed of bacterial exopolysaccharides (EPS) and plant cell wall material (4, 5). It is through infection threads that rhizobia invade and colonize the root interior (1). Infection thread initiation and development require that S. meliloti propagate in the infection thread and produce both NF and the EPS succinoglycan (1). Infection threads in root hairs are extended to the base of these cells and through each cell layer, eventually delivering the bacteria to proliferating cells of the nodule primordium (6, 7). Succinoglycan production by S. meliloti 1021 is required for this bacterium to induce infection threads on host plants (8). S. meliloti strains that do not produce succinoglycan, such as the exoY mutant (9), are able to...
colonize root surfaces and become tightly enclosed within curled root hairs but fail to initiate infection threads (10).

Rhizobial acidic EPSs are either required for or enhance host invasion in multiple rhizobial symbiont-host plant pairs (11–25). In some cases, determining the importance of a symbiotic EPS in host invasion has been complicated by production of multiple EPSs by a single bacterial strain. However, in the S. meliloti 1021-M. truncatula host-symbiont pair, succinoglycan is the only EPS produced in sufficient quantities and in a functional form that can enable infection thread formation (8, 19, 21, 23, 26) (see Discussion). It has also been demonstrated that increased succinoglycan production by S. meliloti leads to an increase in symbiotic productivity of inoculated M. truncatula plants (27). Acidic EPSs of bacterial pathogens of plants can also be virulence determinants in plant disease. Many of these negatively charged polysaccharides have been shown to suppress plant defense activation by sequestering Ca\(^{2+}\) and preventing a signaling cascade (28). Both the EPS xanthan of Xanthomonas campestris pv. campestris (29, 30) and the EPS alginate of Pseudomonas syringae (31, 32) enhance host plant infection by these pathogens and exacerbate disease symptoms. It is not yet known if acidic EPSs of rhizobial plant symbionts and of plant pathogens perform any similar functions in host invasion.

It is also not known why in S. meliloti 1021, succinoglycan is required for infection thread initiation and progression or how it might influence conditions within colonized curled root hairs to facilitate these processes. The succinoglycan monomer is an octasaccharide composed of 1 galactose residue and 7 glucose residues, with acetyl, succinyl, and pyruvyl modifications (33). Negatively charged carboxylates on the succinyl and pyruvyl groups render the polysaccharide acidic. This structure, along with the gene product that catalyzes each step in the biosynthetic pathway, is shown in Fig. 1 (33–37). It has recently been determined that in Mesorhizobium loti R7A, an acidic octasaccharide EPS with some structural similarities to succinoglycan interacts with the Epr3 receptor-like kinase in its plant host Lotus japonicus (16). An M. loti mutant that cannot make this EPS can successfully invade and nodulate L. japonicus (13), but exoU mutants that produce a truncated pentasaccharide EPS cannot invade wild-type plants (13). Thus, there is a striking difference between the M. loti-L. japonicus system and the S. meliloti-M. truncatula system: EPS-deficient mutants of M. loti can invade their host (13), whereas S. meliloti succinoglycan-deficient mutants cannot invade M. truncatula. S. meliloti exoY and exoA mutants, which produce no succinoglycan (9, 34, 38), do not invade host plants (10, 38) and in the case of exoY have been shown to fail in initiating infection threads (10).

S. meliloti strains that are completely succinoglycan deficient are not the only type of succinoglycan mutant with a symbiotic defect. An exoH mutant that produces succinoglycan lacking the succinyl groups (Fig. 1) also cannot invade alfalfa roots (39) and has previously been shown to initiate a reduced number of infection threads on alfalfa and to abort all of the infection threads that are initiated (10). exoH mutants produce only the high-molecular-weight (HMW) form because the glycanses ExoK and ExsH cannot cleave the unsuccinylated form (40). A long-standing question about the nature of the defect in S. meliloti exoH mutants is whether they fail to invade the host because the succinoglycan they produce is unsuccinylated or because they produce only the HMW form of succinoglycan (40). It has not previously been determined if there is a mechanism independent of ExoK and ExsH cleavage for production of low-molecular-weight (LMW) succinoglycan; however, the existence of alternate routes to the LMW form has been proposed: either through cleavage by another enzyme or through direct export of LMW forms (41, 42). If LMW succinoglycan cannot be produced in the absence of the ExoK and ExsH glycanses, and if LMW succinoglycan is required for infection thread formation, then a double mutant with mutations in both glycanses enzymes should have a very severe symbiotic defect similar to that of strains with a mutation in the exoH-encoded succinyltransferase. Conversely, if LMW succinoglycan is not required for infection thread formation, strains deficient in both glycanses should not have a severe symbiotic defect. Thus, it is critical to determine both the symbiotic phenotype of double glycansase mutants and whether or not these strains produce any residual LMW succinoglycan.

