Role of the Glycanases ExoK and ExsH in Regulating the Molecular Weight of the *Rhizobium meliloti* Exopolysaccharide Succinoglycan

by

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ABSTRACT

The *Rhizobium meliloti* exopolysaccharide succinoglycan plays a crucial role in establishment of nitrogen fixing symbiosis between *R. meliloti* and alfalfa, and low molecular weight (LMW) forms of succinoglycan in particular are proposed to be symbiotically active. We have been interested in determining how *R. meliloti* produces LMW succinoglycan as part of a larger effort to understand the nature of the symbiotic role played by succinoglycan.

We report here that *R. meliloti* expresses two endo-1,3-1,4-beta-glycanases, ExoK and ExsH, that contribute in the production of LMW succinoglycan. We have determined that both glycanases accumulate and function extracellularly, and that secretion of ExsH (though not ExoK) is dependent upon the *prsD* and *prsE* genes, which encode the ABC-type transporter and membrane fusion protein, respectively, of a type I secretion system. Genetic analyses indicate that ExoK and ExsH cleave high molecular weight (HMW) succinoglycan to yield LMW succinoglycan in cultures.

Somewhat to our surprise, we observed that succinoglycan that has accumulated in cultures is a poor substrate for cleavage by purified ExoK and ExsH. However, heat-treatment of succinoglycan converts the polysaccharide to forms that are more susceptible to cleavage. By adding purified ExoK and ExsH to cultures of an *R. meliloti exoK exsH* mutant, we demonstrated directly that these glycanases can convert HMW succinoglycan to LMW forms, but that this conversion only occurs if *R. meliloti* cells are present and are actively synthesizing succinoglycan. Our results imply that succinoglycan undergoes a transition from a glycanase-susceptible form to a glycanase-refractory form as it accumulates in cultures. This transition may reflect changes in the conformation or aggregation state of succinoglycan molecules.

Additional genetic analyses revealed that the absence of the acetyl modification of succinoglycan increases the susceptibility of the nascent polysaccharide to cleavage, whereas the absence of the succinyl modification decreases the susceptibility of the nascent polysaccharide to cleavage.

We suggest several approaches for experimentally manipulating the molecular weight distribution of succinoglycan. We also propose a model for how the acyl modifications of succinoglycan and glycanases influence the molecular weight distribution of this symbiotically important polysaccharide.

Thesis Supervisor: Graham C. Walker
Title: Professor of Biology
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Chapter 1

Introduction
I. Nitrogen fixation: Background and significance

Nitrogen fixation, the conversion of dinitrogen gas to ammonia, is a process of fundamental importance to all organisms. Although dinitrogen gas comprises 78% of the earth's atmosphere and therefore is present in great abundance, this form of nitrogen is biologically inaccessible to most types of organisms because it is highly inert. In contrast, the product of nitrogen fixation, ammonia, can be assimilated readily by enzymatic pathways common to many types of organisms to yield amino acids, nucleotides, and other forms of biologically useful nitrogen. In terms of the nitrogen geochemical cycle, nitrogen fixation is the key limiting step in the generation of biologically useful nitrogen.

Nitrogen fixation can be mediated by biological, industrial, and natural, non-biological processes. Biological nitrogen fixation is accomplished by a group of evolutionarily diverse bacterial strains that share one distinguishing trait; they express the nitrogen fixing enzyme nitrogenase (119). Examples of nitrogen fixing bacterial strains include free-living bacteria (Azotobacter, Klebsiella, and Clostridium), cyanobacteria (Nostoc and Anabaena), and bacteria that fix nitrogen in symbiosis with plants of the legume family (Rhizobium, Bradyrhizobium, and Azorhizobium, collectively referred to as rhizobia) (119). Industrial nitrogen fixation is based on the Haber process, whereby dinitrogen gas and hydrogen gas are converted to ammonia on a metal catalyst at extremely high temperatures and pressures (108). Natural, non-biological nitrogen fixation is simply nitrogen fixation caused by lightning (151).

Since the discoveries of symbiotic nitrogen fixation by Hellriegel and Wilfarth in 1888 (121, 135) and of the Haber process by Fritz Haber in 1905 (108), researchers have focused on nitrogen fixation as a fascinating subject for both basic and applied research. Hellriegel and Wilfarth's research on the effect of varying the supply of fixed nitrogen to a variety of plants led them to a remarkable insight; certain soil bacteria can colonize organs, termed nodules, on the roots of legumes and can provide these legumes with a source of biologically useful nitrogen derived from the atmosphere (121, 135). Their research unveiled new systems for the study of complex microbial-plant interactions. Ironically, the sponsors of Hellriegel and Wilfarth's
research, members of the German sugar beet industry, were quite disappointed with the outcome of the research because it seemed irrelevant to the problem of improving sugar beet yields (135). However, the agricultural research community quickly grasped the possible applications of Hellriegel and Wilfarth's insight (121, 135). Cultivation of legumes in soil containing the appropriate nitrogen fixing bacterial strain alleviates the need to apply nitrogen fertilizer to these crops and even replenishes the supply of fixed nitrogen in the soil. Haber's discovery of a non-biological process for the conversion of dinitrogen gas to ammonia enabled researchers to initiate studies of the catalytic mechanism of this process (108). Although German industrialists first used the Haber process for the production of explosives during World War I (108), since the 1940's the more benevolent use of this technology in the industrial production of nitrogen fertilizer has become widespread (151).

Nitrogen fixation technologies have had a tremendous impact on human society and the environment in the past 100 years. In this period, nitrogen fixation technologies, which include both industrial production of nitrogen fertilizer and the deliberate cultivation of legumes for agricultural purposes, have come to account for almost half of the nitrogen fixed in terrestrial ecosystems (151). Naturally occurring biological nitrogen fixation accounts for most of the other half of the nitrogen fixed in terrestrial ecosystems (151), while lightning accounts for the balance of the nitrogen that is fixed (< 4%) (151). Nitrogen fixation technologies have yielded great benefits to society in terms of dramatically increasing global agricultural production, thus contributing to the "Green Revolution" (109, 151). However, nitrogen fixation technologies have also been associated with heavy environmental costs. Industrial nitrogen fixation is an extremely energy intensive process that is driven by, and thus dependent on, the consumption of non-renewable fossil fuels (151). Also, the dramatic increase in the rate of global nitrogen fixation associated with nitrogen fixation technologies is not being offset by increased rates of denitrification, or the conversion of nitrate to dinitrogen gas by bacteria in the soil (151). The consequences of the overload of fixed nitrogen in the environment are serious. Excess nitrates leach from the soil and cause damage to freshwater and marine ecosystems (151). Excess nitrous
oxide, which is a potent greenhouse gas, and nitric oxide, which contributes to tropospheric ozone production and acid rain, escape to the atmosphere (151). Much current applied research on nitrogen fixation is aimed toward maximizing agricultural output while simultaneously minimizing the introduction of fixed nitrogen into soil (23, 109). Basic research on symbiotic nitrogen fixation continues to provide new ideas that may prove useful in accomplishing these goals, while also generating new information that is interesting in unforeseen ways.

II. Symbiotic nitrogen fixation

Molecular basis for microsymbiont-host range specificity and nodule development

The fact that rhizobial strains and host plants exhibit a remarkable degree of specificity in terms of their ability to interact and establish a productive symbiosis has been recognized by researchers practically since the discovery of symbiotic nitrogen fixation, but significant progress in understanding the molecular basis of this specificity began only recently. Groups of closely related rhizobial strains typically interact exclusively with groups of closely related legumes (87). Examples of specific symbiotic partners include Rhizobium meliloti and alfalfa, Bradyrhizobium japonicum and soybean, Rhizobium leguminosarum bv. viciae and pea, R. leguminosarum bv. trifolii and clover, and R. leguminosarum bv. phaseoli and bean (87). Certain rhizobia that exhibit broad host ranges (e.g. Rhizobium spp. NGR234) and certain plants that can undergo symbiosis with many types of rhizobia (e.g. Arachis) have also been identified, but the lack of specificity associated with these rhizobia and host plants is uncommon (87).

Research on nitrogen fixing symbioses in the first half of the twentieth century was dominated by light-microscopy analyses of root nodule structures and biochemical analyses of the enzymatic activity of nitrogenase as purified from bacteria in nodules (27). While these analyses proved useful by encouraging researchers to think of the establishment of symbiosis as a series of discrete steps, they provided few clues about how microsymbionts and hosts interact at the molecular level. It was not until the application of molecular genetic techniques to the study of symbiosis in the late 1970's that researchers began to develop highly productive approaches
Steps in the establishment of nitrogen fixing symbioses

As viewed from a "pre-molecular biology" perspective, the establishment of symbiosis involves a series of steps in which the rhizobia and host plants interact and undergo distinct morphological changes (Fig. 1). The presence of rhizobia in close proximity to the root of a legume can trigger two distinct responses in a homologous host plant, the curling of root hair cells on the surface of roots near the root apical meristem and the initiation of cell divisions in the plant root cortex (87, 105). These responses are only triggered during homologous microsymbiont-host interactions, though, which implies that mechanisms for prevention of heterologous microsymbiont-host interactions function from the earliest stages of symbiosis (87, 105). Rhizobia can trigger these responses without making physical contact with the plant, implying the involvement of diffusible rhizobial signals, termed Nod factors, in this process (87, 149). Over the course of one to several weeks, the dividing plant cells gradually give rise to a root nodule (87). At the time of initiation of nodule development, rhizobia migrate toward the plant root and colonize the curled tips of the root hair cells (87). For any given colonized, curled root hair, an invagination can form at the curled tip (87). The invagination develops into a tube, termed an infection thread, that grows within the root hair cell and extends into the developing nodule (87). Rhizobia travel within the growing infection thread and are eventually released into plant cells located within the developing nodule (87). Upon release into the plant cells, the rhizobia become surrounded by a special membrane of plant origin, such that rhizobia do not actually come in contact with the plant cell cytosol (87). The rhizobia then differentiate to their bacteroid form [so named by researchers who initially thought that these bacteria-like structures actually corresponded to endogenous components of plant cells (121)] (87). The bacteroids proceed to express nitrogenase and fix nitrogen (87).

This general description applies well to symbioses established between a wide range of rhizobial strains and legumes; different symbioses have much in common. One characteristic
that distinguishes symbioses, though, is whether or not the meristem of root nodules is fated to
terminally differentiate (87, 118). Legumes can be classified according to whether they form
indeterminate nodules, for which the meristem does not terminally differentiate, or determinate
nodules, for which the meristem does terminally differentiate (118).

Indeterminate nodules are typically cylindrical and have a meristem located at their distal
tip (87, 118). The meristem continuously divides and generates new proximal cells, so
indeterminate nodules continuously grow at their distal tip (118). A lateral cross section of an
indeterminate nodule thus represents different stages in nodule development, with the distal
region of the nodule representing earlier stages and the proximal region representing later stages
(118) (Fig. 1). In the region of the nodule just proximal to the meristem, rhizobia are released
from infection threads into the new plant cells (118). In the more proximal central region of the
nodule, the rhizobia in plant cells differentiate to their bacteroid form and fix nitrogen (118). In
the most proximal region of older nodules, the plant cells senesce and rhizobial nitrogen fixation
ceases (118). Examples of legumes that form indeterminate nodules are alfalfa, pea, and clover
(118).

Determinate nodules are typically spherical and have a central meristem that divides a
limited number of times and then terminally differentiates (87, 118). Some of the differentiated
plant cells continue to divide. When plant cells that are already infected with rhizobia divide
they give rise to new plant cells that are also infected with rhizobia (118). Examples of legumes
that form determinate nodules are bean and soybean (118).

**Rhizobial mutants can be used to analyze steps in the establishment of symbiosis**

With the application of molecular genetic techniques to the study of symbiosis, it became
possible to analyze the steps of symbiosis at a much higher level of resolution. In a ground-
breaking study, Meade *et al.* (110) used a transposon mutagenesis technique to generate a pool of
*R. meliloti* mutants and then screened these mutants to isolate those that exhibit symbiotic
defects. Meade *et al.* (110) identified two classes of mutants, those that were blocked in the
induction of nodules and those that could successfully induce nodules but that failed to fix nitrogen. Meade et al. (110) were able to correlate these symbiotic defects with specific transposon mutations, enabling the cloning and characterization of bacterial genes that play crucial roles at particular steps in symbiosis. Since then, researchers have used variations on this general approach to isolate numerous additional rhizobial mutants blocked at these and other steps in symbiosis (24, 133). For example, mutants have been isolated that are defective in the initiation of nodule development (nod, nol) (104), nodule invasion (exo, exp, rkp, ndv) (65, 94, 104, 129), survival upon release into plant cells (bacA) (64), and nitrogen fixation (nif, fix) (42, 111).

Analyses of bacterial mutants that are blocked in the earliest steps in symbiosis have proved invaluable in efforts to unravel the complex molecular basis of microsymbiont-host range specificity. These efforts have revealed that rhizobial lipochitooligosaccharides (LCOSs) are the biologically active component of Nod factors and that LCOSs serve as the primary determinants of host range specificity in symbiosis (105). These efforts have also revealed that distinct rhizobial extracellular polysaccharides (EPSs) play a crucial role in mediating root nodule invasion in particular legumes (21, 31, 34, 94) and that EPSs may also serve as determinants of host range specificity (46). At this point a much clearer understanding exists with regard to the symbiotic functions of LCOSs than of EPSs. It is now generally accepted that LCOSs function as bacterial signals that are detected by plants (105). The signaling roles played by LCOSs serve as one model for understanding how EPSs may also function in symbiosis.

III. Rhizobial lipochitooligosaccharides trigger nodule initiation and serve as the primary determinants of host range specificity in nitrogen fixing symbioses

Research on rhizobial LCOSs exemplifies the use of molecular genetic techniques as a starting point for the eventual structural determination of molecules that cause discrete biological effects. The rhizobial nod mutants isolated by Meade et al. (110) and other groups (24, 133) in the early 1980's were presumed to be defective in production of diffusible, nodule inducing
factors (149), termed Nod factors, but there was no indication of the chemical identity of the Nod factors. Comparisons of nod genes that had been identified and cloned from diverse rhizobial strains revealed that nod genes come in two varieties, common and host specific (105). Common nod genes (nodABC) are highly conserved and are ubiquitous among rhizobial strains, whereas host-specific nod genes differ between different rhizobial strains (105). Analyses of transcriptional regulation of nod genes in R. meliloti revealed that the nod genes are normally transcriptionally silent, but that cultivation of the bacterium in the presence of root exudate from homologous host plants results in high levels of nod gene transcription (89). These observations led to the discovery that specific flavonoids induce nod gene transcription via the flavonoid-binding transcriptional activator NodD (89). This improved understanding of the regulation of nod gene expression was instrumental in accomplishing the production and purification of Nod factor in quantities sufficient for structural analyses, culminating in the report of Lerouge et al. (96) in 1990 that LCOSs are the biologically active component of Nod factor. Lerouge et al. (96) demonstrated that LCOSs produced by R. meliloti specifically elicit root hair deformation from the homologous host alfalfa, but not from the heterologous host vetch, when added in nanomolar concentrations.

**Rhizobial LCOSs are involved in host range determination**

LCOSs are directly involved in determining the host range of rhizobial strains; differences in the structure of LCOSs produced by different rhizobial strains largely account for the ability of strains to induce the initiation of nodule development on homologous host plants but not on heterologous host plants (105). Comparisons of LCOSs produced by diverse rhizobial strains have revealed the shared motif of a short, highly modified chitooligosaccharide chain (105). LCOSs produced by different strains vary in terms of the length of the chitooligosaccharide chain (typically 3 to 5 sugar units), the length and degree of saturation of an N-acyl modification at the non-reducing end of the chitooligosaccharide, and the presence of additional modifications (e.g., sulfate, methyl fucose, acetate, glycerol) at the reducing and non-
reducing ends of the chitooligosaccharide chain (105). Most rhizobial strains produce only one LCOS or a few structurally similar LCOSs, and in general different rhizobial strains produce structurally different LCOSs (105). By testing the nodulation phenotypes of recombinant rhizobial strains (19, 45, 142) and by synthesizing modified LCOSs in vitro (144) it has been possible to correlate subtle differences in the structure of LCOSs with dramatic differences in their biological activity (105).

**Rhizobial LCOSs may function as molecular signals**

LCOSs have been proposed to function as molecular signals that are recognized with a high degree of specificity by receptors on the surface or inside of plant cells (105). This idea is supported by the observations that purified LCOSs can trigger morphological changes and developmental responses in plants in the absence of rhizobia and that LCOSs can function at extremely low concentrations (105). LCOS-mediated nodulation is likely to be more complicated than a simple one signal/one receptor system, though (105). A study by Ardourel *et al.* (2), based on the use of a series of rhizobial strains exhibiting increasing defects in acylation of LCOSs, indicated that a wider range of structural variants of LCOSs is able to induce the initiation of root cortical cell divisions than is able to mediate successful establishment of symbiosis. Ardourel *et al.* (2) speculated that plants express at least two receptors for LCOSs, a low stringency receptor that triggers nodule initiation in preparation for symbiosis, and a high stringency receptor that acts as a checkpoint to guard against interactions with inappropriate rhizobial strains at a subsequent step during the course of nodule development.

Much effort is currently being focused on determining how LCOSs trigger developmental responses in homologous host plants. So far there have been no reports of plant receptors proven to mediate the biological effects of LCOSs (105), but a mutant strain of pea, termed *sym2*, is a promising candidate for a host plant that expresses a receptor with an altered specificity for LCOSs (60). Plant mRNAs that are expressed in response to LCOSs have been cloned and characterized as a means of gaining insights into how plant cells respond to LCOSs (105). Also,
a recent report by Erhardt et al. (53), based on the use of calcium-binding dyes, indicates that LCOSs derived from *R. meliloti*, but not LCOSs derived from *R. leguminosarum* or unmodified chitooligosaccharides, can trigger intracellular spikes of calcium in alfalfa root hair cells. Ultimately these types of analyses should provide a clearer picture of the molecular processes that occur downstream of LCOS signaling during nodule development.