We have now characterized the polysaccharides produced by strains deficient in both the ExoK and ExsH glycanses and determined that these strains do not produce any LMW succinoglycan. We have also determined that these “double glycansase” mutants invade M. truncatula roots and establish a productive symbiosis, albeit with less efficiency than wild-type S. meliloti 1021. This demonstrates that the LMW form of succinoglycan is not required for host invasion. This also indicates that successful symbiosis requires succinylation of succinoglycan for a reason that is independent of the effect of succinylation on susceptibility to glycansase cleavage.

RESULTS

Strains deficient in the exsH-encoded succinoglycan glycansase do not have a significant reduction in symbiotic productivity.

We have previously constructed a nonpolar deletion mutant of the succinoglycan glycansase-encoding gene exoK (see below) and found that this strain can invade M. truncatula and form functional nodules, but it does so less efficiently than the wild type (43). To determine if loss of an additional succinoglycan glycansase encoded by exsH also has an effect on symbiosis with M. truncata-

ula, we tested mutants carrying a Tn5-233 transposon insertion in exsH and found that they do not have a statistically significant defect in symbiotic productivity measured by shoot fresh weight, but they do have a small statistically significant reduction in the
number of pink, functional nodules (see Fig. S1 in the supplemental material). The pink color of root nodules induced by rhizobial infection is due to the production of leghemoglobin by the host plant and is indicative of a functional symbiosis (44, 45). This very small effect on the symbiosis is consistent with the lack of detectable expression of the exsH glycanase gene in S. meliloti during host invasion (Fig. 2). Using strains that carry both an exsH:uidA β-glucuronidase (GUS) reporter fusion and a complete copy of exsH in the genome, we found that expression of exsH cannot be detected in S. meliloti in infection threads or root nodules at 14 days postinoculation (Fig. 2A to D), a time point at which strong expression of the operon containing exoK can be detected (43). GUS is expressed in exsH reporter strains when they are grown on M9 medium, demonstrating that the reporter is functional (see Fig. S2 in the supplemental material). Taken together, our results show that expression of the exsH-encoded glycanase is not detectable during host invasion, and loss of this glycanase does not have a significant effect on the symbiotic productivity of the association with M. truncatula.

Strains deficient in both the exoK- and exsH-encoded succinoglycan glycanases invade host roots and form functional nodules. ExsH and ExoK are the only glycanases that have been demonstrated to cleave succinoglycan to generate the LMW form in the 1021 strain of S. meliloti (41, 46 [also see reference 47]). To determine whether or not LMW succinoglycan is required for successful host invasion of M. truncatula, it is necessary to determine the symbiotic phenotype of exoK exsH glycanase double mutants and to determine whether or not these double glycanase mutants produce any LMW succinoglycan. The nonpolar exoK deletion strains described by Mendis et al. (43) (Kdel-trpexoL strains) were constructed as part of a series of strains in which the downstream exoLAMON genes are under identical regulatory control despite alterations to the upstream exoHK region. The design of these nonpolar exoK deletion strains and the “modified wild-type” control strains (trpexoL strains) is shown in Fig. S3 in the supplemental material. To construct the double glycanase mutants, we transduced the exsH::Tn5-233 insertion into the Kdel-trpexoL strains, generating 6 independently isolated double glycanase mutants (strains 1325, 1326, 1328, 1329, 1332, and 1333). We also transduced exsH::Tn5-233 into the trpexoL strains to make exsH single mutants in the “modified wild-type” background. Symbiotic phenotypes of double glycanase mutants are shown in Fig. 3, along with symbiotic phenotypes of exoK single mutants and exsH single mutants. Figure 3A shows average shoot fresh weights of M. truncatula plants inoculated with each S. meliloti strain. The Kdel-trpexoL exsH double glycanase mutants (here, referred to as ExoK ExsH double glycanase mutants) have a small, but statistically significant reduction in plant productivity relative to S. meliloti 1021 wild-type and “modified wild-type” exoK control strains and relative to the trpexoL exsH single mutants (Fig. 3A). (Fig. 3B shows a representative M. truncatula plant inoculated with wild-type S. meliloti 1021 versus an uninoculated plant.) The symbiotic performance of the double glycanase mutants is similar to that of Kdel-trpexoL single glycanase mutants (Fig. 3A). Most of the Kdel-trpexoL single mutants and the ExoK ExsH double glycanase mutant strains also have a small, but statistically significant reduction in the number of pink, functional nodules (Fig. 3C). These results demonstrate that ExoK ExsH double glycanase mutants, like Kdel-trpexoL exoK single mutants, have reduced symbiotic productivity relative to wild-type strains but are still able to form a functional symbiosis on M. truncatula.

Strains deficient in both the ExoK and ExsH succinoglycan glycanases do not produce any detectable LMW succinoglycan. In order to determine whether or not these symbiosis-functional ExoK ExsH double glycanase mutants produce any LMW succinoglycan. We had previously determined that Kdel-trpexoL exoK single mutants produce a reduced but substantial amount of LMW succinoglycan that can be seen in an LMW succinoglycan-diffusion “halo” assay using the fluorescent dye Calcofluor (43). To determine if the ExsH glycanase is the source of the LMW succinoglycan in these
Kdel-trpexoL single mutants, we compared the ExoK ExsH double glycanase mutants with the single glycanase mutants and control strains in a Calcofluor halo assay (Fig. 4). We found that after 12 days of growth on GMS (glutamate mannitol salts medium) medium containing 0.02% Calcofluor, ExoK ExsH double glycanase mutants did not produce a visible halo of LMW succinoglycan (Fig. 4E). This contrasts with the abundant LMW succinoglycan produced by S. meliloti 1021 wild-type, to “modified wild-type” trpexoL control strains, and to exsH single mutants. The symbiotic performance of double glycanase mutants is similar to the symbiotic productivity of Kdel-trpexoL exoK single mutants (43) (Fig. 4C). Thus, it appears that the majority of LMW succinoglycan is made by ExoK cleavage of the polymer with some contribution by ExsH cleavage that is apparent in the absence of ExoK.

It had previously been determined in another study (41) that strains carrying transposon insertions in both exoK and exsH do not produce a visible halo of LMW succinoglycan in a Calcofluor assay (Fig. 4). We found that after 12 days of growth on GMS (glutamate mannitol salts medium) medium containing 0.02% Calcofluor, ExoK Exsh double glycanase mutants did not produce a visible halo of LMW succinoglycan (Fig. 4E). This contrasts with the abundant LMW succinoglycan produced by S. meliloti 1021 wild-type and trpexoL “modified wild-type” strains (Fig. 4A). It also contrasts with the large amount of LMW material made by exsH single mutants in both the 1021 and the trpexoL backgrounds (Fig. 4D) and the intermediate amount of LMW material already demonstrated to be made by Kdel-trpexoL exoK single mutants (43) (Fig. 4C). Thus, it appears that the majority of LMW succinoglycan is made by ExoK cleavage of the polymer with some contribution by ExsH cleavage that is apparent in the absence of ExoK.

It had previously been determined in another study (41) that strains carrying transposon insertions in both exoK and exsH do not produce an LMW succinoglycan “halo” in a Calcofluor assay, but that these strains do produce a residual amount of LMW polysaccharide material that can be detected with the anthrone-sulfuric acid assay for hexose sugars (48). However, the identity of the LMW, hexose-containing material produced by these strains was not established in this previous study. In order to determine whether any of the LMW polysaccharide produced by ExoK ExsH double glycanase mutants is succinoglycan, we characterized LMW polysaccharide from these strains by size separation and sugar composition analysis. We isolated culture supernatant from GMS minimal medium cultures of wild-type 1021 and two independently isolated ExoK ExsH double glycanase mutants, 1325 and 1328. Culture supernatant from the succinoglycan-deficient exoY mutant (34) served as a negative control. We used total culture supernatant rather than alcohol-precipitated polysaccharide because it has been reported that alcohol precipitation is inefficient in isolating LMW forms of succinoglycan (49). Table 1 shows the quantification of polysaccharide calculated from the anthrone-positive material per milliliter from each culture supernatant normalized to the cell density of the culture measured at optical density at 600 nm (OD600). The two ExoK ExsH double glycanase mutant strains tested, 1325 and 1328, produce 60 to 70% of the amount of polysaccharide produced by the wild type (Table 1). In contrast, the succinoglycan-deficient exoY mutant (34) served as a negative control. We used total culture supernatant rather than alcohol-precipitated polysaccharide because it has been reported that alcohol precipitation is inefficient in isolating LMW forms of succinoglycan (49). Table 1 shows the quantification of polysaccharide calculated from the anthrone-positive material per milliliter from each culture supernatant normalized to the cell density of the culture measured at optical density at 600 nm (OD600). The two ExoK ExsH double glycanase mutant strains tested, 1325 and 1328, produce 60 to 70% of the amount of polysaccharide produced by the wild type (Table 1). In contrast, the succinoglycan-deficient exoY mutant produces 10% of the amount of polysaccharide produced by the wild type. This demonstrates that even the exoY mutant produces a small amount of hexose-containing polysaccharide, while the wild-type and the double glycanase mutants produce a large quantity of hexose-containing material.