Research on LCOSs may prove useful for understanding signaling that occurs in a wide variety of other contexts. For example, LCOSs derived from a rhizobial strain, as well as preparations of a plant-derived chitinase, were found to restore embryonic development to a temperature-sensitive mutant carrot cell line (39, 40). Also, chemically synthesized LCOSs have been shown to enhance tobacco protoplast division rates (131). These observations suggest the possibility that LCOSs may play a role in plant development outside the context of nodulation. Furthermore, genes that encode products homologous to the rhizobial chitooligosaccharide synthase NodC have been cloned from *Xenopus*, zebrafish, and mouse (138, 139). For *Xenopus* and zebrafish, the expression of these genes can be detected early in embryogenesis and the production of chitooligosaccharides can be detected in cell extracts derived from embryos (139). In addition, injection of fertilized zebrafish eggs with antisera that block the activity of these chitooligosaccharide synthases causes severe defects in embryogenesis (4). These observations suggest the possibility that chitooligosaccharides may also play a role in animal development.

IV. **Rhizobial exopolysaccharides may function as signals in mediating rhizobial invasion of root nodules**

Research on rhizobial EPSs exemplifies the use of molecular genetic approaches as a tool to test molecules of known structure for potential biological activities. In the early 1980's, rhizobial EPSs were considered one of many possible candidates for rhizobial factors involved in nodule initiation (1), given that rhizobial strains typically produce copious amounts of EPS (at least during cultivation in the lab) and given that EPSs produced by different rhizobial strains are structurally diverse (29). In the mid 1980's, several research groups, including the Walker lab,
generated rhizobial mutants defective in production of EPSs as a means to test this idea (21, 31, 34, 94). These groups discovered that the production of EPS is crucial for establishment of effective symbiosis for certain rhizobial strains, such as *R. meliloti* (94), *R. leguminosarum* bv. *viciae* (21), *R. leguminosarum* bv. *trifolii* (31), and *Rhizobium* spp. NGR234 (34), though not due to a role of EPS in the initiation of nodule development. Mutants of these rhizobial strains that are blocked in EPS production actually can induce the formation of nodules on the roots of homologous host plants, but these nodules lack infection threads and generally are devoid of bacteria (21, 31, 34, 94). EPS deficient mutants can successfully enter individual nodules at a very low frequency, but in these cases the rhizobia are generally localized intercellularly rather than intracellularly (94). Thus for these rhizobial strains, EPS is crucial for infection thread development and for rhizobial invasion of root nodules through infection threads (94).

**Rhizobial EPS seems to be required for host plants that form indeterminate nodules**

Rhizobial EPS is not required in all symbioses. In contrast to the symbioses established between *R. meliloti* and alfalfa, *R. leguminosarum* bv. *viciae* and pea, and *R. leguminosarum* bv. *trifolii* and clover, which all require rhizobial EPS (21, 31, 94), the symbioses established between *R. leguminosarum* bv. *phaseoli* and bean (21) and *B. japonicum* and soybean (91) do not require rhizobial EPS. Infection thread formation is an integral part of the normal invasion process in all cases (87); thus, mechanisms for nodule invasion that are independent of infection thread formation do not account for the difference between the two groups.

The type of nodules formed by a particular legume turns out to be a good predictor of whether that legume requires rhizobial EPS for establishment of symbiosis (87). In general, plants that form indeterminate nodules (such as alfalfa and pea) exhibit a requirement for rhizobial EPS, whereas plants that form determinate nodules (such as bean and soybean) do not (87). For example, Hotter and Scott (81) tested the symbiotic proficiency of EPS deficient mutants derived from a strain of *Rhizobium loti*, and determined that these mutants fail to establish symbiosis with the homologous host *Leucaena leucocephala*, which forms
indeterminate nodules. However, the same mutants succeed in the establishment of symbiosis with another homologous host, *Lotus pedunculatus*, which forms determinate nodules. One exception to this rule regarding the determinance fate of nodules and the requirement for rhizobial EPS has been reported though; Djordjevic et al. (46) observed that EPS deficient mutants of the broad host range strain *Rhizobium* spp. NGR234 fail to form effective symbiosis with the homologous host siratro, even though this host plant forms determinate nodules.

**Specific structural features of rhizobial EPSs are crucial for their symbiotic functions**

Specific structural features seem to be crucial for the symbiotic function of EPSs. For example, the structurally distinct polysaccharides produced by *Rhizobium* spp. NGR234 and *R. leguminosarum* bv. *trifolii* are not functionally interchangeable for establishment of symbiosis by EPS deficient mutants of these strains on their homologous hosts (46). Furthermore, *R. meliloti* mutants that produce forms of EPS that are altered in terms of their molecular weight and acyl substituents exhibit symbiotic defects similar to those of strains that are completely blocked in EPS production (as described in greater detail below) (69, 93).

**Possible roles for rhizobial EPSs in symbiosis**

Determining precisely how rhizobial EPSs mediate root nodule invasion has been, and remains, a central challenge in the field of research on rhizobial EPSs. One possibility is that EPSs play a physical role, for example by contributing a crucial structural component to the molecular matrix inside infection threads or by providing the microsymbiont with a barrier against plant defense responses (87). However, it is difficult to reconcile a physical role for EPSs with the apparent symbiotic specificities of EPSs produced by diverse rhizobial strains and with the apparent lack of a requirement for EPSs for particular symbioses. A second, more attractive possibility is that rhizobial EPSs act as receptor-mediated signals that promote plant developmental responses or that inhibit or modulate plant defense responses. Signaling roles for EPSs would be consistent with their symbiotic specificities, and there are already precedents for
thinking that carbohydrates can function as signals in plants (105). A signaling mechanism for EPSs based on triggering a developmental response would be analogous to the role of LCOSs in triggering root hair curling and the initiation of nodule development (105). A signaling mechanism for EPSs based on modulating plant defense responses would be similar in principle, though opposite in effect, to the role of heptaglucosyl fragments derived from the cell walls of fungal pathogens in triggering defense responses in infected host plants (140, 141). Clearly, research on this topic is still at an early stage. One approach that may help to advance understanding of the role of EPSs in symbiosis is to focus on determining which structural features of specific EPSs are crucial for their symbiotic activities. The Walker lab and others have invested great effort in conducting these types of analyses with regard to symbiotically relevant polysaccharides produced by *R. meliloti*.

V. Succinoglycan, the major exopolysaccharide produced by *R. meliloti*, is a useful model system for studying the symbiotic roles of exopolysaccharides

Succinoglycan is the major EPS produced by *R. meliloti*, and as such it was the focus of the first studies on a potential symbiotic role for *R. meliloti* EPS in symbiosis. Succinoglycan is a polymer of an octasaccharide repeating unit, consisting of galactose, glucose, acetate, succinate, and pyruvate, in a ratio of approximately 1:7:1:1:1 (1, 83). The structure of the carbohydrate backbone (1, 83) and the positions of the noncarbohydrate substituents (123) have been determined (Fig. 2).

The first genetic screen aimed specifically at isolating *R. meliloti* mutants defective in production of succinoglycan was based on the use of the fluorescent dye Calcofluor, which binds to many types of polysaccharides containing β-1,4 linkages, including succinoglycan, and fluoresces brightly when bound (94). Leigh *et al.* (94) determined that colonies of the wild-type strain Rm1021 fluoresce brightly when cultivated on growth medium containing Calcofluor and visualized under UV-light, due to Calcofluor binding to the succinoglycan at the surface of these colonies. To isolate mutants defective in the production of succinoglycan, Leigh *et al.* (94)
conducted transposon mutagenesis of the wild-type strain and screened for mutants that form non-fluorescing colonies: 26 non-fluorescing colonies comprising six complementation groups were isolated (94). Leigh et al. (94) determined that each mutant that is blocked in production of succinoglycan is also blocked in root nodule invasion, thus establishing a strong correlation between the two phenotypes. These results were consistent with the contemporaneous report of Finan et al. (57), that particular spontaneous mutants of *R. meliloti* that had been isolated based on the loss of epitopes to specific monoclonal antibodies were also defective in both the production of succinoglycan and in the establishment of symbiosis. At this point the Walker lab and others independently initiated thorough genetic analyses of the pathway for biosynthesis of succinoglycan in an effort to identify structural features of succinoglycan that are crucial for its symbiotic activity, and, ultimately, to determine the precise role that succinoglycan plays in the establishment of symbiosis.

**Model for synthesis of the octasaccharide repeating unit of succinoglycan**

A comprehensive model describing the steps in synthesis and modification of the octasaccharide repeating unit of succinoglycan has been proposed (126), based on a combination of biochemical and genetic analyses. Tolmasky et al. (148) established a foundation for understanding the steps in succinoglycan biosynthesis from a biochemical perspective by developing a system for isolation and analysis of radiolabelled, lipid-linked repeating units of succinoglycan biosynthesis. This system is based on the introduction of UDP-[14C]-galactose into *R. meliloti* cells by use of a "freeze-thaw" method followed by incubation of cells to enable incorporation of radiolabel into biosynthetic intermediates of succinoglycan (148). Lipid-linked biosynthetic intermediates are recovered from aliquots of cells by an organic extraction technique, and the intermediates are released from the lipid carrier by acid hydrolysis (148). The structure of free intermediates can then be determined by thin layer chromatography and additional analytical techniques (148). To increase the efficiency of incorporation of radiolabel and to prevent conversion of UDP-galactose to UDP-glucose inside cells, Tolmasky et al. (148)
used an *R. meliloti* strain defective in expression of UDP-glucose-4-epimerase activity. By this approach, Tolmasky *et al.* (148) determined that the first step in octasaccharide synthesis is the addition of UDP-galactose to a lipid carrier, and that subsequent steps entail the addition of seven UDP-glucose residues to yield a linear octasaccharide repeating unit.

The genetic analysis of succinoglycan production was greatly facilitated by the discovery that most of the genes (termed *exo*) involved in the synthesis and processing of succinoglycan, as well as genes involved in the regulation of succinoglycan production, are linked (94, 103). The linkage of *exo* genes first became apparent based on the results of complementation tests; particular cosmid clones derived from an *R. meliloti* genomic DNA library can restore succinoglycan production to *exo* mutants that belong to distinct complementation groups (94, 103). Subsequent molecular analyses confirmed that the restoration of succinoglycan production in these strains corresponds to true complementation rather than multi-copy suppression by a non-linked gene (103). Long *et al.* (103) conducted intensive mutagenesis of the genomic DNA spanning these linked *exo* genes and identified a total of 12 complementation groups representing three classes of mutants: 1) mutants that are completely blocked in succinoglycan production (*exoA, exoB, exoF, exoL, exoM, exoP*, and *exoQ*), 2) mutants that produce succinoglycan but exhibit unusual Calcofluor phenotypes (*exoH, exoK*, and *exoN*), and 3) mutants that exhibit altered regulation of succinoglycan production (*exoG* and *exoJ*) (103). Determination of the nucleotide sequence of the entire *exo* region ultimately revealed the presence of 19 genes (10, 11, 12, 25, 67, 68, 114). Most of the original complementation groups correspond to single genes, and, in most cases, the genes were named according to the corresponding complementation groups (10, 11, 12, 25, 67, 68, 114) (Fig. 3). However, the *exoG* complementation group was renamed *exoX* (122), and the *exoJ* complementation group was found to correspond to the intergenic region between the *exoX* and *exoY* genes (122). Further mutant analyses indicated that mutants defective in the *exoW* or *exoV* genes are blocked in production of succinoglycan (67, 68) and that mutants defective in the *exoZ* gene exhibit a delay in the onset of Calcofluor fluorescence (25, 125). Nucleotide sequence analyses provided some clues regarding the
possible functions of the 19 exo genes by revealing, for example, which genes are likely to encode glycosyl transferases involved in synthesis of the octasaccharide repeating unit of succinoglycan (10, 11, 12, 67). However, the conventional approaches of analyzing Calcofluor phenotypes of colonies and quantifying extracellular carbohydrate produced by mutants offered little promise in helping to order the exo genes according to their roles in biosynthesis of succinoglycan repeating units.

Reuber and Walker (126) used an elegant approach to solve this problem; they used the radiolabelling approach developed by Tolmasky et al. (148) to isolate and analyze the intermediates of succinoglycan biosynthesis that accumulate in particular exo mutant strains, thus enabling identification of the biosynthetic steps that are blocked in many of the exo mutants (126). For these experiments, an exoB exoR strain was used as the wild-type control (126). The exoB (also termed galE) mutation blocks UDP-glucose-4-epimerase activity (25, 28), which enables highly specific labeling of intermediates of succinoglycan biosynthesis (148), and the exoR mutation causes up-regulation of succinoglycan synthesis (47), which causes increased production of the radiolabeled biosynthetic intermediates (126). Individual exo mutations were then transduced into an exoB exoR strain, and the lipid-linked biosynthetic intermediates that accumulate in mutant backgrounds were isolated and analyzed (126). It is important to note that the "freeze-thaw" technique disrupts the ability of R. meliloti cells to polymerize succinoglycan repeating units (126); thus, the analysis of Reuber and Walker (126) provided specific information regarding the order of steps in succinoglycan biosynthesis up to but not beyond completion of the octasaccharide repeating unit (126).

Based on this type of analysis and based on the nucleotide sequence of the exo genes, Reuber and Walker (126) proposed a comprehensive model describing the steps of octasaccharide synthesis (Fig. 4). According to this model, the ExoY glycosyl transferase catalyzes the first step in octasaccharide synthesis, the transfer of UDP-galactose to a lipid carrier (126). This step also depends on the ExoF protein, though the precise role played by ExoF is not known (124). The subsequent additions of six UDP-glucose residues to the lipid-linked
Saccharides are catalyzed individually by ExoA, ExoL, ExoM, ExoO, ExoU, and ExoW, in that order (124). The identity of the glycosyl transferase responsible for the addition of the final UDP-glucose residue is not known (126). The ExoZ, ExoH, and ExoV proteins were proposed to mediate the addition of the acetyl, succinyl, and pyruvyl modifications, respectively (93, 125, 126). These modifications are added as the octasaccharide repeating unit is being synthesized (126). Interestingly, exoV mutants, but not exoZ or exoH mutants, are blocked in biosynthesis of the succinoglycan polymer, suggesting that pyruvylation of the octasaccharide repeating unit may be crucial for the subsequent polymerization of succinoglycan or that ExoV may play an additional role in succinoglycan biosynthesis (68). The ExoP, ExoQ, and ExoT proteins were proposed to play roles in polymerization and transport of succinoglycan (126), based on the observations that exoB exoR exoP, exoB exoR exoQ, and exoB exoR exoT mutants each produce normally-modified octasaccharide repeating units when analyzed by the radiolabelling technique (126), whereas exoP, exoQ, and exoT mutants produce little or no polymer of succinoglycan when analyzed by conventional techniques (68, 103).

**Low molecular weight forms of succinoglycan may be symbiotically active**

Along with the development of a model for synthesis of succinoglycan, much effort was focused on determining whether particular structural features of succinoglycan are crucial for the symbiotic activity of the polysaccharide. One aspect of the structure of succinoglycan that has emerged as being important in determining symbiotic activity is the degree of polymerization of polysaccharide chains (8, 93, 150). *R. meliloti* has the capacity to produce succinoglycan in low molecular weight (LMW) forms and high molecular weight (HMW) forms that are easily separable by gel filtration chromatography or ethanol precipitation techniques (92). There is currently some debate about the precise composition of LMW succinoglycan. Previously reported results of reducing end analyses indicate that LMW forms consist of monomers, trimers, and tetramers of the octasaccharide repeating unit (8), but the results of recent mass spectrometry analyses indicate instead that LMW forms consist of monomers, dimers, and trimers of the
octasaccharide repeating unit (Lai-Xi Wang, personal communication). The HMW forms of succinoglycan consist of substantially longer multimers of repeating units (8); gel filtration chromatography analyses indicate that HMW succinoglycan samples exhibit an average molecular weight on the order of 10^6 daltons, which suggests that single molecules of HMW succinoglycan may contain hundreds to thousands of octasaccharide repeating units (36). When grown in a particular minimal medium (termed GMS), the wild-type strain produces approximately half of its succinoglycan in HMW forms and half in LMW forms (92). The osmolarity of growth media can dramatically affect this molecular weight distribution though; with increasing osmolarity, *R. meliloti* exhibits a shift toward production of more HMW succinoglycan and less LMW succinoglycan (22). As described below, several lines of evidence suggest that LMW forms of succinoglycan, but not HMW forms of succinoglycan, specifically promote root nodule invasion, and that these LMW forms of succinoglycan may function as signals in mediating this process (8, 93, 150).

**exoH gene is required for production of LMW succinoglycan**

Prior to the large scale screen for the identification of *exo* genes conducted by Long *et al.* (103), *exoH* mutants had already been isolated by another approach (93). Leigh *et al.* (93) isolated a series of allelic mutants, based on their unusual Calcofluor phenotype, and designated these mutants *exoH*. Colonies of *exoH* mutants fluoresce brightly like wild-type colonies (when cultivated on growth medium supplemented with Calcofluor and visualized under UV-light) but fail to produce the fluorescent halos (termed Calcofluor halos) that are characteristic of wild-type colonies (93). Calcofluor halos are presumed to correspond to diffusible forms of succinoglycan (93).

Interestingly, *exoH* mutants exhibit a defect in invasion of root nodules that is of the same severity as the defect associated with those *exo* mutants that are completely blocked in production of succinoglycan (93, 94). This is the case, despite the fact that *exoH* mutants produce seemingly normal levels of Calcofluor binding polysaccharide (93). This observation
suggests the possibility that \textit{exoH} mutants produce altered, symbiotically inactive forms of succinoglycan. Leigh \textit{et al}. (93) determined that the polysaccharide produced by \textit{exoH} mutants contains glucose and galactose in a ratio of 7:1, like normal succinoglycan, implying that \textit{exoH} mutant succinoglycan has the same carbohydrate backbone as wild-type succinoglycan. However, the succinoglycan produced by the \textit{exoH} strain completely lacks the succinyl modification that is present once per repeating unit on normally-modified succinoglycan (93) (Fig. 4). Furthermore, Leigh and Lee (92) determined that \textit{exoH} mutant succinoglycan accumulates in cultures almost entirely in HMW forms; thus, \textit{exoH} mutants exhibit a defect in production of LMW succinoglycan (92). These observations suggest the possibility that there may be a causal link between the absence of the succinyl modification on succinoglycan and the failure of a strain to produce LMW succinoglycan and that there may be an additional causal link between the failure of a strain to produce LMW succinoglycan and the failure of the strain to produce a Calcofluor halo (92, 93). Furthermore, these results suggest that succinylation of succinoglycan and/or production of LMW succinoglycan are crucial for successful root nodule invasion (92, 93).

\textbf{The \textit{exoK} gene encodes an endo-1,3-1,4-\textbeta- glycanase}

The extensive genetic analysis conducted by Long \textit{et al}. (103) revealed a second group of allelic mutants, designated \textit{exoK}, that exhibit another unusual Calcofluor phenotype. Colonies of \textit{exoK} mutants fluoresce brightly but they produce a Calcofluor halo with a delay relative to wild-type colonies (103). Nucleotide sequence analyses revealed that the \textit{exoK} gene encodes a protein homologous to bacterial endo-1,3-1,4-\textbeta-glycanases (10, 68); this enzymatic activity would be predicted to depolymerize succinoglycan to yield monomers or multimers of the octasaccharide repeating unit, structurally identical to repeating units produced during succinoglycan biosynthesis (148). The presence of a gene encoding a putative succinoglycan depolymerase within a cluster of genes implicated in succinoglycan biosynthesis suggests the possibility that this depolymerase may play some role in the processing of succinoglycan (10, 68). The delayed
Calcofluor halo phenotype of \textit{exoK} mutants suggests that ExoK normally contributes to production of Calcofluor halos, presumably by depolymerizing HMW succinoglycan to yield forms that are sufficiently short to diffuse from colonies (10, 68). Taken together, these analyses suggest that ExoK is a good candidate for a protein involved in production of LMW succinoglycan. However, \textit{exoK} mutants are symbiotically proficient, implying that ExoK is not crucial for symbiosis (103).

\textbf{Succinoglycan can mediate invasion in trans}

Klein \textit{et al.} (88) helped to refine understanding of the symbiotic role of succinoglycan by determining that succinoglycan can function \textit{in trans} to facilitate root nodule invasion. They conducted coinoculations of \textit{exo}+ and \textit{exo} mutant strains on alfalfa and determined that the presence of \textit{exo}+ strains is sufficient to suppress the invasion defect associated with those \textit{exo} mutants that are defective in synthesis of succinoglycan (88). These results imply that succinoglycan that is being produced by \textit{exo}+ cells is capable of mediating invasion by \textit{exo} cells that are not synthesizing succinoglycan (88). Of the various models for the function of rhizobial EPS in symbiosis, these results are perhaps easiest to reconcile with a signaling role for succinoglycan.

\textbf{LMW forms of succinoglycan are capable of mediating symbiosis in trans}

Subsequent analyses conducted by Battisti \textit{et al.} (8) lend support to both the idea that LMW forms of succinoglycan are symbiotically active and the idea that succinoglycan functions as a signal that mediates root nodule invasion. Battisti \textit{et al.} (8) determined that a fraction of LMW succinoglycan, tentatively identified as highly charged tetramers of the octasaccharide repeating unit based on reducing end analyses, could restore root nodule invasion by \textit{exoH} mutants and by various \textit{exo} mutants that are defective in synthesis of succinoglycan. In contrast, HMW succinoglycan was not capable of mediating root nodule invasion (8). The observation that even very low concentrations of LMW succinoglycan (1 \textmu M) were effective in mediating
root nodule invasion again suggests that succinoglycan mediates root nodule invasion by a signaling mechanism (8).

VI. *R. meliloti* has the capacity to produce two additional symbiotically relevant polysaccharides

The extensive characterizations of *R. meliloti* have revealed that this strain has the capacity to produce several structurally diverse, symbiotically active polysaccharides, instead of just one type of polysaccharide. In addition to succinoglycan, *R. meliloti* can produce a second EPS, termed EPS II (65), and a capsular polysaccharide, termed KPS (128). Like the case for succinoglycan, EPS II and KPS can be produced in symbiotically active or inactive forms (65, 86, 128), and the symbiotically active forms seem to correspond to LMW forms of these polysaccharides (69, 129). Production of the symbiotically active forms of any one of the three polysaccharides alone is sufficient to mediate root nodule invasion (8, 65, 69, 94, 129).

The capacity of *R. meliloti* to express three parallel pathways for production of symbiotically active polysaccharides might easily have confounded analyses of the symbiotic functions of any single one of these polysaccharides. However, it turned out quite serendipitously that the wild-type *R. meliloti* strain used in the initial genetic analyses of EPS production, Rm1021, is repressed for synthesis of EPS II (65) and is capable of producing KPS only in symbiotically inactive forms (129). It is therefore dependent on production of succinoglycan in symbiotically active forms for efficient nodule invasion (94). In retrospect it is clear that these particular characteristics of Rm1021 greatly facilitated the initial discovery that succinoglycan plays an important role in symbiosis (94). The capacity of *R. meliloti* strains to produce symbiotically active forms of EPS II and KPS (depending on the genetic background of the strain) only became apparent through subsequent research (65, 128).

Low molecular weight forms of EPS II are sufficient to mediate root nodule invasion

The latent capacity of Rm1021 and the virtually identical strain Rm2011 to produce EPS
II was discovered independently in several different contexts. The wild-type strain produces EPS II when it carries the cosmids pEX1 or pEX2, both of which were isolated from an *R. meliloti* genomic DNA library (65). Strains containing the spontaneous mutation expR101 (65) or transposon mutations in the *mucR* gene (86, 106) also produce EPS II. Genetic analyses indicate that the partially overlapping genomic DNA fragments cloned in pEX1 and pEX2 span a cluster of genes, designated the *exp* region, that are implicated in the synthesis of EPS II (16, 65). The presence of extra copies of either the *expA* or *expE* gene seem to be sufficient to upregulate EPS II synthesis in an otherwise wild-type strain (65). MucR is a DNA binding protein involved in repression of *exp* gene expression (17, 18, 86). It has not been determined yet how the *expR101* mutation causes increased production of EPS II.

The structure of EPS II has been determined (79). The polysaccharide is a polymer of a modified disaccharide repeating unit, consisting of galactose, glucose, acetate, and pyruvate in a ratio of 1:1:1:1 (79). Thus, EPS II is structurally distinct from succinoglycan. The synthesis of succinoglycan and EPS II are accomplished by the products of different sets of genes (*exo* versus *exp*) (65, 103). However, the *exoB* UDP-glucose-4-epimerase gene is required for production of both polysaccharides, since both succinoglycan and EPS II contain galactose (1, 65, 79).

A thorough genetic analysis of EPS II synthesis strongly implicates LMW forms of EPS II in mediating successful root nodule invasion (69). The introduction of the *expR101* mutation (by use of a transductionally linked transposon as a selectable marker) into an *exo* strain that produces no succinoglycan restores the ability of the *exo* mutant to invade nodules, and this restoration depends on the ability of the strain to produce EPS II (65). In contrast, introduction of the *mucR* mutation into an *exo* strain does not restore symbiotic proficiency to the mutant (86). To determine the basis of the difference between the symbiotic phenotypes of *expR101 exo* and *mucR exo* mutants, González et al. (69) compared the structures of the EPS II produced by the two strains. Although no differences in terms of substituents or linkages were detected, a clear difference in terms of the molecular weight distribution of EPS II was observed (69). The *expR101* strain produces both LMW and HMW EPS II, whereas the *mucR* strain produces only
HMW EPS II, suggesting that LMW forms of EPS II are the symbiotically active forms of this polysaccharide too (69). González et al. (69) confirmed this point directly, by testing the ability of LMW and HMW fractions of EPS II to restore invasion by exo and exo mucR mutants (69). As expected, LMW forms of EPS II, but not HMW forms of EPS II, are capable of mediating invasion (69).

**Low molecular weight forms of KPS may also be symbiotically active**

A remarkably similar story also seems to be developing with regard to the R. meliloti capsular polysaccharide KPS. The ability of R. meliloti to produce a symbiotically active capsular polysaccharide was discovered by Reuhs et al. (128), based on the observation that the exoB mutant AK631, derived from the wild-type strain Rm41, is capable of invading nodules despite being blocked for synthesis of both succinoglycan and EPS II (158). In contrast, exoB mutants derived from the wild-type strain Rm1021 had previously been shown to be blocked in establishment of symbiosis (94). Reuhs et al. (129) reconciled these apparently contradictory results upon determining that both Rm41 and Rm1021 have the capacity to produce KPS, but that the rkpZ (previously termed lpsZ) gene is specifically required for production of KPS in symbiotically active forms. The rkpZ gene is present in the Rm41 genome but absent in the Rm1021 genome (158). Introduction of the rkpZ gene into exoB mutants of Rm1021 restores symbiotic proficiency to this strain (129, 157). Furthermore, the presence of the rkpZ gene correlates with the ability of R. meliloti strains to produce LMW forms of KPS, suggesting that LMW forms are the symbiotically active forms of this polysaccharide (127, 129).

**VII. Two possible mechanisms for production of low molecular weight succinoglycan: direct synthesis and depolymerization**

How does R. meliloti produce LMW forms of its symbiotically relevant polysaccharide succinoglycan? The answer to this question may provide the key for understanding the symbiotic role of rhizobial EPSs in general. The results of genetic analyses and root nodule
invasion restoration assays imply that LMW forms of succinoglycan are symbiotically active (8, 93, 150), but more rigorous testing should be conducted before strong conclusions are drawn. The model for biosynthesis of octasaccharide repeating units of succinoglycan (126) serves as an excellent foundation for studies aimed at understanding the mechanisms used by \textit{R. meliloti} for control of the molecular weight of succinoglycan. And genetic and biochemical analyses have already provided several clues about genes that may be involved in controlling production of HMW and LMW succinoglycan (\textit{exoP}, \textit{exoQ}, \textit{exoT}, and \textit{exoK}) (68, 103, 126).

The more general issue of how bacteria control the molecular weight distribution of their polysaccharides is also interesting for several reasons. First, although the process by which bacteria synthesize lipid-linked oligosaccharide repeating units of heteropolymeric polysaccharides (such as O-antigen polysaccharide, xanthan gum, and succinoglycan) is fairly well defined (156), the subsequent steps of polymerization and transport of polysaccharides remain poorly understood. A better understanding of bacterial control of polysaccharide polymerization might in turn explain the basis for the polydispersity typically associated with bacterial polysaccharide samples. Second, the observation that mutations and environmental factors can influence the molecular weight distribution of polysaccharides (6, 7, 22, 93) suggests that bacteria exert some degree of active control in determining polysaccharide molecular weight. Third, the molecular weight distribution of polysaccharides affects their biological activity. As described above, LMW but not HMW forms of rhizobial polysaccharides seem to play a crucial role in establishment of nitrogen fixing symbiosis (8, 69). In contrast, longer rather than shorter chains of O-antigen polysaccharides are crucial for pathogenesis associated with enteric bacteria (155). Finally, molecular weight distributions affect the physical properties (e.g. viscosity) of solutions of polysaccharides. Development of the capability to engineer polysaccharides within discrete ranges of molecular weight could yield practical benefits regarding the use of bacterial polysaccharides in commercial products and industrial processes.

Two distinct mechanisms could account for control of the molecular weight distribution of succinoglycan by \textit{R. meliloti}, and, more specifically, for control of production of LMW
succinoglycan: 1) regulation of the extent of polymerization of succinoglycan repeating units, and 2) expression of glycanases that depolymerize succinoglycan. The two mechanisms are not mutually exclusive, and indeed the Walker lab has generated evidence in support of both (70, 162, 163).

Regulation of the extent of polysaccharide polymerization: O-antigen polysaccharide as a model

The polymerization of O-antigen polysaccharides of enteric bacterial strains is perhaps the most thoroughly developed model system for the study of polymerization of heteropolymeric polysaccharides (155), and as such it may prove useful to consider polymerization of succinoglycan in the context of this system. O-antigen polysaccharide is a component of lipopolysaccharide (LPS). LPS is a component of the outer layer of the outer membrane of Gram negative bacteria; LPS molecules are oriented such that the O-antigen polysaccharides protrude above the surface of the cell, where they provide a protective coating for the bacterium (155, 156). The O-antigen polysaccharides are highly variable in two respects. First, the carbohydrate backbones of repeating units vary widely between different bacterial strains (155, 156). Second, for a given bacterial strain, O-antigen polysaccharides exhibit polydispersity, such that the degrees of polymerization of individual molecules vary around one to several modes (155, 156). The basis of this polydispersity is the subject of intense research (155), and a comprehensive model for polymerization of O-antigen will need to convincingly account for this polydispersity.

A working model for the steps in polymerization of O-antigen polysaccharide has been proposed (155). According to this model, Wzx (RfbX) may be a "flippase" that catalyzes the transfer of completely synthesized lipid-linked oligosaccharide repeating units from the cytoplasmic face of the inner membrane to the periplasmic face (101, 155). In the periplasmic space, Wzy (Rfc) polymerizes O-antigen repeating units, and the chain length determinant protein Wzz (Rol, CLD) regulates this process (6, 7, 155). To account for the polydispersity of O-antigen polysaccharides, Bastin et al. (6) have proposed that Wzy and Wzz may form a
complex that has two states, E for extension of the O-antigen polysaccharide and T for transfer of the O-antigen to core polysaccharide. In this complex, Wzz is proposed to act as a molecular clock that shifts the complex from the E state to the T state after a particular amount of time (rather than when each O-antigen polysaccharide reaches a particular size) (6). In some cases the complex may become reset, leading to the production of polysaccharide chains distributed around a higher mode (6). This model is particularly attractive because it accounts for both uni-modal and multi-modal size distributions of O-antigen polysaccharides and for the polydisperse distributions of polysaccharides around each mode (6). Genes encoding homologs of Wzz have been identified within many clusters of genes involved in the synthesis of a wide range of structurally diverse polysaccharides (155), suggesting the possibility that at least some aspects of O-antigen polysaccharide polymerization will prove relevant to the study of a wide range of other polysaccharides.

**Low molecular weight succinoglycan can be synthesized directly**

The production of both LMW and HMW succinoglycan by *R. meliloti* could conceivably be regulated by a mechanism that involves controlling the degree of polymerization of succinoglycan at the step of polysaccharide polymerization. Such a mechanism might be directly analogous to the chain length determinant model for O-antigen polysaccharide polymerization (6); perhaps a CLD-like protein of *R. meliloti* either rapidly directs the release of monomers or short polymers from a succinoglycan polymerase, resulting in the production of LMW succinoglycan, or slowly directs the release of polymers, resulting in the production of HMW succinoglycan. Alternately, an entirely different mechanism may occur in *R. meliloti*. For example, the bacterium may express distinct pathways for polymerization of LMW and HMW succinoglycan. Recent reports are beginning to provide a basis for distinguishing between these possibilities (14, 15, 70).

Analyses conducted by Becker *et al.* (14) suggest that ExoP plays a role in modulating the molecular weight distribution of succinoglycan. ExoP consists of an N-terminal periplasmic
domain and a C-terminal cytoplasmic domain, as predicted by hydrophobicity plots and confirmed by analyses of exoP-TnphoA fusions (11, 14). Nucleotide sequence analyses indicate that the N-terminal domain of ExoP includes a motif that is present in Wzz proteins involved in determination of O-antigen polysaccharide chain length, and that the C-terminal domain of ExoP includes a potential ATP/GTP binding motif (11, 14).

To gain insights into the role of ExoP in succinoglycan production, Becker et al. (14) constructed a truncated ExoP protein, termed ExoP*, that lacks the C-terminal domain. Becker et al. (14) determined that the exoP* strain exhibits a dramatic increase in the ratio of LMW versus HMW succinoglycan that accumulates in cultures relative to the wild-type strain. The exoP* strain also produces approximately 17-fold less total succinoglycan than the wild-type strain (14), but even upon introduction of an exoR mutation, which causes increased production of succinoglycan (47), the exoP* strain still exhibits this shift toward production of LMW succinoglycan (14). Thus, the shift toward production of LMW succinoglycan associated with the exoP* strain is not strictly an indirect effect associated with the production of a lower total amount of succinoglycan (14). Becker and Pühler (15) determined that increasing osmolarity causes a shift toward greater production of HMW succinoglycan by the exoP* mutant, as is the case for the wild-type strain (22), but at any given osmolarity the exoP* strain still exhibits a greater ratio of LMW versus HMW succinoglycan in comparison to the wild-type strain (15). These results suggest that ExoP normally plays a role in modulating production of LMW and HMW succinoglycan in cultures, perhaps by regulating the extent of polymerization of succinoglycan (14, 15). Apparently, truncation of the ExoP protein alters this modulation, resulting in a shift toward production of LMW succinoglycan (14). An important caveat to these studies is that the phenotype of the exoP* mutant may reflect a disruption of the normal polymerization machinery of cells rather than reflecting a specific role for ExoP in regulating the degree of polymerization of succinoglycan.