LMW succinoglycan produced by wild-type S. meliloti 1021
FIG 4 There is no LMW succinoglycan detectable from ExoK ExsH double glycanase mutants with the Calcofluor-fluorescence halo assay. After 12 days of growth on GMS medium containing 0.02% Calcofluor, S. meliloti 1021 wild-type and trpxeL “modified wild-type” strains (A) produce a large halo of diffused LMW succinoglycan. Negative-control, succinoglycan-deficient exoY and exoA strains (B) are “Calcofluor dark,” showing that they do not produce succinoglycan. (C) Kdel-trpxeL exoK single mutants have a reduced halo diameter, consistent with production of an intermediate amount of LMW succinoglycan. (D) exsH single mutants in both the 1021 and the trpxeL backgrounds have halos of similar size to the wild type, suggesting that loss of exsH has little effect on production of LMW succinoglycan. (E) ExoK ExsH double glycanase mutants produce no detectable Calcofluor halo, suggesting that no LMW succinoglycan is produced in the absence of these two glycanases. has previously been determined to consist of monomers, dimers, and trimers of the octasaccharide (42, 50) with calculated molecular masses of 1.5 to 1.7, 3.1 to 3.5, and 4.6 to 5.2 kDa, respectively. (The molecular mass range is due to variability in degree of succinylation of each succinoglycan monomer.) In order to isolate LMW material, we collected solutes smaller than 10 kDa in size by filtering the culture supernatants through a 10-kDa molecular mass cutoff (MMCO) membrane. Table 1 shows the percentage of total polysaccharide produced by each strain that is smaller than 10 kDa in size. The succinoglycan-deficient exoY mutant and both ExoK ExsH double glycanase mutants produce similar percentages (30 to 40%) of total polysaccharide as species smaller than 10 kDa, while the wild type produces a much greater percentage of total polysaccharide in forms smaller than 10 kDa (70%).

The LMW fraction of each sample was further fractioned by size exclusion on a Superdex 75 column and the hexose sugar content of the fractions analyzed by anthrone-sulfuric acid assays. The results are shown in Fig. S4 in the supplemental material. Wild-type LMW polysaccharide from the Superdex 75 column was collected for finer fractionation on a Superdex 30 column. Wild-type LMW material was resolved on the Superdex 30 column (Fig. 5A) into 2 major peaks composed of fractions 25 to 28 (peak 2) and fractions 36 to 41 (peak 4) and 2 minor peaks of fractions 20 to 23 (peak 1) and fractions 29 to 34 (peak 3) (Fig. 5A). Vitamin B$_{12}$ (1.35 kDa) served as LMW marker in all column runs (Fig. 5). Samples of succinoglycan-deficient exoY mutant and ExoK ExsH double glycanase mutants 1325 and 1328 were also separated on the Superdex 30 column (Fig. 5B). What appears to be a single large polysaccharide peak was detected at an identical position in fractions 25 to 34 in both ExoK ExsH double glycanase mutants and in the succinoglycan-deficient exoY mutant. This peak is centered on fraction 30, which is the same position as peak 3 from the wild type. The fact that ExoK ExsH double glycanase mutants have an LMW polysaccharide profile nearly identical to the succinoglycan-deficient exoY mutant suggests that LMW polysaccharides produced by the ExoK ExsH double glycanase mutants are not succinoglycan.

In order to establish the identity of these peaks, the glucose/galactose ratio of each peak was determined. Sugar composition of the peaks from fractions 25 to 28 (peak 2), 29 to 34 (peak 3), and 36 to 41 (peak 4) from wild-type S. meliloti 1021 and of the peaks centered on fraction 30 from the mutants was determined by the alditol acetate method at the University of Georgia Complex Carbohydrate Research Center, and the results are summarized in Table 2. The succinoglycan monomer has previously been determined by mass spectrometry to contain 7 glucose sugars and 1 galactose sugar (33). In contrast, cyclic β-glucans are pure glucose, and in S. meliloti are close in size to the succinoglycan dimer (51). The other polysaccharide that may be produced by S. meliloti 1021 in very small quantities, EPSII (also known as galactoglucan) has a repeating unit of 1 galactose:1 glucose (52, 53). The sugar composition analysis described below indicates that the peaks from the wild type are composed chiefly of succinoglycan, while the peaks centered on fraction 30 from the exoY mutant and from the ExoK ExsH double glycanase mutants are cyclic β-glucans.