Recent analyses conducted by González et al. (70) have helped to clarify the distinct roles played by ExoP, ExoQ, and ExoT in succinoglycan production and have provided support for the
model for direct synthesis of LMW succinoglycan. González *et al.* (70) applied an improved technique for introduction of radiolabelled UDP-galactose into *R. meliloti* cells, based on electroporation of cells (137) rather than the "freeze-thaw" technique (148), that does not disrupt the ability of cells to polymerize succinoglycan. By applying this technique in the analysis of *exoP, exoQ,* and *exoT* mutants, González *et al.* (70) determined that *exoB exoR exoQ* mutants are specifically blocked in production of HMW succinoglycan, that *exoB exoR exoT* mutants are specifically blocked in production of trimers and tetramers of succinoglycan, and that *exoB exoR exoP* mutants are blocked in production of all of these polymerized forms of succinoglycan. Thus, these results implicate ExoQ and ExoT in direct synthesis of HMW succinoglycan and trimers/tetramers of succinoglycan, respectively, and implicate ExoP in polymerization of both forms of succinoglycan (70). These results, as well as the previous observation that *exoP* mutants produce no detectable succinoglycan in cultures (103), cast some doubt on the idea that ExoP functions primarily as a Wzz-like chain length determinant for succinoglycan; *wzz* mutants exhibit a shift in the molecular weight distribution of O-antigen polysaccharides relative to wild-type enteric bacterial strains (6, 7), not a complete block in polymerization like that associated with *exoP* mutants (103) and *exoB exoR exoP* mutants (70). It is not clear at this point why *exoB exoR exoT* mutants can produce HMW succinoglycan during *in vitro* polymerization analyses (70), but *exoT* mutants fail to produce succinoglycan in cultures (68), or why *exoB exoR exoQ* mutants can produce trimers and tetramers of succinoglycan during *in vitro* polymerization analyses (70), but *exoQ* mutants are blocked in establishment of symbiosis (103). One possible explanation is that the absence of the ExoT or ExoQ proteins causes a substantial though not complete disruption of the succinoglycan polymerization or export machinery, resulting in a dramatic decrease in succinoglycan production *in vivo* (70). Further analyses may clarify these issues.

**Depolymerization is a second mechanism that may also contribute to production of low molecular weight succinoglycan**
A second mechanism that might account for production of LMW succinoglycan by *R. meliloti* is the expression of glycanases, such as ExoK (10, 68), that depolymerize HMW succinoglycan to yield LMW succinoglycan. Such a mechanism could conceivably function during polymerization of individual polysaccharide chains, if the polysaccharide chains are accessible to glycanases during polymerization. Such a mechanism could also function after completion of polymerization, during release of polysaccharides from cells or as polysaccharide accumulates extracellularly.

The phenomenon of a bacterial strain expressing a glycanase that depolymerizes the strain's own EPS has been reported previously (49, 50, 116). For example, *B. japonicum* expresses an extracellular glycanase that can partially depolymerize EPS isolated from cultures of this strain (49), *Pseudomonas aeruginosa* expresses a glycanase that depolymerizes alginate produced by this strain (50), and *Pseudomonas marginalis* expresses a cell-associated glycanase that depolymerizes a galactoglucomannan produced by this strain (116). In each of these cases, the biochemical activities of the glycanases have been analyzed based on purification of the glycanases from cells or from culture supernatants (49, 50, 116), but the genes encoding these glycanases have not been cloned and mutants defective in expression of these glycanases have not been isolated. In the absence of even preliminary genetic analyses, it is difficult to evaluate the biological relevance of these glycanases. Genetic analyses of the role of *R. meliloti* glycanases in the production of LMW succinoglycan can serve as a useful counterpoint to biochemical analyses conducted on other systems, in terms of advancing understanding of the biological relevance of glycanases.

*R. meliloti* expresses two glycanases, ExoK and ExsH, that contribute to production of low molecular weight succinoglycan

I have determined that *R. meliloti* expresses a second endo-1,3-1,4-β-glycanase, ExsH, in addition to ExoK, and that ExoK and ExsH both contribute to production of LMW succinoglycan by *R. meliloti* (162). Given that *exoH* mutants fail to produce Calcofluor halos (93) and fail to
produce LMW succinoglycan (92), I reasoned that defects in production of Calcofluor halos by *R. meliloti* mutants might generally correlate with defects in production of LMW succinoglycan. Given that *exoK* mutants are delayed in production of Calcofluor halos (103), I reasoned that the ExoK glycanase normally contributes to production of Calcofluor halos. And given that *exoK* mutants eventually produce Calcofluor halos (103), I reasoned that *R. meliloti* might express glycanases in addition to ExoK. Thus, I conducted a genetic screen to identify additional glycanases, based on mutagenesis of an *exoK* mutant and screening for mutants with even more severe defects in production of Calcofluor halos (162). I identified three genes, which I designated *prsD, prsE,* and *exsH,* that are absolutely required for production of Calcofluor halos in an *exoK* mutant background, but that are not required for production of Calcofluor halos in strains carrying a wild-type copy of the *exoK* gene (162). Nucleotide sequence analyses indicate that *prsD* and *prsE* encode the ABC-type transporter and membrane fusion protein, respectively, of a Type I secretion system (55), and that *exsH* encodes a protein with an N-terminal domain typical of proteins secreted by Type I secretion systems (55, 154) and a C-terminal domain homologous to endo-1,3-1,4-β-glycanases (78). These data suggest that ExsH is a glycanase that is secreted by PrsD/PrsE (162). I determined that together the *exoK* gene and the *prsD/prsE/exsH* genes contribute to production of LMW succinoglycan (162). In comparison to the wild-type strain, which produces approximately 50% of its total succinoglycan in LMW forms (92), *exoK prsD, exoK prsE,* and *exoK exsH* mutants produce only approximately 3% of their total succinoglycan in LMW forms (162). Interestingly, none of these genes is required for establishment of symbiosis between *R. meliloti* and alfalfa (103, 162). This genetic analysis is described more thoroughly in Chapter 2.

In reconciling apparently contradictory results of biochemical analyses and genetic analyses of the activities of ExoK and ExsH, I made the unexpected discovery that ExoK and ExsH cleave nascent succinoglycan, but not succinoglycan that has accumulated in culture supernatants, to yield LMW succinoglycan (163). Genetic analyses had convincingly implicated the *exoK* and *exsH* genes in the process of production of LMW succinoglycan, implying that
ExoK and ExsH cleave HMW succinoglycan to yield LMW succinoglycan in *R. meliloti* cultures (162). Yet, in trying to reconstitute depolymerization reactions *in vitro*, I observed that ExoK and ExsH, expressed and purified from *Escherichia coli*, could generate virtually no LMW succinoglycan by cleavage of HMW succinoglycan prepared from *R. meliloti* cultures (163). I found that neutral/heat-treatment and acid/heat-treatment convert succinoglycan to forms that can be cleaved extensively by ExoK and ExsH, indicating that the preparations of ExoK and ExsH purified from *E. coli* were not simply inactive (163). By analyzing the products of cleavage of acid/heat-treated succinoglycan, I confirmed that ExoK and ExsH are indeed endo-1,3-1,4-β-glycanases (163). I determined that ExoK and ExsH expressed by *R. meliloti* exhibit essentially the same activities as ExoK and ExsH expressed and purified from *E. coli*, ruling out the possibility that the expression or purification technique alters the ability of ExoK or ExsH to cleave succinoglycan (163). By supplementing cultures of an *exoK exsH* mutant with purified ExoK or ExsH, I determined that both enzymes actually can convert HMW succinoglycan to LMW succinoglycan, but only in cultures that are actively synthesizing succinoglycan (163). My results imply that nascent succinoglycan is susceptible to extensive cleavage by ExoK and ExsH, but that succinoglycan undergoes a transformation to a glycanase-refractory form as it accumulates in cultures (163). The results of these biochemical analyses are described more thoroughly in Chapter 3.

I have determined that the succinyl and acetyl modifications of succinoglycan influence the susceptibility of succinoglycan to cleavage by ExoK and ExsH (164). The defect in production of LMW succinoglycan associated with *exoH* mutants suggested the possibility that the non-succinylated succinoglycan produced by this strain is a poor substrate for cleavage by ExoK and ExsH (92, 93). And although no analyses had been done to determine whether the non-acetylated succinoglycan produced by *exoZ* mutants might also be cleaved to a different extent that normally-modified succinoglycan, previous research on a different rhizobial polysaccharide indicated that the absence of the acetyl group increases the susceptibility of that polysaccharide to cleavage by specific lyases (80). To test whether the succinyl and acetyl
modifications of succinoglycan actually do influence the susceptibility of succinoglycan to cleavage by ExoK and ExsH, I constructed strains representing each combination of wild-type or mutant allele for the \textit{exoK}, \textit{exsH}, \textit{exoH}, and \textit{exoZ} genes (164). I then determined that \textit{exoZ} mutants exhibit a shift toward production of more LMW succinoglycan and less HMW succinoglycan in comparison to the wild-type strain (164). This shift is dependent on the \textit{exoK}+ and \textit{exsH}+ genes, implying that the negative effect on production of LMW succinoglycan associated with the \textit{exoH} mutation outweighs the positive effect associated with the \textit{exoZ} mutation (164). By adding increasing amounts of ExoK or ExsH to cultures of \textit{exoK exsH}, \textit{exoH exoK exsH}, \textit{exoZ exoK exsH}, and \textit{exoH exoZ exoK exsH} strains, and measuring the accumulation of LMW succinoglycan, I determined that ExoK and ExsH cleave non-acetylated succinoglycan with higher efficiency relative to normally-modified succinoglycan, and that ExoK and ExsH cleave non-succinylated succinoglycan with lower efficiency (164). I have also determined that increasing conversion of HMW succinoglycan to LMW succinoglycan in cultures correlates with a decreasing average degree of polymerization of the remaining HMW succinoglycan (164). In other words, the ExoK and ExsH glycanases affect the molecular weight distribution of succinoglycan not just by contributing to the production of LMW succinoglycan but also by decreasing the average length of HMW succinoglycan chains that accumulate in cultures (164). The results of these analyses are described more thoroughly in Chapter 4.

During the course of analyzing the biochemical activities of ExoK and ExsH, I developed a method for measuring glycanase-mediated depolymerization of succinoglycan in solution, based on the use of Congo Red dye. Congo Red can be used to detect depolymerization of many structurally diverse polysaccharides, such as chitin, barley glucan, and xylan, based on the observation that Congo Red binds HMW forms, but not depolymerized forms, of these polysaccharides and that upon binding Congo Red exhibits an increase in OD525 (corresponding to a shift in color from dull orange to bright red) (159). Several assays have been described for the use of Congo Red in the detection of polysaccharide depolymerase activity on solid substrates, such as agar growth medium or polyacrylamide gels (147, 159). I became interested
in developing a method for detection of depolymerization of succinoglycan in solution, to complement other depolymerization analyses based on detection of increases in the concentration of reducing ends in glycanase reactions (100). Unexpectedly, I determined that Congo Red dye binds native, normally modified succinoglycan very slowly or not at all, but that neutral/heat-treatment converts succinoglycan to a form that can be bound by Congo Red fairly rapidly, over a time course of minutes to hours, and that acid/heat-treatment of succinoglycan converts succinoglycan to a form that can be bound by Congo Red even more rapidly, over a time course of seconds to minutes. I determined that increasing glycanase-mediated depolymerization of succinoglycan can be detected based on a decreasing rate and extent of binding of Congo Red to polysaccharide in solution. My results suggest that this assay may be useful for distinguishing between endo-1,3-1,4-β-glycanases that cleave succinoglycan with a high degree of processivity versus those that cleave succinoglycan with a low degree of processivity. This assay is also particularly useful for measuring glycanase activity in bacterial culture supernatants. The assay is described in Chapter 5.
Fig. 1. Diagram of the establishment of the *Rhizobium meliloti*-alfalfa nitrogen fixing symbiosis. In this diagram, the mature nodule represents an example of an indeterminate nodule.
Fig. 2. Structure of the octasaccharide repeating unit of succinoglycan. Glc: glucose, Gal: galactose.
Fig. 3. Map of the \textit{exo} region.
Fig. 4. Model for biosynthesis and modification of octasaccharide repeating unit of succinoglycan. According to this model, ExoP, ExoQ, and ExoT are involved in polymerization and/or transport of succinoglycan.
Chapter 2

The *Rhizobium meliloti* exo*K* Gene and *prsD/prsE/lexsH* Genes

Are Components of Independent Degradative Pathways

Which Contribute to Production of Low Molecular Weight Succinoglycan*

ABSTRACT
When grown on medium supplemented with the succinoglycan-binding dye Calcofluor and visualized under UV light, colonies of *Rhizobium meliloti* *exoK* mutants produce a fluorescent halo with a delayed onset relative to wild-type colonies. By conducting transposon mutagenesis of *exoK* mutants of *R. meliloti* and screening for colonies with even more severe delays in production of these fluorescent halos, we identified three genes, designated *prsD*, *prsE*, and *exsH*, which are required for the eventual production of fluorescent halos by *exoK* colonies. Nucleotide sequence indicates that the *prsD* and *prsE* genes encode homologs of ABC transporters and membrane fusion proteins of Type I secretion systems, respectively, whereas *exsH* encodes a homolog of endo-1,3-1,4-β-glycanases with glycine rich nonameric repeats typical of proteins secreted by Type I secretion systems. The *exoK* gene and the *prsD/prsE/exsH* genes were shown to be components of independent pathways for production of extracellular succinoglycan degrading activities and for production of low molecular weight succinoglycan by *R. meliloti*. Based on these results, we propose that ExsH is a succinoglycan depolymerase secreted by a Type I secretion system composed of PrsD and PrsE, and that the ExsH and ExoK glycanases contribute to production of low molecular weight succinoglycan.
INTRODUCTION

Bacterial exopolysaccharides (EPS) are crucial for establishment of nitrogen fixing symbioses between rhizobial strains, such as *Rhizobium meliloti*, and corresponding host plants that form indeterminate nodules, such as alfalfa (21, 31, 34, 94). Bacterial mutants that fail to produce EPS elicit root nodules on their specific host plants but fail to invade these nodules (57, 94, 95). Although a variety of bacterial EPS have been implicated in mediating bacterial invasion of root nodules, the precise roles played by bacterial EPS are not well understood.

Wild-type *R. meliloti* produces an acidic EPS, termed succinoglycan (1, 83, 123). Wild-type colonies grown on medium supplemented with the laundry whitener Calcofluor fluoresce brightly when visualized under UV light, due to Calcofluor binding to the succinoglycan produced by these colonies (94). This observation served as a basis for screens to identify genes necessary for succinoglycan production. A series of nonfluorescing *exo* mutants that fail to produce succinoglycan were isolated; most of the *exo* genes are located in a cluster, which has been sequenced (10, 11, 12, 67, 68, 94, 103, 113). Those *exo* mutants that are defective in succinoglycan production are also blocked in invasion of root nodules, implying that succinoglycan mediates invasion (57, 94, 103, 113).

*R. meliloti* also has the capacity to produce a second exopolysaccharide, termed EPS II or galactoglucon (65, 79, 85, 166). Two mutations, *expR101* (65) and *mucR* (85, 166), permit the synthesis of this otherwise cryptic exopolysaccharide. Intriguingly, the *expR101* mutation, but not the *mucR* mutation, restores nodule invasion ability to *exo* mutant strains that are unable to produce succinoglycan (65, 86). The basis for this distinction is that while both *expR101* and *mucR* strains produce high molecular weight (HMW) EPS II, only the *expR101* strain produces low molecular weight (LMW) EPS II (69). A specific size class of LMW EPS II, but not HMW EPS II, is sufficient to mediate invasion (69).

Analogously to EPS II, a certain size class of LMW succinoglycan may be the form of succinoglycan crucial for mediating root nodule invasion (8, 150). Succinoglycan is a polymer of an octasaccharide repeating unit; each unit is composed of one galactose and seven glucose
residues and carries approximately one succinyl, acetyl, and pyruvyl group (1, 123). When wild-type *R. meliloti* is cultured in minimal medium, succinoglycan accumulates in both HMW and LMW forms (22, 92). The HMW forms are polymers of thousands of succinoglycan repeating units, and the LMW forms are monomers and short oligomers of the repeating units (8). Strains mutated in the *exoH* gene produce succinoglycan lacking the succinyl modification (93), produce HMW but not LMW succinoglycan (92), and are deficient in the invasion of root nodules (93). In addition, Battisti *et al.* (1992) reported that a highly charged, oligomeric form of LMW succinoglycan, but not HMW succinoglycan, is sufficient to restore invasion of root nodules by *exo* mutants when the EPS is added exogenously to plant roots. Based on these observations, it was proposed that LMW succinoglycan plays a role in promoting nodule invasion.

Two general mechanisms could account for production of LMW succinoglycan in cultures: direct synthesis of LMW forms and/or bacterial production of enzymes that degrade succinoglycan to yield LMW forms. Regarding direct synthesis, the ExoP protein has been proposed to regulate polymerization of succinoglycan (14), and thus could play a role in direct synthesis of LMW succinoglycan. Regarding degradation of succinoglycan, the *R. meliloti exoK* gene, located within the *exo* region (103), encodes a protein homologous to endo-1,3-1,4-β-glycanases (10, 68). This class of enzyme cleaves β-1,4-linkages adjacent to β-1,3-linkages. Since one β-1,3,1,4-linkage occurs per octasaccharide repeating unit of succinoglycan (1), the ExoK protein has been predicted to depolymerize HMW succinoglycan (10, 68), to yield products that are multimers and/or monomers of the octasaccharide repeating unit structurally identical to that which serves as an intermediate in succinoglycan polymerization (148).

Distinctions between the phenotypes of colonies of wild-type, *exoH*, and *exoK* strains led us to hypothesize that *R. meliloti* produces one or more succinoglycan degrading enzymes in addition to ExoK, and that these enzymes may be involved in production of LMW succinoglycan. When grown on medium containing Calcofluor and visualized under UV light, wild-type colonies fluoresce brightly and, over the course of several days, produce fluorescent halos (Calcofluor halos) which correspond to succinoglycan diffusing from colonies (94). *exoH*
colonies fluoresce brightly but produce no Calcofluor halos (93). Since *exoH* mutants are also
defective in production of LMW succinoglycan (92), we reasoned that there may be a general
correlation between production of Calcofluor halos and production of LMW succinoglycan. *exoK* colonies fluoresce brightly and exhibit Calcofluor halos, but the onset of these halos is
delayed with respect to that of wild-type colonies (10, 68, 103), suggesting that the ExoK
glycanase normally cleaves HMW succinoglycan to lower molecular weight forms which diffuse
from colonies to form Calcofluor halos. The eventual production of halos by *exoK* mutants
suggests that *R. meliloti* may encode at least one additional succinoglycan degrading activity. We hypothesized that *exoK* and additional genes involved in production of Calcofluor halos
might also contribute to production of LMW succinoglycan by *R. meliloti*.

Here we report the identification of three genes, designated *prsD*, *prsE*, and *exsH*, which
are necessary for the eventual production of Calcofluor halos by *exoK* mutants. We present
evidence that the *exoK* gene and *prsD/prsE/exsH* genes are components of two independent
pathways for production of extracellular succinoglycan depolymerases, and that both pathways
are implicated in production of LMW succinoglycan.
MATERIALS AND METHODS

Strains, growth media, antibiotics, and testing for effective nodulation of plants. 

*R. meliloti* strains used in this study are listed in Table 1. The following growth media were used: LB (107), MGS (potassium phosphate (100 mM, pH 6.8), mannitol (55 mM), monosodium glutamate (5 mM), and sodium chloride (8 mM)), and GMS medium (containing mannitol at a final concentration of 27.5 mM) (165). MGS and GMS were also supplemented with magnesium sulfate (1 mM), calcium chloride (0.25 mM), biotin (1 mg/l for MGS and 10 μg/l for GMS) and thiamine (100 μg/l for GMS) after autoclaving of media. Agar (1.5%) was included for solid media. Antibiotics were used in LB medium at the following concentrations: for *R. meliloti*, gentamicin, 50 μg/ml; neomycin, 200 μg/ml; oxytetracycline, 750 ng/ml; spectinomycin, 100 μg/ml; streptomycin, 500 μg/ml; trimethoprim, 500 μg/ml to 1 mg/ml, and for *E. coli*, ampicillin; 100 μg/ml; chloramphenicol, 20 μg/ml; gentamicin, 5 μg/ml; kanamycin, 25 μg/ml, nalidixic acid; 50 μg/ml, spectinomycin, 100 μg/ml; and tetracycline, 10 μg/ml. In some cases, antibiotics were used at lower concentrations when used in combination (66). The same concentrations of antibiotics were used in MGS medium as in LB medium, except for gentamicin (160 μg/ml) in combination with spectinomycin (400 μg/ml), and for neomycin (67 μg/ml) in combination with tetracycline (3 μg/ml). Detection of Calcofluor binding to *R. meliloti* colonies was conducted as previously described (94). Tests for effective nodulation of the *Medicago sativa* cultivars Iriquois (Agway) and GT13R-plus (ABI Alfalfa) by *R. meliloti* strains were conducted as previously described (66).

Tn5 and Tn5-233 mutagenesis of *R. meliloti* and characterization of mutants. Tn5 and Tn5-233 mutagenesis of *R. meliloti* strains already carrying transposons derived from Tn5 was conducted as described (66). An *R. meliloti* genomic library (62) was used for the cloning of genes by complementation. In general, plasmids were moved to and from *R. meliloti* strains by a triparental mating procedure (66). Previously described methods were used to isolate plasmid DNA (107) and genomic DNA (3). Restriction mapping, cloning of restriction fragments, and
Southern hybridization analyses were conducted by standard methods (107). Probes were generated by the use of $^{32}$P-dNTP with the NEBlot random priming kit (New England Biolabs).

**Plasmid construction, nucleotide sequencing, and localization of** $prsD$ and $prsE$ **to pRmeSU47b.** pG108 and pG115 were constructed by cloning the 4.2 kb $PstI$ fragment and the 1.9 kb $EcoRI$ fragment of pG100, respectively, into the $PstI$ or $EcoRI$ site of pSW213 (33). pG307 was constructed by cloning the 3.0 kb $EcoRI$ fragment of pD56 (103) into the $EcoRI$ site of pSW213. Tn5 and mini-Tn5 Km (41) mutagenesis of DNA cloned in plasmids, and homogenotization and transduction of these mutations, were conducted as described (66). We constructed nested deletions of plasmids for nucleotide sequencing using the *double-stranded* Nested Deletion Kit (Pharmacia Biotech). In general, we sequenced *R. meliloti* DNA cloned in the vectors pBluescript II KS+ and pUC19 by use of Perkin Elmer ABI sequencers. Nucleotide sequence data of the 3.9 kb of DNA including the $prsD/prsE$ genes and the 3.0 kb of DNA including the $exsH$ gene have been submitted to the GenBank database under accession numbers U89163 and U89164. To determine whether $prsD/prsE$ might be localized to pRmeSU47a or pRmeSU47b, we used a previously described Southern hybridization analysis (82). DNA cloned in pG115 hybridizes to DNA isolated from the *Agrobacterium tumefaciens* strain At125, which contains *R. meliloti* pRmeSU47b, but not to DNA from the isogenic strains At128, which contains pRmeSU47a, or GMI9023, which contains no megaplasmid.

**Saturation mutagenesis of the R. meliloti genome.** Because we used transposons to mutagenize *R. meliloti*, we would not expect our screen to have yielded mutations in i) genes required for viability, since Tn5 and Tn5-233 insertions in genes typically yield null mutations, or ii) genes encoding proteins that can be functionally substituted by other proteins, since transposon mutagenesis typically yields only one new mutation per strain. Also, since we introduced the new Tn5 and Tn5-233 mutations into exoK strains by transduction, we would expect derivatives in which the new mutation is transductionally linked to the exoK mutation to
be underrepresented, due to cotransduction.

Except for these caveats, the recovery of multiple independent mutant alleles of \textit{prsD} and \textit{prsE} (five and seven mutations per gene, respectively) is consistent with our having accomplished saturation mutagenesis of the vast portion of the \textit{R. meliloti} genome not transductionally linked to the \textit{exoK} gene. Regarding genes linked to \textit{exoK}, the recovery of only one \textit{exsH} mutant allele and zero \textit{exoH} mutant alleles is consistent with the fact that the \textit{exsH} gene is 55% transductionally linked to the \textit{exoK445} mutation and that the \textit{exoH} gene is immediately adjacent to the \textit{exoK} gene. Also, site directed mutagenesis of the \textit{exs} region did not reveal any complementation groups other than \textit{exsH} that are crucial to production of Calcofluor halos.

**Extracellular suppression of Calcofluor halo defects of \textit{R. meliloti} strains.** To test for extracellular suppression of the Calcofluor halo defects of normally haloless colonies, we cultivated colonies of a tester strain at distances ranging from 3 to 10 mm from colonies of a normally haloless indicator strain. We observed the indicator colonies periodically over the course of two weeks and scored whether a given tester strain could cause the appearance of a Calcofluor halo around a normally haloless indicator strain. Extracellular suppression can occur whether or not indicator and tester colonies physically contact each other.

**Congo Red dye assays.** The assay for detection of diffusion of polysaccharide degrading activities from colonies is based on a previously described protocol (159). Succinoglycan, barley glucan, carboxymethylcellulose, or laminarin were added to 1 mg/ml to MGS top agar (0.7% agar) prior to autoclaving of the top agar. Colonies were incubated on MGS agar medium for three to five days. Then MGS top agar supplemented with polysaccharide was poured over colonies. Colonies were incubated for an additional 8 hours to 48 hours. Top agar was stained by pouring excess Congo Red (1 mg/ml) solution onto plates, incubating plates 10 minutes at 37°C, rinsing plates with excess sodium chloride solution (1 M), and incubating plates for 10 minutes at 37°C.
Analyses of extracellular proteins in culture supernatants. We used previously described approaches for cultivating strains in GMS medium (8). Cultures were incubated until freshly saturated (approximately 48 hours). Cells were removed from culture supernatants by centrifugation (19000 g, 20 min). Proteins were isolated from supernatants by precipitation in the presence of ammonium sulfate (70%) followed by centrifugation (13000 g, 20 min) (all steps at 4°C). Protein pellets were dissolved in 25 mM potassium phosphate (pH 6.8), dialyzed against 10 mM potassium phosphate (pH 6.8), and concentrated by lyophilization. Protein samples (12 μg) were separated by discontinuous SDS PAGE (8% polyacrylamide separating gels) and visualized by staining with Coomassie blue dye, as previously described (3).

Analyses of extracellular carbohydrate in culture supernatants. We used previously described approaches for cultivating strains in GMS medium (8), and for conducting Biogel A5 (92) and Biogel P4 gel filtration chromatography (8). Cells were removed from culture supernatants by centrifugation (27000 g, 20 min); samples which appeared viscous were diluted in five volumes of distilled water and subjected to further centrifugation (20000 g, 20 min). In general, carbohydrate was quantified by the anthrone-sulfuric acid method (102). For Biogel A5 chromatography, samples consisting of 2 mg carbohydrate from lyophilized culture supernatants dissolved in 20 ml of column buffer (sodium phosphate (50 mM, pH 7.0) sodium chloride (100 mM)) were applied to the column. For Biogel P4 chromatography, samples consisting of 100 μg to 500 μg carbohydrate in 1 ml of column buffer (pyridine acetate (100 mM pH 5)) were applied to the column. The succinoglycan depolymerase of Cytophaga arvensicola was prepared by a variation on a previously described method (75), and succinoglycan samples were depolymerized by treatment with the depolymerase in a potassium phosphate (100 mM, pH 5.8) buffer at 37°C. Thin layer chromatographic analyses of depolymerized succinoglycan were conducted as previously described (126).
RESULTS

Isolation of thirteen mutants of an \textit{exoK} strain that are haloless on medium containing Calcofluor. To identify genes besides \textit{exoK} that encode succinoglycan degrading activities, we conducted random mutagenesis of \textit{exoK} mutants and screened for mutants exhibiting even more severe delays or defects in production of Calcofluor halos. Two different approaches were employed.

In our first approach, we conducted random Tn5 and Tn5-233 mutagenesis of a population of the wild-type strain Rm1021, and transduced pools of these insertion mutations into populations of \textit{exoK}445::Tn5-233 and \textit{exoK}445::Tn5 strains, respectively. We used this transduction technique to mutagenize \textit{exoK} mutants in order to circumvent the problem that Tn5 based transposons do not transpose efficiently when introduced into \textit{R. meliloti} strains with a resident Tn5 based transposon (66). We selected for colonies of double mutants, \textit{i.e.} strains carrying both a newly transduced transposon insertion and the parental \textit{exoK} transposon mutation, on the rich growth medium (LB) we typically use for transductions. We then transferred colonies to a mannitol-glutamate-salts agar minimal medium (MGS) supplemented with Calcofluor, on which \textit{R. meliloti} colonies produce large quantities of succinoglycan and large Calcofluor halos. Using this approach, we screened $1.4 \times 10^4$ transductants and isolated four strains that failed to produce Calcofluor halos.

In a second approach, we also conducted random Tn5 mutagenesis of Rm1021, but then transduced these mutations into the \textit{exoX}363::Tn5-233 \textit{exoK}445::Tn5-Tp strain. The \textit{exoX}363 mutation causes increased succinoglycan production by \textit{R. meliloti} strains (122), and makes it easy to detect the Calcofluor halos produced by colonies grown on LB medium. Using this approach, we screened $3.6 \times 10^4$ transductants directly on LB medium and isolated nine additional strains that failed to produce Calcofluor halos.

Thus, in total we screened $5 \times 10^4$ transductants and isolated 13 haloless derivatives of \textit{exoK} or \textit{exoK} \textit{exoX} parental strains (Table 1). Each of the 13 derivatives carries a single, newly-acquired transposon mutation which we provisionally designated \textit{hal-1} through \textit{hal-13}, to
Transductional analyses indicate that hal mutations cause a haloless phenotype in the exoK mutant background, but not in the wild-type background. We transduced each hal transposon mutation into both Rm1021 and exoK strains, and observed that each hal single mutant produced a Calcofluor halo indistinguishable from that of Rm1021, whereas each hal exoK double mutant produced no Calcofluor halo (Fig. 1). These results confirmed that the transposon mutation in each hal strain is linked to, and causes, the haloless phenotype in an exoK mutant background. Furthermore, the results implied that, in each hal single mutant, the exoK+ gene is sufficient to mediate production of a normal halo. Consistent with this inference, transfer of the plasmid pEX154 (103), which contains the exoK+ gene, into the 13 haloless strains also restores production of normal halos (Table 2), whereas a derivative of pEX154 with a Tn5 mutation in the exoK gene (103) fails to restore halos. Although the exoX363 mutation helped facilitate the isolation of haloless mutants, our results indicated that the mutation is not required for manifestation of the haloless phenotype of any of the hal exoK mutants. Therefore, we conducted all further analyses of the hal mutations in strains carrying the exoX+ gene.

The mutations hal-1 through hal-12 correspond to two complementation groups involved in Calcofluor halo production. We cloned the genes mutated in the strains hal-1 through hal-12 by complementation. A cosmid library of genomic DNA derived from Rm1021 was transferred to the hal-1 exoK strain by transconjugation, and ten colonies with restored Calcofluor halos were isolated from several thousand colonies screened. We determined that nine of these cosmids restored Calcofluor halo production by complementing the exoK mutation. The remaining cosmid, which we designated pG100, restored halo production by complementing the hal-1 mutation (Table 2). By transferring pG100 into the other hal exoK strains, we determined that pG100 also complements the haloless phenotypes of the 11 mutants hal-2 exoK through hal-12 exoK, but not hal-13 exoK (Table 2). In each case, colonies of complemented
strains produce Calcofluor halos with the delayed onset typical of an *exoK* single mutant. By conducting Southern hybridization analyses, we confirmed that in these cases restoration of halo production is due to complementation, rather than multicopy suppression, and that each of the mutations *hal-1* through *hal-12* represent different transposon insertion events and thus are independent alleles (data not shown). We identified two distinct complementation groups within the cosmid pG100 by subcloning DNA from it to yield the plasmid pG108, which complements the mutations *hal-1* through *hal-5*, and the plasmid pG115, which complements the mutations *hal-6* through *hal-12* (Fig. 2, Table 2).

Using a previously described method based on Southern hybridization (82), we localized the two complementation groups to the *R. meliloti* megaplasmid pRmeSU47b (32). This replicon also contains the *exo* region, a cluster of genes (including *exoK* and *exoX*) encoding products involved in synthesis and modification of succinoglycan (10, 11, 12, 67, 68, 103, 114). However, we determined that the mutations *hal-1* through *hal-12* are not transductionally linked to the *exo* region.

We sequenced 3.9 kb of DNA derived from pG100 and identified two long ORFs. ORF1 is 1746 bp and corresponds to the mutations *hal-1* through *hal-5*. ORF2 is 1317 bp and corresponds to the mutations *hal-6* through *hal-12*. The two ORFs are oriented in the same direction, and the start codon of ORF2 overlaps the stop codon of ORF1 by 2 bp.

**ORF1, designated prsD, encodes a product homologous to ABC transporter components of Type I secretion systems.** ORF1 is predicted to encode a 62 kDa protein. BLAST homology searches revealed that this protein is homologous to proteins of the ATP Binding Cassette (ABC) transporter family, and more specifically to the PrtD and HlyB protein transporters of Type I secretion systems (55). This subfamily of transporters has been implicated in N-terminal signal peptide independent secretion of various proteins, particularly proteases and hemolysins. AprD of *Pseudomonas aeruginosa* (51), PrtDSM of *Serratia marcescens* (98), and PrtD of *Erwinia chrysanthemi* (97), all of which are proteins implicated in secretion of proteases,
exhibit high degrees of similarity with the protein encoded by ORF1 (approximately 44% amino acid identity in each case) (Fig. 3). A series of hemolysin secretion proteins, including HlyB of *Escherichia coli* (106), exhibit lower degrees of similarity. The positions and sequences of the predicted ATP binding sites Walker box A and B, which are conserved among ABC transporters (55), are also conserved in the ORF1 protein. Hydropathy plots indicate that the N-terminus of the ORF1 protein contains a highly hydrophobic domain, which may constitute several membrane spanning sequences like those previously identified in PrtD (44) and predicted for AprD and PrtDSM.

During the course of our experiments, we learned that the ORF1 protein is also highly homologous to the product of a recently identified *R. leguminosarum* bv. *viciae* gene (69% amino acid identity) (58). We and Finnie *et al.* (58) have adopted consistent nomenclature and have designated the homologous genes from *R. meliloti* and *R. leguminosarum* bv. *viciae* as *prsD*, since the products of both genes are proposed to mediate protein secretion (see below).

**ORF2, designated *prsE*, encodes a product homologous to membrane fusion proteins of Type I secretion systems.** ORF2 is predicted to encode a 48 kDa protein. BLAST homology searches revealed that this protein is homologous to the PrtE and HlyD membrane fusion proteins, crucial for the transport process for Type I secretion systems (55). To date, the genes encoding these membrane fusion proteins have invariably been found to be directly adjacent to, and downstream of, the genes encoding the partner ABC type transporter protein; this is the case for ORF2. PrtE (97), AprE (51), and PrtESM (98) each exhibit the same degree of similarity with the ORF2 protein (approximately 34% amino acid identity in each case) (Fig. 4) while proteins involved in secretion of hemolysins, such as HlyD (106), exhibit lower degrees of similarity. Hydropathy analyses predict the presence of a short hydrophobic domain near the N-terminus of the protein encoded by ORF2; this corresponds to a transmembrane segment in PrtE (98).

The ORF2 protein is also highly homologous to the product of a recently identified
The *hal-13* mutation is transductionally linked to the *exo* region and the *exsA* and *exsB* genes. During the course of conducting transductional analyses on the *hal* mutations, we determined that the *hal-13* mutation is transductionally linked to genes of the *exo* region. Measurement of frequencies of cotransduction between *hal-13* and the mutations *exoK445* (55%), *exoX363* (63%), and *exoB94* (86%) localized the *hal-13* mutation to a position several kb from the *exsA* and *exsB* genes (13) (Fig. 5).