Wild-type fractions 25 to 28 (peak 2) contain 80.0% glucose and 16.6% galactose with small quantities of other sugars (for full results, see Table S2 in the supplemental material), which is a glucose/galactose ratio of ~5:1. Wild-type fractions 36 to 41 contain 81.3% glucose and 16.4% galactose, which is also a glucose/galactose ratio of 5:1. Although a glucose/galactose ratio of 7:1 rather than 5:1 is predicted based on succinoglycan structure (33), a 5:1 ratio is very close to values previously detected for succinoglycan monomer by the alditol acetate method (e.g., see Fig. 1 in reference 42, in which the monomer peak glucose/galactose ratio

<table>
<thead>
<tr>
<th>Strain</th>
<th>Amt of an anthrone-positive material normalized to culture density OD$<em>{600}$ of anthrone per ml/OD$</em>{600}$ cell density</th>
<th>% of total anthrone-positive material of &lt;10 kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. meliloti 1021 wild type</td>
<td>5.00</td>
<td>70</td>
</tr>
<tr>
<td>exoY::Tn5 mutant (no succinoglycan)</td>
<td>0.46 (10% of wild type)</td>
<td>40</td>
</tr>
<tr>
<td>1325 ExoK ExsH double glycanase mutant</td>
<td>2.81 (60% of wild type)</td>
<td>40</td>
</tr>
<tr>
<td>1328 ExoK ExsH double glycanase mutant</td>
<td>3.57 (70% of wild type)</td>
<td>30</td>
</tr>
</tbody>
</table>
was ~5.5:1) (42). In contrast, wild-type fractions 29 to 34 (peak 3) contain 88.6% glucose and 8% galactose, which is a glucose/galactose ratio of 11:1 and is also similar to the value previously determined for peaks that are a mixture of succinoglycan and cyclic β-glucans (42). Based on the elution positions of Superdex 30 column peaks, sugar composition analysis, and comparisons with earlier work (42, 50), we conclude that wild-type fractions 25 to 28 (peak 2) are succinoglycan dimer, fractions 29 to 34 (peak 3) are a mixture of cyclic β-glucans and succinoglycan, and fractions 36 to 41 (peak 4) are succinoglycan monomer. The sugar composition of fractions 20 to 23 (peak 1) was not tested since it has such a small amount of hexose-positive material, but its elution position is consistent with succinoglycan trimer. Compared with previous observations, we isolated a smaller quantity of trimer relative to the quantity of dimer and monomer (50). One possible explanation for this is that by not alcohol precipitating polysaccharide, we

**TABLE 2** Glycosyl composition of LMW polysaccharide fractions

<table>
<thead>
<tr>
<th>Strain</th>
<th>µg glucose equivalents of hexose sugars (anthrone/sulfuric acid assay)</th>
<th>mol% of glycosyl residues in sample</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. meliloti</em> 1021 wild type</td>
<td>142 (peak 2)</td>
<td>92 (peak 3)</td>
</tr>
<tr>
<td><em>exoY</em>::Tn5 mutant (no succinoglycan)</td>
<td>171</td>
<td>497</td>
</tr>
<tr>
<td>1325  ExoK ExsH double glycanease mutant</td>
<td>189</td>
<td>450</td>
</tr>
<tr>
<td>1328  ExoK ExsH double glycanease mutant</td>
<td>288</td>
<td>674</td>
</tr>
</tbody>
</table>

* The fraction numbers shown are from the Superdex 30 column.
* Some samples contained <2.2% each mannose, xylose, and/or arabinose. Full glycosyl composition results are shown in Table S2 in the supplemental material.
* ND, not determined (no polysaccharide peak).
retained a larger percentage of the dimer and monomer present in the culture supernatant. This is consistent with the observation that precipitation of LMW succinoglycan is inefficient (49).

LMW polysaccharide material from the succinoglycan-deficient exoY mutant and from both ExoK ExsH double glycanase mutants appears to be a single broad peak centered on fraction 30. However, based on the Superdex 30 separation alone, we could not exclude the possibility that there were multiple peaks in these fractions representing multiple hexose-containing species. Therefore, we separately analyzed fractions 25 to 28 and fractions 29 to 34 as we had done for the peaks from the wild type. The results are summarized in Table 2. The material from fractions 25 to 28 and from fractions 29 to 34 from ExoK ExsH double glycanase mutants are 95% glucose and 1.6% galactose, indicating that these mutants do not produce a significant amount of galactose-containing polysaccharide of this size. In fact, these fractions from the ExoK ExsH double glycanase mutants have less galactose than the same fractions from the succinoglycan-deficient exoY mutant, which strongly suggests that these mutants do not produce any LMW succinoglycan. The size of oligosaccharides from all these mutants and the fact that they are composed almost exclusively of glucose, strongly suggest that they are cyclic β-glucans.