The *hal-13* mutation corresponds to a third complementation group, consisting of a single ORF designated *exsH*. Having mapped the position of the *hal-13* mutation, we transferred the cosmids pD56 and pD2, which had been isolated during previous efforts to characterize the *exo* region (94, 103), into the *hal-13 exoK* strain. These cosmids span the region mutated in the *hal-13* strain and do not carry the *exoK* gene (Fig. 5). As expected, both cosmids complemented the haloless phenotype of the *hal-13 exoK* strain, restoring the delayed halo phenotype of the *exoK* single mutant (Table 2). We confirmed that this is complementation, rather than multicopy suppression, by conducting Southern hybridization analyses (data not shown).

To localize the complementation group, we tested a previously described set of 20 Tn5 mutagenized pD56 derivatives (103) for the ability to complement the *hal-13 exoK* haloless phenotype (Fig. 5). Three derivatives, designated 308, 334, and 343, failed to complement the *hal-13* mutation. We also tested for the presence of additional linked complementation groups involved in Calcofluor halo production, by homogenotizing each of the set of 20 mutations in pD56 into the *R. meliloti* genome and then transducing an *exoK* mutation into each strain, but we did not find evidence for any additional complementation groups. We subcloned a 3 kb fragment
of DNA, which overlaps *hal-13* and the mutations 308, 334, and 343, to yield a plasmid designated pG307 which is sufficient to complement the *hal-13* mutation.

We sequenced the 3 kb fragment derived from pG307 and identified one long ORF of 1395 bp. Based on its proximity to the recently characterized *exs* genes, we have designated this ORF as the *exsH* gene. We determined the orientation of the *exsH* gene with respect to the linked *exo* and *exs* genes. Specifically, we determined the exact positions of mutations 308 and 334 (103) by sequencing outward from one end of Tn5 for both mutations to map the positions of these Tn5 insertions in the *exsH* gene. This allowed us to orient the *exsH* gene with respect to the previously determined positions of these two Tn5 insertions in pD56. The *exsH* gene is transcribed in the same direction as the linked *exsA* and *exsB* genes.

**exsH encodes a domain typical of proteins secreted by Type I secretion systems.** The *exsH* gene is predicted to encode a 50.2 kDa protein. BLAST homology searches revealed that the N-terminal half of ExsH includes a domain homologous to the RTX (repeats in toxin) domains of various proteins, such as hemolysins, secreted by Type I secretion systems (55, 154). The characteristic features of RTX domains are glycine rich nonapeptide repeats that approximately match the consensus LXGGXG(N/D)DX, where X is any amino acid (154). ExsH contains four such repeats (Fig. 6). Various biochemical and protein crystallographic analyses have implicated these nonapeptide repeats in calcium binding and in mediating the calcium dependent lytic activity of hemolysins (9, 132). The nonapeptide repeats may also facilitate secretion of hemolysins and other proteins; while such repeats are not part of the minimal signal necessary for secretion of C-terminal truncations of proteins such as *E. coli* HlyA (84) and *E. chrysanthemi* PrtB (43), the repeats are necessary for secretion of a higher molecular weight CyaA-PrtB fusion protein (99), which, in terms of its size, is more similar to the full length proteins normally secreted by these pathways (55).

It is also noteworthy that the sequence of the last four amino acids of ExsH, DWQI, is similar to the consensus DUUU (where U is any hydrophobic amino acid) which occurs at the
last four amino acids of several proteases which are targets of Type I secretion systems (63). The presence and precise position of this motif has been demonstrated to be essential for secretion of PrtG (63), and thus may prove to be crucial for secretion of these other proteins, including ExsH.

**ExsH has a glycanase domain consistent with succinoglycan depolymerase activity.** BLAST homology searches also revealed that the C-terminal half of ExsH encodes a domain conserved among glycanases. This domain is most highly similar to the endo-1,3-1,4-β-glycanases of *Rhodothermus marinus* (143) and *Clostridium thermocellum* (136), and also has similarities to endo-1,3-β-glycanases of *Thermotoga neapolitana* (37) and *Bacillus circulans* (160, 161) (Fig. 6). As determined by a method for grouping glycosyl hydrolases according to similarities in predicted secondary structure (77, 78), these glycanases belong to one group within Family 16 of the glycosyl hydrolases (38). The ExoK glycanase belongs to a second group within this same family. These two groups share common predicted structural features but a low degree of similarity at the amino acid level. In general, members of both groups are predicted or have been demonstrated to have an N terminal signal peptide (20, 143, 160) that mediates export to the periplasmic space and/or secretion from cells by the sec-dependent Type II pathway (120). For example, ExoK is predicted to have an N-terminal signal peptide (10). ExsH is unique among proteins reported to belong to these two groups in that it apparently lacks an N-terminal signal peptide but instead contains features typical of proteins secreted by Type I secretion systems. These data suggest the possibility that ExsH is secreted by the PrsD/PrsE proteins.

**Genetic evidence that the prsD/prsE/exsH genes are components of a novel, exoK-independent pathway for production of Calcofluor halos.** Our analyses of the 13 hal mutations indicate that we have likely achieved saturation mutagenesis of the *R. meliloti* genome in terms of identifying genes crucial for Calcofluor halo production which our screening method could be expected to reveal (see Materials and Methods). To test whether the prsD/prsE/exsH
genes function together in a single pathway for Calcofluor halo production or function in distinct, parallel pathways, we constructed \( \text{prsD exsH} \) and \( \text{prsE exsH} \) double mutants. Like the \( \text{prsD}, \text{prsE}, \) and \( \text{exsH} \) single mutants, the double mutants exhibited no detectable delay in production of Calcofluor halos. This is consistent with the three genes functioning in one pathway.

We also tested whether multiple copies of the \( \text{prsD/prsE/exsH} \) genes could restore Calcofluor halo production to haloless mutants by multicopy suppression, as opposed to complementation. The results of our complementation analyses had already indicated that extra copies of the \( \text{prsD}^+ \) gene do not restore Calcofluor halo production to a \( \text{prsE exoK} \) mutant, that extra copies of the \( \text{prsE}^+ \) gene do not restore Calcofluor halo production to a \( \text{prsD exoK} \) mutant, and that extra copies of the \( \text{prsD}^+/\text{prsE}^+ \) genes do not restore Calcofluor halo production to an \( \text{exsH exoK} \) mutant (Table 2). However, we determined that extra copies of the \( \text{exsH}^+ \) gene can partially suppress the haloless defects of \( \text{prsD exoK} \) and \( \text{prsE exoK} \) strains (Table 2). Specifically, we transferred the plasmids \( \text{pD56} \) and \( \text{pG307} \), which carry the \( \text{exsH}^+ \) gene, into the \( \text{prsD exoK} \) and \( \text{prsE exoK} \) strains, and observed that both \( \text{pD56} \) and \( \text{pG307} \) enable both of these strains to produce Calcofluor halos, though with a delay more severe than that of an \( \text{exoK} \) single mutant. Derivatives of \( \text{pD56} \) with \( \text{Tn5} \) insertions in the \( \text{exsH} \) gene fail to restore halo production, implying that the \( \text{exsH}^+ \) gene, rather than another gene on \( \text{pD56} \) or \( \text{pG307} \), is responsible for restoration of Calcofluor halos. Thus, the \( \text{prsD} \) and \( \text{prsE} \) gene products facilitate, but are not absolutely required for, mediation of Calcofluor halo production by ExsH, when the \( \text{exsH} \) gene is present in multiple copies.

**Succinoglycan of haloless \( \text{prsD exoK}, \text{prsE exoK}, \) and \( \text{exsH exoK} \) colonies, but not haloless \( \text{exoH} \) colonies, can be converted to a diffusible, Calcofluor halo form by glycanases diffusing from adjacent colonies.** To test whether PrsD and PrsE may mediate secretion of ExsH, we devised an assay to detect production of extracellular glycanases by \( R. \text{meliloti} \). We cultivated colonies of haloless indicator strains in close proximity to colonies of tester strains, and observed whether particular tester strains could mediate extracellular suppression of the
haloless phenotype of an indicator strain. We reasoned that if a strain was haloless due to its inability to produce or secrete glycanases, then glycanases diffusing from adjacent colonies should degrade succinoglycan at the surface of the normally haloless colony, releasing diffusible succinoglycan and causing a Calcofluor halo to form. Alternately, if a strain was haloless due to properties of the succinoglycan produced by that strain, then additional glycanase activity provided in trans should not cause a Calcofluor halo to form.

We used the following haloless strains as indicators: \textit{prsD exoK}, \textit{prsE exoK}, \textit{exsH exoK}, \textit{exoH}, \textit{exoH exsH}, \textit{exoH exoK}, and \textit{exoH exsH exoK}. The results, summarized in Table 3, indicate that the haloless phenotype of the \textit{exoH} mutant is distinct from that of the \textit{prsD exoK}, \textit{prsE exoK}, and \textit{exsH exoK} strains. Apparently, \textit{prsD exoK}, \textit{prsE exoK}, and \textit{exsH exoK} mutants fail to produce or secrete activities that convert succinoglycan at the surface of colonies to a diffusible form, but the succinoglycan produced by these strains can be converted to a diffusible Calcofluor halo form by activities provided in trans. In contrast, the nonsuccinylated form of succinoglycan produced by \textit{exoH} mutants can not be converted to a diffusible Calcofluor halo form, but \textit{exoH} mutants do secrete activities capable of degrading normal succinoglycan.

**Diffusion of succinoglycan degrading activities from \textit{R. meliloti} colonies is dependent on the \textit{exoK} and the \textit{prsD/prsE/exsH} genes.** We conducted a second, independent test to detect diffusion of polysaccharide degrading activities from colonies, based on a previously-described approach (159). Top agar supplemented with the polysaccharide of interest is poured over mature colonies, the colonies are incubated further to enable enzymes diffusing from the colonies to degrade polysaccharide in the top agar, and then the top agar is treated with Congo Red dye (159). In general, Congo Red binds HMW but not LMW forms of polysaccharides that contain \(\beta\)-1,3 and/or \(\beta\)-1,4 linkages, such as succinoglycan, and Congo Red appears bright red when bound (159). The top agar stains bright red where HMW polysaccharide remains and is clear where polysaccharide degrading activities have diffused from colonies.

Using MGS agar as growth medium, we tested the following substrates: barley glucan
(β-1,3-1,4-linkages), carboxymethylcellulose (β-1,4-linkages), laminarin (β-1,3-linkages), and succinoglycan. Our results, which are summarized in Table 4, indicate that exoK mutants exhibit a delay in production of diffusible succinoglycan degrading activities with respect to wild-type, prsD, prsE, and exsH strains. prsD exoK, prsE exoK, and exsH exoK mutants produce no detectable succinoglycan degrading activities. Wild-type strains, but not exoK mutants, also mediate slow degradation of barley glucan. No diffusible carboxymethylcellulose or laminarin activities were detected from colonies of any R. meliloti strains. Thus, our results imply that ExoK and ExsH degrade succinoglycan with a high degree of specificity, that ExoK is apparently better able to cleave succinoglycan than is ExsH, and that no additional succinoglycan degrading activities can be detected diffusing from R. meliloti colonies grown on MGS medium.

**prsD and prsE mutants exhibit defects in production of several extracellular proteins.** Our analyses clearly implicate the prsD and prsE genes in production of an extracellular glycanase. To further clarify the roles played by these genes in this process, we compared the SDS PAGE profiles of proteins prepared from supernatants of exoY, exoY prsD, exoY prsE, and exoY exsH strains. We used exoY strains, which fail to produce succinoglycan and thus yield culture supernatants of relatively low viscosity, to improve the efficiency of removal of cells from culture supernatants by centrifugation. At least two proteins (140 kDa and 60 kDa) which are clearly present in exoY and exoY exsH supernatants are absent in exoY prsD and exoY prsE supernatants (Fig. 7). In this analysis, we have not been able to unambiguously assign a particular protein band to the ExsH protein, perhaps because R. meliloti produces additional proteins which migrate similarly to ExsH in SDS PAGE analyses or because ExsH is produced at relatively low levels. Nonetheless, it is clear that the profiles of extracellular proteins produced by exoY prsD and exoY prsE mutants is consistent with PrsD/PrsE mediating secretion of several proteins. The simplest model consistent with the rest of our data is that PrsD/PrsE also mediate secretion of the ExsH succinoglycan depolymerase.
The *exoK* and *prsD/prsE/exsH* genes contribute to production of LMW succinoglycan in cultures. We reasoned that if wild-type *R. meliloti* produces succinoglycan depolymerases, these enzymes may contribute to production of LMW succinoglycan in cultures. Thus, we determined the molecular weight distribution of succinoglycan in cultures of *R. meliloti* strains to examine whether defects in production and secretion of glycanases correlate with an increase in the ratio of HMW to LMW succinoglycan in cultures.

For these experiments, we cultured strains in a glutamate-mannitol-salts minimal medium (GMS medium). GMS is a lower osmolarity medium in comparison to MGS. In GMS medium the extracellular carbohydrate produced by Rm1021 consists almost entirely of succinoglycan, except for traces of cyclic-β-glucans (92). In contrast, we observed that in MGS medium, in addition to producing succinoglycan, the strain Rm1021 produces large quantities of other extracellular carbohydrates (data not shown). Thus, we used GMS medium to simplify our analyses of succinoglycan produced in cultures.

The molecular weight distribution of succinoglycan produced by Rm1021 cultured in GMS medium has been reported previously (8, 92). About half of the succinoglycan occurs as HMW forms and about half occurs as LMW forms, as determined by fractionation of extracellular carbohydrate from cultures by Biogel A5 gel filtration chromatography (92). Furthermore, fractionation of LMW succinoglycan by Biogel P4 chromatography revealed that the LMW forms consist of monomers and oligomers of the octasaccharide repeating unit (8).

Using these same techniques to assay *R. meliloti* cultures, we determined that mutations in the *exoK*, *prsD*, *prsE*, and *exsH* genes cause an increase in the ratio of HMW to LMW succinoglycan that accumulates in cultures (Table 5). The *prsD exoK*, *prsE exoK*, and *exsH exoK* double mutants exhibit the most severe phenotypes, each accumulating approximately 97% of their extracellular carbohydrate as HMW succinoglycan.

As controls for these experiments, we determined that each of the strains produces approximately equal amounts of extracellular carbohydrate in GMS medium (data not shown) and that this carbohydrate consists almost entirely of succinoglycan. To do this, we treated
extracellular carbohydrate from culture supernatants of each strain with succinoglycan depolymerase purified from *Cytophaga arvensicola* (75) and determined that for each sample approximately 97% of the carbohydrate eluted from a Biogel P4 gel filtration column at the same position as monomers of the octasaccharide repeating unit of succinoglycan. Furthermore, treatment of HMW carbohydrate from each strain with the succinoglycan depolymerase converted each sample to a form that migrated in TLC analyses like the fully modified octasaccharide repeating unit of succinoglycan (data not shown). Thus our results indicate that the decrease in accumulation of LMW succinoglycan in cultures of strains mutated in the *exoK*, *prsD*, *prsE*, and *exsH* genes is more easily explained by a decrease in conversion of succinoglycan from HMW forms to LMW forms rather than by a defect in direct synthesis of LMW forms or by a switch to production of novel polysaccharides.

The *prsD/prsE/exsH* genes are not required for establishment of symbiosis in wild-type or *exoK* strain backgrounds. Since LMW succinoglycan has been implicated in *R. meliloti* invasion of alfalfa root nodules, and since cultures of the *exsH exoK* mutant accumulate little or no LMW succinoglycan, we tested whether the *prsD, prsE, and exsH* genes are required for establishment of symbiosis. We determined that the *prsD, prsE, and exsH* single mutants, and the *prsD exoK, prsE exoK, and exsH exoK* double mutants, all establish an effective symbiosis over the same time course as Rm1021 and *exoK* strains. Our results imply that neither the PrsD/PrsE secretion system, nor the proposed ExoK and ExsH succinoglycan depolymerases, are required for establishment of symbiosis between *R. meliloti* and alfalfa.
DISCUSSION

The *R. meliloti* prsD and prsE genes are proposed to encode components of a Type I secretion system which mediates secretion of the *exsH* gene product. By mutagenizing *exoK* mutants and isolating colonies which fail to produce Calcofluor halos, we have identified three genes, designated prsD, prsE, and exsH, which contribute to production of Calcofluor halos by *R. meliloti*, independently of the *exoK* gene. We have determined that the prsD and prsE genes encode homologs of the ABC transporter and the membrane fusion protein, respectively, of Type I secretion systems, and that the *exsH* gene encodes a glycanase with features typical of proteins secreted by Type I secretion systems. We have also determined that the *exoK* gene and the prsD/prsE/exsH genes are components of parallel pathways for production of Calcofluor halos, production of extracellular succinoglycan degrading activities, and production of LMW succinoglycan. On the basis of our results, we propose that i) ExsH is a succinoglycan depolymerase which is secreted by a Type I secretion system composed of PrsD/PrsE, and ii) the ExoK and ExsH succinoglycan depolymerases both contribute to production of LMW succinoglycan.

According to the current model for Type I secretion pathways, the transport system consists of three components: an ABC type transporter, a membrane fusion protein, and an outer membrane component (55, 152). In general, mutants lacking any one of the three components of the secretion system exhibit a phenotype such that the proteins normally targeted for secretion instead accumulate in the cytoplasm (152). At this point, the PrsD/PrsE Type I secretion system we propose conspicuously lacks a homolog of the outer membrane component. It is formally possible that the *R. meliloti* PrsD/PrsE system functions without such a component, but this seems unlikely since no Type I secretion system described to date has been proven to function without such a component. It seems more likely that the *R. meliloti* PrsD/PrsE system also includes an outer membrane component, but that our transposon mutagenesis screen failed to reveal gene(s) encoding such a component. Possible explanations for this could be that the gene encoding the outer membrane component is required for viability or that multiple genes exist
which encode functionally interchangeable outer membrane components. In either case, our screening method, based on transposon mutagenesis, would not be expected to be useful in identifying such genes.

Additional evidence for Type I secretion systems in rhizobial strains. Evidence for Type I secretion systems in rhizobial strains has been reported previously. The NodO protein of *R. leguminosarum* bv. *viciae* contains glycine rich repeats similar to those conserved among RTX proteins (52). When expressed in *E. coli*, the NodO protein is secreted in a manner dependent upon the presence of the *hlyB/hlyD/tolC* genes or the *prtD/prtE/prtF* genes (134). Furthermore, when the cloned *nodO* + gene was expressed in various rhizobial strains, including *R. meliloti*, NodO protein was secreted (134). Thus, it was proposed that Type I secretion systems exist in the various rhizobial strains (134).

Recently, homologs of PrsD and PrsE have been identified in *Rhizobium leguminosarum* bv. *viciae* and in *R. leguminosarum* bv. *trifolii*. Finnie *et. al.* (58) identified homologous genes of *R. leguminosarum* bv. *viciae* by conducting a mutant screen to identify genes necessary for secretion of the NodO protein and demonstrated that secretion of NodO and several other proteins, including an EPS degrading enzyme, is dependent upon these genes. The nucleotide sequence of homologous genes, designated *resD* and *resE*, from *Rhizobium leguminosarum* bv. *trifolii* has also been reported recently (90).

Additional *R. meliloti* genes encoding homologs of Type I secretion proteins, which are distinct from *prsD* and *prsE*, have also been reported recently. The nucleotide sequence of the *exp* gene cluster, implicated in EPS II production, has been determined (16). The *expD1* and *expD2* genes encode homologs of the ABC transporter and the membrane fusion protein, respectively, of a Type I secretion system (16). Furthermore, the *expE1* gene, which is located immediately downstream of the *expD1* and *expD2* genes, encodes a protein homologous to NodO (16). Although the *expD1*, *expD2*, and *expE1* genes are crucial for EPS II production (16, 65), the precise roles they play in this process have not been determined. It also has not been
determined whether the previously reported secretion of NodO protein by *R. meliloti* is dependent upon the *prsD/prsE* genes or the *expD1/expD2* genes, but it seems likely that at least one and perhaps both pairs of these genes are involved. Regarding this point, it is noteworthy that the *exp* genes are transcribed at low levels in a wild-type background but at high levels in *expRI01* and *mucR* backgrounds (16, 65, 86).

**ExsH is a succinoglycan depolymerase.** Both the *exsH* and *exoK* genes encode homologs of endo-1,3-1,4-β-glycanases of Family 16 of glycosyl hydrolases (38). The presence of either the *exsH*+ gene or the *exoK*+ gene correlates with diffusion of succinoglycan degrading activities from colonies on MGS medium. The presence of either the *exsH*+ gene or *exoK*+ gene also correlates with the accumulation of LMW succinoglycan in GMS culture supernatants. Based on these parallels between the functions and sequences of the *exoK*+ and *exsH*+ genes, we conclude that ExsH, like ExoK, is most likely a endo-1,3-1,4-β-glycanase which depolymerizes succinoglycan. It is intriguing that the *exoK*+ gene has a greater effect than the *exsH*+ gene in terms of mediating degradation of succinoglycan on MGS agar medium, but that the *exsH*+ gene has a greater effect than the *exoK*+ gene in terms of mediating accumulation of LMW succinoglycan in GMS culture medium. These differences may reflect different mechanisms for regulation of the *exoK* and *exsH* genes or different optimal conditions for activity of ExoK and ExsH.

Our results indicate that the *prsD* and *prsE* genes play an important role in facilitating the process of ExsH mediated production of Calcofluor halos and LMW succinoglycan, and thus imply that to a large extent ExsH mediated depolymerization of succinoglycan occurs beyond the cytoplasm. However, while in most respects *prsD* and *prsE* mutants are phenotypically identical to *exsH* mutants in terms of expression of succinoglycan depolymerase activity, we did observe one difference. Extra copies of the *exsH*+ gene, provided on a cosmid, partially suppress the haloless Calcofluor phenotype of *prsD exoK* and *prsE exoK* mutants, whereas extra copies of the *prsD*+ and *prsE*+ genes do not suppress the *exsH exoK* mutant haloless phenotype. This genetic
distinction implies that, even in the absence of the PrsD/PrsE secretion system, ExsH can mediate depolymerization of succinoglycan to a small extent when the \textit{exsH}+ gene is expressed at a higher level than normal. This activity may be due to cellular lysis associated with overexpression of ExsH or to a low level of secretion of ExsH by other ABC type transport systems expressed by \textit{R. meliloti}, such as the proposed ExpD1/ExpD2 system. In addition, it is formally possible that ExsH may accomplish a minor amount of succinoglycan depolymerization within the cytoplasm, if HMW succinoglycan is present there.

Analyses of the Congo Red and Calcofluor phenotypes of \textit{R. meliloti} colonies imply that, like ExsH, the ExoK succinoglycan depolymerase reaches the extracellular environment. ExoK is not secreted in a PrsD/PrsE dependent manner, but instead may be secreted in a Type II \textit{sec}-dependent manner, based on the occurrence of a predicted N-terminal signal peptide in the \textit{exoK} gene product (10). It is not yet clear to what extent the extracellular localization of ExoK is crucial for its activity.

\textbf{Molecular mechanisms for production of low molecular weight succinoglycan and their relationship to nodule invasion.} If a highly charged, oligomeric form of LMW succinoglycan is indeed crucial for mediating nodule invasion, as proposed by Battisti \textit{et al.} (1992), then there must be a mechanism for generating it independently of ExoK and ExsH, since \textit{exsH exoK} mutants are proficient in establishment of symbiosis. For example, it may be the case that \textit{R. meliloti} can express additional parallel pathways for production of LMW succinoglycan under different growth conditions than those tested here.

Growth conditions can dramatically affect the ratio of HMW to LMW succinoglycan produced by \textit{R. meliloti}; high osmolarity conditions correlate with production of predominantly HMW succinoglycan whereas low osmolarity conditions correlate with production of predominantly LMW succinoglycan (22). Analyses of succinoglycan produced by wild-type, \textit{exoK}, \textit{exsH}, and \textit{exsH exoK} strains, cultured in growth media of a broad range of osmolarities, could reveal the extent to which ExoK, ExsH, and additional parallel pathways contribute to
production of LMW succinoglycan under different growth conditions.

There is increasing evidence that \textit{R. meliloti} is also capable of producing LMW succinoglycan by direct synthesis. The \textit{exoP}, \textit{exoQ}, and \textit{exoT} genes have been proposed to be involved in polymerization and transport of succinoglycan (126). \textit{ExoP} in particular may regulate the degree of polymerization of succinoglycan (14). Also, recently González, Semino, and Walker have determined that the \textit{exoQ} and \textit{exoT} gene products may play a specific role in synthesis of HMW succinoglycan and LMW succinoglycan, respectively, and that the \textit{exoP} gene product may be required for polymerization of both forms of succinoglycan (70).

\textit{R. meliloti} exhibits many striking functional redundancies with respect to production and processing of EPS. \textit{R. meliloti} has the capacity to produce succinoglycan, EPS II, and a capsular polysaccharide termed KPS (128), and each polysaccharide alone is capable of mediating nodule invasion in particular \textit{R. meliloti} genetic backgrounds. It is becoming apparent that \textit{R. meliloti} has the capacity to produce LMW succinoglycan by multiple pathways, and may also express multiple protein secretion pathways. Functional redundancy of parallel genetic pathways complicates mutant analyses by increasing the difficulty associated with demonstrating the physiological relevance of any one particular pathway. However, given the large genetic investment made by \textit{R. meliloti} in maintaining these functionally redundant pathways, it seems likely that they have physiological relevance for the bacterium. Further genetic analyses should help to clarify this issue.
ACKNOWLEDGMENTS

We thank C. Finnie and J. A. Downie for sharing unpublished data regarding analyses of the Rhizobium leguminosarum bv. viciae prsD and prsE genes and A. Becker for sharing unpublished data regarding the expD1 and expD2 genes. We also thank Latoya Maynard for construction of the exoK exoH and exsH exoK exoH strains and Sumati Murli and Juan E. González for critical reading of the manuscript. This work was supported by Public Health Service grant GM31030 from the National Institutes of Health.
Fig. 1. Calcofluor halo phenotypes of *Rhizobium meliloti* strains. A fluorescent halo develops around wild-type colonies over the course of several days. The onset of halo formation is delayed for *exoK* colonies. *hal-1* colonies (and *hal-2* through *hal-13* single mutant colonies) produce halos like those of wild-type colonies. *hal-1 exoK* colonies (and *hal-2 exoK* through *hal-13 exoK* double mutant colonies) produce no fluorescent halos.
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<td>hal-1 exoK</td>
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**Fig. 2.** Restriction map of 5.3 kb fragment of cosmid pG100, which complements the *prsD* and *prsE* mutations. Restriction sites for *BamHI* (B), *EcoRI* (E), and *PstI* (P) are shown. Positions of the *prsD* and *prsE* genes and the mutations *hal-1* through *hal-12* are indicated. Additional mutations, designated 21 through 25, were generated by Tn5 or mini-Tn5 Km mutagenesis of cloned DNA. These mutations were homogenotized into the *R. meliloti* chromosome and transduced into an *exoK* mutant. The positions of these mutations are also indicated. (●) indicates Tn5, (▲) indicates Tn5-233, and (▲, ▼) indicate mini-Tn5 Km. Filled symbols indicate that mutations block production of Calcofluor halos in the *exoK* mutant background. Open symbols indicate that mutations have no effect on Calcofluor halo production. Nucleotide sequence was determined for a 3.9 kb fragment, extending from upstream of *prsD* to the end of fragment cloned in pG115.
Fig. 3. Comparison of the deduced amino acid sequences of *E. chrysanthemi* PrtD, *S. marcescens* PrtDSM, *P. aeruginosa* AprD, and *R. meliloti* PrsD. Alignment was generated by PileUp program. Amino acids which are identical among at least three of the proteins are indicated in capitals. The Walker box A site and B site are underlined.
481  
PrtD  igLARA\text{mYGd}  PcLliLDEPN  a\text{LDsEGdQA}  Lm\text{qAIvALqk}  RGaTV\text{vLiTH}  RP\text{aIttlaqk}  i\text{ILheGGqQ}  rm\text{GlardVlt}  
PrtDSM  v\text{ALARALYGs}  PaLVV\text{LDEPN}  a\text{NLDrEGEeA}  Lqr\text{AIeALka}  R\text{GnTivLvTH}  k\text{PaiLattDk}  1\text{LvLtaGQvQ}  hf\text{GpsdaiLk}  
AprD  i\text{ALARALYGa}  PtLVV\text{LDEPN}  s\text{NLDdsGEQA}  Lla\text{AIqALka}  R\text{GcTV\text{vLiTH}}  Rag\text{vLgcaDr}  1\text{LaLnaGQlh}  1y\text{GerdqVLa}  
PrsD  v\text{ALARALYGd}  PfLVV\text{LDEPN}  s\text{NLDaEGEQA}  Lse\text{AImsvrs}  R\text{GgiVivvaH}  R\text{PsalasvDl}  v\text{LmmneGrmQ}  af\text{GpkeqVLg}  
560  
PrtD  e\text{Lq.q.rsAan}  q\text{armnptaam pq}........  
PrtDSM  k\text{LpgfapAaa}  v\text{apantgrsn ggfnvyanf aktasgerkv}  
AprD  a\text{LnnqraAsa sqqradyrva gypaqvva}  prqggve...  
PrsD  q\text{v1rpqqyer qnalkvvaeg qeakg}........
Fig. 4. Comparison of the deduced amino acid sequences of *E. chrysanthemi* PrtE, *S. marcescens* PrtESM, *P. aeruginosa* AprE, and *R. meliloti* PrsE. Alignment was generated by PileUp program. Amino acids which are identical among at least three of the proteins are indicated in capitals.
Fig. 5. Position of the *hal-13* mutation and the *exsH* gene, relative to the *exsA* and *exsB* genes and the *exo* region. pEX154, pD56, and pD2 have been described previously (103). Also shown are 20 Tn5 mutations, designated 308 through 358, which were previously generated in pD56 and homogenotized into the *R. meliloti* chromosome (103). We determined that for the pD56 derivatives containing each mutation 308 through 358, only those containing mutations 308, 334, and 343 fail to restore Calcofluor halo production to the *hal-13 exoK* strain. Also, only mutations 308, 334, and 343 cause a Calcofluor haloless phenotype when transduced into an *exoK* strain.
Fig. 6. (A) Deduced amino acid sequence of the N-terminal half of ExsH (amino acids 1 to 221). Underlined are four repeats similar to the consensus LXGGXG(N/D)DX, where X is any amino acid, which is conserved among RTX proteins. (B) Comparison of the C-terminal half of ExsH (amino acids 222 to 465) and the homologous domains of the endo-1,3-1,4-β-glycanases BglA of *Rhodothermus marinus* and LicA of *Clostridium thermocellum*. Amino acids which are identical among at least two of the proteins are indicated in capitals.
(a) MSKTVLNAV G TPLYYSGST AWFSATGSGP TLHGTA GNDS MGWGSSVNV T MIGGREDDIY YLYSSINRAY EAAGEGVDI T
81 STWMSYTLPA NFENLTVTGS GRPAFGEAAD NIINKGSGTQ TIDORRGNDV LIGACGADTF VFARONGSDL ITDFNYDDIV
161 RLDGYGFTSF EQILSNVAQE GADLRLHLAD GESLVFANTT ADELQAHQFR LSLDRSVLSQ T

(b) 222 fSDEFNtlqL rNgtsgvwd a kWNPapeKGa t lssNGEqQw Yinpsy Joe T a svNsfsvnnG V LItaA pas eaiaqaeinG Y
301 GwWPAfW1LP aDgswe.. ..ppElDvV e mrGqdsntVi aTVHsne...
BglA WSDEFdYsgL p3pek.......WdYdvGg hGwNgB1Qy YTr......A rieNArVggG V LiI.EARhE py.....E Gr
LicA WSDEFWngseI .Nman..... .WsYDdpt nGrwNgeVqs YTq....... .NNAYiKdG aLvi.EARKE ditepsgEtY
381 GwWPAfW1LP aDgswe.. ..ppElDvV e mrGqdsntVi aTVHsne...
BglA eYTSarLvTr GKaSWTYGrF EIRAr1PsGr GtWPAIWMLP dqrtygsayW PdnGEIDIME hvGinPDvVh GTVHtKaynh
LicA hYTSskLiTx GKKSWkYGRF EIRAKMPQGQ GiWPAIWMmP eDepfygt.W PkcGEIDIME l1GhePDkiy GTIHaFgBPb.
461 GwWPAfW1LP aDgswe.. ..ppElDvV e mrGqdsntVi aTVHsne...
BglA llgTrqrgSI rvPtarT... DFHYya1EWT PEEIRWYVdVd sLYy...rf pinelrtdeap dwrhwPFDPQ PnLImNIAVG
LicA ....keSqGty tlPegTfAd DFHYYSIEWe PgEIRWYidG kLYhVAndwy srdpyladdy tyra.FFDQn FFL11NIsVG
501 GwWPAfW1LP aDgswe.. ..ppElDvV e mrGqdsntVi aTVHsne...
BglA GwWGQqgvd peaFPaqlVv DYVRVYrwe .......... LicA GgWpGypD.e ttvPpQVMV DYVRVYqkDk yphrekpake
Fig. 7. SDS PAGE separation and Coomassie blue staining of extracellular proteins prepared from *R. meliloti* culture supernatants. Lines indicate positions of molecular weight markers (kDa). Top two arrows indicate proteins which are clearly present in *exoY* and *exoY exsH* supernatants but are absent from *exoY prsD* and *exoY prsE* supernatants. Arrow with asterisk indicates predicted position of ExsH protein. The presence of additional proteins produced by *R. meliloti* which migrate similarly to ExsH may obscure detection of a band due to ExsH. Alternately, ExsH protein may be produced in quantities too small to detect by this approach. Lanes: 1) *exoY* strain, 2) *exoY prsD* strain, 3) *exoY prsE* strain, 4) *exoY exsH* strain.
Table 1. Strains used in this study.

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+++ = normal Calcofluor halo development  
++ = delayed onset of Calcofluor halo development, typical of exoK mutant  
+ = delayed onset of Calcofluor halo development, more severe than that of exoK mutant  
- = haloless phenotype  
N.D. = not determined

*In these cases restoration of Calcofluor halo development for the hal-1 exoK strain (prsD exoK strain) and the hal-6 exoK strain (prsE exoK strain) was due to suppression mediated by the exsH<sup>+</sup> gene rather than complementation mediated by the prsD<sup>+</sup> or prsE<sup>+</sup> genes.
Table 3. Testing for extracellular suppression of Calcofluor haloless phenotypes of indicator strain colonies, mediated by adjacent tester strain colonies.