Strains deficient in the exoH-encoded succinyltransferase and both succinoglycan glycanases cannot engage in a productive symbiosis. In order to determine whether succinylation of succinoglycan is the critical factor in its symbiotic function, we made strains that are isogenic to the ExoK ExsH double glycanase mutants, except that they are also deleted for the exoH-encoded succinyltransferase. (For deletion design, see Fig. S3 in the supplemental material.) The symbiotic phenotypes of these triple mutant strains that lack both glycanases and the succinyltransferase are shown in Fig. 6. Growth of plants inoculated with any of 6 independently isolated, triple mutant strains (strains 1342, 1343, 1344, 1345, 1348, and 1349) on nitrogen-free medium is completely arrested and is indistinguishable from that of uninoculated plants. (Error bars show SEM.) The number of plants inoculated with each strain is shown.
complete inability to form functional nodules and to support host plant growth, while strains that lack only the glycanses have merely a slight reduction in symbiotic productivity. This demonstrates that succinylation of succinoglycan is required for \textit{S. meliloti} to engage in a functional symbiosis and that this requirement is independent of the effect that succinylation has on susceptibility to glucanase cleavage.

**DISCUSSION**

We have demonstrated that \textit{S. meliloti} 1021 double mutants deficient in both ExoK and ExsH glycanses make only HMW succinoglycan and that these strains can form a productive symbiosis with the host \textit{M. truncatula}. This is the first report providing biochemical evidence that the residual LMW hexose-sugar-containing material produced by \textit{S. meliloti} 1021 ExoK ExsH double glycanase mutants is not succinoglycan but is most likely cyclic \(\beta\)-glucans. This demonstrates that, at least under these growth conditions, production of LMW succinoglycan is completely dependent upon ExoK and ExsH glycanses. Our results also demonstrate that ExoK ExsH double glycanase mutants can form a productive symbiosis with \textit{M. truncatula}, which in this case must be mediated by HMW succinoglycan. In studies that report partial rescue of host invasion by an \textit{S. meliloti} succinoglycan-deficient mutant by coinoculation with succinoglycan, it was the LMW succinoglycan fraction that promoted rescue (50, 54, 55). These findings are not mutually exclusive with our results. A requirement for the LMW form in rescue studies could be specific to experiments in which succinoglycan is exogenously applied to the root hair surface and is not actively being secreted by bacteria in infection threads.

Other symbiotic EPSs can mediate infection thread formation by other strains of \textit{Sinorhizobium meliloti} (19), but these other polysaccharides are not produced in \textit{S. meliloti} 1021. For example, an \textit{expR}101 mutant of \textit{S. meliloti} 1021, \textit{S. meliloti} 8530, produces the EPS galactoglycan (EPSII) in sufficient quantities to mediate infection thread formation on the host alfalfa (19). However, in \textit{S. meliloti} 1021, under phosphate-replete conditions, EPSII is produced in, at most, trace amounts (56–58). Also, EPSII cannot support invasion on the host \textit{M. truncatula} (21). Therefore, EPSII cannot have been responsible for host invasion of \textit{M. truncatula} by the \textit{ExoK ExsH} double glycanase mutants. In addition, the capsular \(K\) antigen of \textit{S. meliloti} strain \textit{Rm}41 can also mediate infection thread formation on the host alfalfa (19), but the \textit{S. meliloti} 1021 strain lacks fully functional paralogs of the critical \textit{Rm}41 \textit{rkpZ} gene and consequently cannot produce \(K\) antigen in a symbiotically functional form (23, 59, 60). Therefore, infection thread formation on \textit{M. truncatula} by \textit{S. meliloti} 1021 is dependent on succinoglycan, and successful host invasion by the \textit{ExoK ExsH} double glycanase mutants is not mediated by EPSII or \(K\) antigen.

This work also answers the long-standing question regarding whether \textit{S. meliloti} strains with a mutation in the \textit{exoH}-encoded succinyltransferase fail to form a productive symbiosis because the succinoglycan they produce is unsuccinylated or, instead, because it cannot be cleaved by the glycanases and is therefore only in the HMW form. ExoK ExsH double glycanase mutants producing HMW succinylated succinoglycan form a successful symbiosis with \textit{M. truncatula}, while triple mutants with mutations in both glycanases and \textit{exoH}, producing HMW unsuccinylated succinoglycan, form neither functional nodules nor a productive symbiosis. Since we have shown that the successful \textit{ExoK ExsH} double glycanase mutants do not produce LMW succinoglycan, this strongly suggests that the symbiotic defect in \textit{exoH} succinyltransferase-deficient mutants is due to the lack of the acidic succinyl group on succinoglycan.

The unsuccinylated succinoglycan produced by an \textit{exoH} mutant of \textit{S. meliloti} lacks 1 to 2 negatively charged substituents per monomer, although it retains the negatively charged pyruvyl group (39). The degree of succinylation of EPS produced by other \textit{S. meliloti} strains has also been proposed to affect the ability of each strain to form a productive symbiosis on a particular \textit{M. truncatula} ecotype (61). The loss of the succinyl groups of succinoglycan results in an increase in viscosity and in polymer chain stiffness, probably due to reduced charge density (62). It is also likely that loss of negative charge would reduce the ability of succinoglycan to interact with positively charged ions in the infection thread matrix (28). Another possibility is that modifications to succinoglycan could alter its ability to quench reactive oxygen species (ROS) in the infection thread matrix (63). These factors might affect the fluidity of the infection thread matrix, thereby affecting infection thread progression (5, 64).

Recent studies on the EPS produced by the \textit{exoU} mutant of \textit{Mesorhizobium loti} R7A also suggest that loss of negative charge on symbiotically active rhizobial EPSs may be important for function (13, 16). The truncated, pentasaccharide EPS produced by the \textit{exoU} mutant lacks one neutral glucose sugar and two negatively charged uronic acid sugars (glucuronic acid and riburonic acid) (13, 16). This truncated \textit{M. loti} \textit{exoU} mutant-produced EPS prevents infection thread formation on the host plant \textit{L. japonicus}, and this blockage is dependent upon the \textit{L. japonicus} \textit{Exr}-encoded receptor-like kinase (13, 16). It is not yet known if it is the loss of negative charge from uronic acids or other structural features of the truncated \textit{exoU} mutant EPS that is critical for blocking infection thread formation. It is possible that \textit{S. meliloti} succinoglycan similarly interacts with an \textit{M. truncatula} ortholog of \textit{Exr3} and that loss of the negatively charged succinyl groups leads to a blockage in infection thread formation.

An important difference between the roles of EPS in \textit{M. loti}-\textit{L. japonicus} symbiosis and \textit{S. meliloti-M. truncatula} symbiosis is that an \textit{M. loti} EPS-deficient \textit{exoB} mutant can invade \textit{L. japonicus} roots and form a functional symbiosis, although it is less efficient, inducing 30 to 50% the number of mature, extended infection threads induced by the wild type at 10 to 14 days postinoculation (13, 16). This contrasts with the requirement for succinoglycan in infection thread formation on plant hosts by \textit{S. meliloti}. A succinoglycan-deficient \textit{S. meliloti} \textit{exoY} mutant forms no extended infection threads on the host alfalfa by 10 to 12 days postinoculation (10). This is consistent with earlier work showing that EPS production by rhizobia is more critical for symbiosis on plant hosts that form indeterminate nodules, such as alfalfa and \textit{M. truncatula}, than on those that form determinate nodules, such as \textit{L. japonicus} (65). In indeterminate nodules, infection threads must be maintained throughout the life of the node to allow bacteria to reach and invade not only cells of the nodule primordium but also the newly divided plant cells behind the persistent nodule meristem (7). The accumulation of aborted infections (10, 66), cytological evidence for plant defense responses (67), and expression of plant defense genes (66) in roots inoculated with succinoglycan-deficient strains of \textit{S. meliloti} provide extensive evidence for a role in succinoglycan in the intimate interaction between bacteria and root cells during invasion. Whether all of
these critical symbiotic interactions between succinoglycan and its host are dependent upon an *M. truncatula* ortholog of *L. japonicus* EPR3 receptor-like kinase remains to be determined.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *S. meliloti* 1021 strains (see Table S1 in the supplemental material) were grown at 30°C in LBMC medium (68), GMS (glutamate manniot salt medium) (41), M9 minimal medium (8), or Jensen’s plant medium with glutamate and manniot (27). Bacterial plates contained 1.5% Bacto agar (BD, Franklin Lakes, NJ). Cal- colfluor polysaccharide indicator plates contained 0.02% Calcolfluor white M2R (fluorescent brightener 28 Sigma, St. Louis, MO) (8). The antibi- otic concentrations were 1 mg/ml or 500 μg/ml streptomycin, 200 μg/ml neomycin, 25 μg/ml gentamicin, and 50 μg/ml spectinomycin.

**Construction of plasmids and *S. meliloti* mutant strains.** Restriction enzymes and polymeases were obtained from New England Biolabs (Ips- swich, MA). Primers were obtained from Eurofins MWG Operon (Hunts- ville, AL). Transductions were performed using phage φM12 (69). All strains, plasmids, and primers and the construction of strains are de- scribed in Table S1 in the supplemental material.

**Plant nodulation assays.** Host plant *Medicago truncatula* cv. “Jema- long A17” was prepared for inoculation with *S. meliloti* as previously described (68). Seedlings were moved to individual Jensen’s medium mi- crocosms and inoculated with *S. meliloti* of the appropriate strain as de- scribed previously (68). Plants were grown in a Percival AR-36L incubator (Perry, IA) at 21°C, with 60 to 70% relative humidity and 100 to 175 μmol m⁻² s⁻¹ light for 7 weeks.

**Detection of β-glucoronidase activity and imaging of roots and nodules.** β-Glucuronidase expression by bacteria was detected by staining whole roots in X-Gluc buffer (1 mM 5-bromo-4-chloro-3-indolyl-β-D- glucuronic acid, cyclohexylammonium salt; 0.02% SDS, 50 mM Na- phosphate [pH 7.1]) (70) for 48 h. Whole roots were imaged on an AZ100 Multi-Zoom microscope equipped with a DS-FI1, 5-megapixel color camera (Nikon Instruments, Melville, NY).

**Polysaccharide fractionation and quantification.** To isolate LMW polysaccharides, total culture supernatant from 5-day GMS cultures was collected by centrifugation for 20 min at 11,000 × g in a Beckman Avanti J-20XP centrifuge. Hexose-sugar-containing polysaccharide was quanti- fied by anthrone-sulfuric acid assays as described previously (27). The optical density at 620 nm (OD₆₂₀) of sample anthrone assays was com- pared to a 2-fold dilution series of glucose. Anthrone assays performed on the appropriate medium served as the blank. Culture supernatant was then vacuum filtered through a 0.2-μm pore filter, followed by pressure filtration through a 10-kDa filter in a stirred cell. After isolation of mate- rial ≤5 kDa, samples were freeze-dried and resuspended in deionized water. Insoluble material was removed by centrifugation. Samples were dialyzed against 0.125 M NaCl–0.125 M Na acetate buffer at 0.5 ml/min. Fractions of 1.2 ml were collected with 0.25 column volume discarded prior to starting fractionation. The average total OD₆₂₀ value measured by the anthrone-sulfuric acid assay for Superdex 30 fractions 10 to 65 from the wild type was 3.26. Sample from the exoY mutant and double glycans mutant 1325 and 1328 that had been size selected between 10 and 0.5 kDa (as described above) was diluted to 3.26 OD₆₂₀ anthrone assay units per ml. One-milliliter aliquots were run on the Superdex 30 column under the same conditions as the wild type.

**Glycosyl composition analysis.** Fractions 25 to 28 of all wild-type samples run on the Superdex 30 column were pooled and dialyzed against deionized water using a 1- to 0.5-kDa MMCO membrane and freeze- dried. Fractions 29 to 34 and fractions 36 to 41 from all wild-type samples were similarly pooled and dialyzed using a 0.5- to 0.1-kDa MMCO mem- brane. The same fraction pools were prepared from the exoY mutant and from double glycans mutant 1325 and 1328. Glycosyl composition analysis was performed by combined gas chromatography-mass spec- trometry (GC/MS) of alditol acetates (AAs) as previously described (71) at the University of Georgia Complex Carbohydrate Resource Center. Com- position analysis was performed using 300 to 500 μg of sample. As the internal standard, 20 μg inositol was added to samples. Samples were hydrolyzed in 2 M trifluoroacetic acid (TFA) for 2 h in a sealed tube at 121°C, reduced with NaBD₄, and acetylated using acetic anhydride-TFA. The resulting AAs were analyzed on an Agilent 7890A gas chromatograph interfaced with a 5975C MSD in electron impact ionization mode. Sepa- ration was performed on a 30-m Supelco SP-2331 bonded-phase fused silica capillary column.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00606-16/-/DCSupplemental.

Text S1, DOCX file, 0.1 MB.

Figure S1, TIF file, 2.4 MB.

Figure S2, TIF file, 2.4 MB.

Figure S3, TIF file, 2.4 MB.

Figure S4, TIF file, 2.4 MB.

Table S1, DOCX file, 0.2 MB.

Table S2, DOCX file, 0.1 MB.

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**REFERENCES**