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<th>Tester strain genotype</th>
<th>Indicator strain genotype</th>
<th>Extracellular suppression of indicator strain haloless phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>exsH exoK</td>
<td>+</td>
</tr>
<tr>
<td>prsD</td>
<td>exsH exoK</td>
<td>+</td>
</tr>
<tr>
<td>prsE</td>
<td>exsH exoK</td>
<td>+</td>
</tr>
<tr>
<td>exsH&lt;sup&gt;a&lt;/sup&gt;</td>
<td>exsH exoK</td>
<td>+</td>
</tr>
<tr>
<td>exoK&lt;sup&gt;a&lt;/sup&gt;</td>
<td>exsH exoK</td>
<td>+</td>
</tr>
<tr>
<td>prsD exoK</td>
<td>exsH exoK</td>
<td>-</td>
</tr>
<tr>
<td>prsE exoK</td>
<td>exsH exoK</td>
<td>-</td>
</tr>
<tr>
<td>exsH exoK&lt;sup&gt;a&lt;/sup&gt;</td>
<td>exsH exoK</td>
<td>-</td>
</tr>
<tr>
<td>wild type</td>
<td>exoH</td>
<td>-</td>
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<tr>
<td>exsH</td>
<td>exoH</td>
<td>-</td>
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<tr>
<td>exoK</td>
<td>exoH</td>
<td>-</td>
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<tr>
<td>exsH exoK</td>
<td>exoH</td>
<td>-</td>
</tr>
<tr>
<td>wild type</td>
<td>exoH exsH exoK</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Introduction of an *exoH* mutation (which converts a strain to haloless phenotype) or an *exoY* mutation (which knocks out succinoglycan synthesis) into the given tester strain did not change the extracellular suppression phenotype.

<sup>b</sup> Identical results were obtained with *prsD exoK* and *prsE exoK* as indicator strains.
Table 4. Testing for diffusion of polysaccharide degrading activities from *R. meliloti* colonies by the Congo Red colony assay.

<table>
<thead>
<tr>
<th>strain</th>
<th>succinoglycan</th>
<th>barley glucan</th>
<th>carboxymethyl-cellulose</th>
<th>laminarin</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>prsD</em></td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>prsE</em></td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>exsH</em></td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>exoK</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>prsD exoK</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>prsE exoK</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>exsH exoK</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

++ = clear halos visible by 8 hours  
+ = clear halos visible by 24 hours  
- = no clear halos visible after 48 hours
Table 5. Ratios of high molecular weight (HMW) to low molecular weight (LMW) extracellular carbohydrate in culture supernatants (expressed as percentage of total carbohydrate).*

<table>
<thead>
<tr>
<th>succinoglycan</th>
<th>Rm1021</th>
<th>exoK</th>
<th>prsD</th>
<th>prsE</th>
<th>exsH</th>
<th>exoK prsD</th>
<th>exoK prsE</th>
<th>exoK exsH</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMW</td>
<td>52**</td>
<td>66**</td>
<td>93</td>
<td>95</td>
<td>91</td>
<td>98</td>
<td>97</td>
<td>97</td>
</tr>
<tr>
<td>LMW</td>
<td>48</td>
<td>34</td>
<td>7</td>
<td>5</td>
<td>9</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

* Data represent average of two samples separated by Biogel A5 column chromatography. Standard deviations were less than or equal to 1%, except where noted.

** Standard deviation less than 3.5%.
Chapter 3

The *Rhizobium meliloti* ExoK and ExsH Glycanases Specifically Depolymerize Nascent Succinoglycan Chains*

ABSTRACT

The *Rhizobium meliloti* ExoK and ExsH glycanases have been proposed to contribute to production of low-molecular-weight (LMW) succinoglycan by depolymerizing high-molecular-weight (HMW) succinoglycan chains in *R. meliloti* cultures. We expressed and purified ExoK and ExsH and determined that neither enzyme can extensively cleave succinoglycan prepared from *R. meliloti* cultures, although neutral/heat-treatment and acid/heat-treatment convert succinoglycan to forms that can be cleaved efficiently by both enzymes. These results were somewhat surprising, given that the *exoK*+ and *exsH*+ genes play a crucial role in production of LMW succinoglycan in *R. meliloti* cultures. We demonstrated by Western blot analyses that *R. meliloti* expresses ExoK and ExsH, that both proteins can be detected extracellularly, and that ExsH secretion is dependent upon the *prsD*+/*prsE*+ genes, consistent with previous predictions based on mutant analyses. Furthermore, we determined that the depolymerization activities associated with purified ExoK and ExsH are comparable to *exoK*+ and *exsH*+-dependent depolymerization activities expressed in *R. meliloti* cultures. We resolved the apparent contradiction between the results of our previous genetic analyses and depolymerization assays by determining that ExoK and ExsH can cleave HMW succinoglycan that is being actively produced by *R. meliloti*, but not succinoglycan that has accumulated in cultures, to yield LMW succinoglycan. We propose that ExoK and ExsH dynamically regulate the molecular weight distribution of succinoglycan by cleaving nascent succinoglycan only during a limited period after its synthesis, perhaps before it undergoes a time-dependent change in its conformation or aggregation state.
INTRODUCTION

The nitrogen fixing symbioses established between soil bacteria of the *Rhizobiaceae* and leguminous plants are complex developmental processes involving the exchange of multiple signals between the bacteria and their hosts (61, 105). For rhizobial-legume symbioses that involve the formation of indeterminate root nodules, such as the *Rhizobium meliloti*-alfalfa symbiosis, bacterial production of extracellular polysaccharides (EPS) is crucial for successful invasion of root nodules (21, 31, 34, 57, 94). The precise roles played by bacterial EPS in the invasion process are not known, but evidence that particular forms of EPS exhibit specificity for particular symbioses (46, 65) and that small quantities of EPS are sufficient to mediate invasion by EPS deficient mutants (8, 46, 69) suggests the possibility that EPS plays a signaling role in invasion.

*R. meliloti* has the capacity to produce two types of EPS, succinoglycan (1, 83) and EPS II (65, 79, 85, 123, 166), and a capsular polysaccharide, KPS (128). Each of these polysaccharides can be produced in either symbiotically active or symbiotically inactive forms (65, 69, 93, 94, 128). Of these polysaccharides, the wild-type strain Rm1021 produces only succinoglycan in a symbiotically active form and thus relies on succinoglycan production for invasion (57, 94). The structure of succinoglycan has been determined: succinoglycan is a polymer of an octasaccharide repeating unit, consisting of galactose, glucose, acetate, succinate, and pyruvate in a ratio of approximately 1:7:1:1:1 (1, 83). In cultures, *R. meliloti* produces succinoglycan in high-molecular-weight (HMW) forms, consisting of hundreds to thousands of octasaccharide repeating units, and low-molecular-weight (LMW) forms, consisting of monomers, trimers, and tetramers of the octasaccharide repeating unit (8, 22, 92). LMW forms of succinoglycan have been proposed to be the forms crucial for establishment of symbiosis (8, 150).

The genetic analysis of succinoglycan production by *R. meliloti* has been greatly facilitated by the use of Calcofluor, a fluorescent dye that binds succinoglycan. When colonies of the wild-type strain are cultivated on growth medium supplemented with Calcofluor and are
visualized under UV light, the colonies fluoresce brightly due to Calcofluor binding to the succinoglycan produced by the colonies (94). Mutagenesis of the wild-type strain, followed by screening for nonfluorescing colonies, enabled the isolation of many mutants defective in production of succinoglycan (94, 103, 113). A group of genes involved in regulation, synthesis, and processing of succinoglycan have been cloned and sequenced, and most of these genes are located in a cluster termed the \textit{exo} region (10, 11, 12, 67, 68, 103, 113). Based on analyses of the nucleotide sequence of the \textit{exo} genes and characterization of the succinoglycan biosynthetic intermediates that accumulate in the cells of \textit{exo} mutants, a model for succinoglycan biosynthesis has been proposed (126).

How \textit{R. meliloti} controls the molecular weight distribution of succinoglycan is less well understood. Two simple models are that the bacteria produce LMW succinoglycan 1) by direct synthesis or 2) by expressing glycanases that cleave HMW succinoglycan to yield LMW forms. These two models are not mutually exclusive; indeed evidence has been reported in support of both. Regarding the first model, truncation of the \textit{exoP} gene, proposed to be involved in polymerization of succinoglycan, causes a marked decrease in the ratio of HMW:LMW succinoglycan produced by \textit{R. meliloti} (14). Also, González \textit{et al.} have obtained evidence for genetically separable systems for the direct synthesis of LMW and HMW succinoglycan (J. E. González, C. E. Semino, L. E. Castellano-Torres, and G. C. Walker; unpublished results).

Regarding the second model, we have previously reported that \textit{R. meliloti} strains with transposon mutations in the \textit{exoK} gene and either the \textit{exsH}, \textit{prsD}, or \textit{prsE} gene exhibit a dramatic defect in production of LMW succinoglycan (162). Based on nucleotide sequence analyses, ExoK and ExsH are predicted to belong to two distinct subclasses of endo-1,3-1,4-β-glycanases (10, 68, 162). Enzymes of this type would be predicted to cleave HMW succinoglycan to yield monomers of the octasaccharide repeating unit, structurally identical to the octasaccharide subunits generated during succinoglycan biosynthesis (148), or multimers of the repeating unit. Thus, ExoK and ExsH are excellent candidates for proteins directly involved in depolymerization of HMW succinoglycan to yield LMW forms. Both ExoK and ExsH are predicted to accumulate
extracellularly (162). In the case of ExsH in particular, its extracellular localization is predicted to be crucial for its activity, given that the prsD and prsE genes encode the ABC-type transporter and the membrane fusion protein, respectively, of a Type I secretion system, that the exsH gene encodes a domain typical of proteins secreted by Type I secretion systems, and that mutations in any one of these three genes cause defects in production of LMW succinoglycan of the same magnitude (162).

To refine our model for how glycanases contribute to production of LMW succinoglycan, we tested ExoK and ExsH in reconstituted succinoglycan-depolymerization reactions. We demonstrated that ExoK and ExsH can depolymerize succinoglycan prepared from R. meliloti cultures, though we found that treatments that alter the physical properties of succinoglycan greatly affect the extent to which ExoK and ExsH can cleave the polysaccharide. Strikingly, we also determined that ExoK and ExsH can cleave succinoglycan produced by actively growing cells, but not succinoglycan in cell-free culture supernatants, to yield LMW succinoglycan. We infer that the physiologically relevant role of these glycanases is to cleave nascent succinoglycan chains rather than to cleave succinoglycan which has accumulated extracellularly.
MATERIALS AND METHODS

Strains, Growth Media, and Preparation of Succinoglycan. The *R. meliloti* strains Rm1021 (wild-type), Rm7210 (exoY), Rm8832 (exoY exoK), Rm8833 (exoY exsH), Rm8834 (exoY exoK exsH), Rm8835 (exoY prsD), and Rm8836 (exoY prsE) (162), and the *Escherichia coli* strain BL21(DE3) (145) have been described. *R. meliloti* strains were cultivated at 30°C as described (8). The following growth media were used: Luria-Bertani (LB) (107), M9 (107), MGS (pH 7.4) (162), and GMS (162). Succinoglycan was prepared from MGS cultures of Rm1021 as previously described (130).

Expression, Recovery, and Analysis of ExoK and ExsH. To construct pEXOK and pEXSH, we amplified the complete *exoK* and *exsH* ORFs by PCR while simultaneously introducing restriction sites (*Nde*I and *Bcl*I for *exoK* and *Nde*I and *Bam*HI for *exsH*) at the ends of the fragments, and then cloned these fragments into the vector pET5a (Promega), which had been digested with *Nde*I and *Bam*HI. We used previously described techniques to induce expression of ExoK and ExsH in the strain BL21(DE3) (145). For preparation of ExoK, ExsH, and soluble protein from BL21(DE3)/pET5a, cells from 100 ml of culture were resuspended in buffer (50 mM KCl, 50 mM HEPES (pH 7.5), 1 mM EDTA, 0.5% Nonidet P-40, 1 mM DTT) and were lysed by sonication. ExoK and ExsH were isolated by denaturing insoluble cellular proteins in buffer (8 M urea, 50 mM HEPES, 50 mM DTT), dialyzing samples against buffer (5 M urea, 25 mM HEPES, 100 mM KCl) for 2 hours, and removing urea and DTT by further staged dialysis against buffer (25 mM HEPES, 100 mM KCl). All protein preparation steps were conducted at 4°C. Protein samples were separated by discontinuous SDS-PAGE (10% polyacrylamide separating gels) and visualized by staining with Coomassie brilliant blue, as previously described (3).

Carbohydrate Analyses. For *in vitro* depolymerization assays, succinoglycan (approximately 0.3 mg/ml) was dissolved in reaction buffer (50 mM potassium phosphate (pH
7.0), 1 mM MgSO₄, 0.25 mM CaCl₂), treated with ExoK, ExsH, or soluble protein from BL21(DE3)/pET5a (0.16 µg/ml to 20 µg/ml final concentrations) and incubated at 30°C for 24 hours. Carbohydrate concentrations and relative reducing end concentrations were determined by the anthrone-sulfuric acid method (102) and the Lever method (100), respectively. We used previously described methods for Biogel A5-m and Biogel P4 column chromatography (8, 92, 162).

To determine the reducing end generated by ExoK and ExsH-mediated cleavage of acid/heat-treated succinoglycan, enzyme-digested samples were passed over a Biogel P4 column and monomers of octasaccharide were recovered. 150 µg samples were then lyophilized, dissolved in 1 ml water with 2 mg sodium borohydride, incubated 12 hours at room temperature, treated with 30 µl 50% acetic acid, and desalted by passage over a Biogel P4 column. Reduced monomer samples were acid hydrolyzed (2 N trifluoroacetic acid, 121°C for 2 hours), lyophilized to remove the acid, dissolved in water, and analyzed by high pressure anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD, Dionex). To confirm that extracellular carbohydrate present in cultures was succinoglycan, we used essentially the same method as described above, except that 1) extracellular carbohydrate was completely depolymerized as described previously (162) and 2) the step of reduction by sodium borohydride was omitted.
RESULTS

Expression of ExoK and ExsH in Escherichia coli. The *R. meliloti* exoK and exsH genes have been proposed to encode extracellular endo-1,3-1,4-β-glycansases of 30 kDa and 50 kDa, respectively, that depolymerize high-molecular-weight (HMW) succinoglycan to yield low-molecular-weight (LMW) succinoglycan in *R. meliloti* culture supernatants (10, 68, 162). In order to reconstitute these depolymerization reactions *in vitro*, we used the pET expression system to generate large quantities of ExoK and ExsH. Specifically, we amplified the complete *exoK* and *exsH* open reading frames (ORFs) by PCR, cloned the amplified ORFs into the vector pET5a, confirmed that the two cloned ORFs were free of nucleotide sequence errors, and designated the two resulting plasmids pEXOK and pEXSH. We transferred each of the plasmids pEXOK, pEXSH, and pET5a into the *E. coli* strain BL21(DE3) and cultivated the resulting strains under conditions that induce expression of ORFs cloned into pET5a. We determined that the strain containing pEXOK expresses a pair of ca. 31 kDa proteins that are unique to this strain (Fig. 1). These proteins correspond to full length or processed forms of ExoK. The strain containing pEXSH expresses a 51 kDa protein that corresponds to ExsH (Fig. 1). We have confirmed these assignments by Western blot analyses (see below). When expressed by the pET system, ExoK and ExsH are predominantly associated with proteins that remain insoluble after sonication of cells. We purified ExoK and ExsH to a substantial degree simply by subjecting these insoluble protein fractions to a denaturation/renaturation treatment followed by centrifugation to remove insoluble proteins. ExoK and ExsH comprise a high proportion of the remaining soluble proteins derived from the pEXOK and pEXSH containing strains, respectively.

Native succinoglycan is highly refractory to depolymerization by ExoK and ExsH. We proceeded to test these soluble ExoK and ExsH preparations for succinoglycan depolymerase activity *in vitro*, under conditions relevant to the function of these enzymes *in vivo*. Reactions were incubated at 30°C, the standard growth temperature for *R. meliloti*. The substrate for these
reactions was HMW succinoglycan purified from wild-type *R. meliloti* culture supernatants (native succinoglycan). And, since our previous analyses of *R. meliloti* strains implied that ExoK and ExsH function extracellularly, and that for ExsH in particular its extracellular localization is crucial for its activity, we used an assay buffer that simulates typical *R. meliloti* growth media.

We made the striking observation that under these conditions ExoK and ExsH can cleave succinoglycan only to a slight extent. Treatment of native succinoglycan (375 µg/ml) with ExoK protein (20 µg/ml) for 24 hours yielded a 2.5 fold increase in the concentration of reducing ends, as determined by the Lever reducing end assay (Fig. 2), and converted about 3% of the HMW succinoglycan to LMW forms, as determined by Biogel P4 gel filtration chromatography. Similar treatment with ExsH protein (20 µg/ml) yielded a 1.2 fold increase in the concentration of reducing ends (Fig. 2) and also converted about 3% of the HMW succinoglycan to LMW forms. Thus, neither ExoK nor ExsH were cleaving succinoglycan extensively. For comparison, succinoglycan that had been depolymerized completely to monomers of the octasaccharide repeating unit by treatment with succinoglycan depolymerase from *Cytophaga arvensicola* (75) exhibited a 12 fold increase in the concentration of reducing ends (Fig. 2). It is worth noting that the Lever assay overestimates the concentration of reducing ends for polysaccharides, presumably due to alkaline hydrolysis of polysaccharide chains during the course of the assay (74); thus we use the Lever assay here not to measure the precise degree of polymerization of samples but to provide a qualitative comparison of the extent of depolymerization among samples.

We observed that ExoK and ExsH remained active over the 24 hour time course of the depolymerization reactions described above, and that extending the reaction incubation times or supplementing reactions with additional ExoK or ExsH caused little or no increase in the extent of cleavage of succinoglycan. Thus our results implied that a large proportion of the succinoglycan in these samples was refractory to cleavage by ExoK and ExsH. To gain insights into why the succinoglycan was refractory to cleavage, we heated succinoglycan solutions to 100°C for several minutes at neutral pH (neutral/heat-treatment) or to 100°C for 90 minutes in 50
mM oxalic acid (acid/heat treatment), followed by cooling of the succinoglycan solutions to room temperature (and neutralization and dialysis of acid/heat-treated samples). These treatments irreversibly converted succinoglycan to forms that were more susceptible to cleavage by ExoK and ExsH (Fig. 2). For example, incubation of acid/heat-treated succinoglycan with ExoK (20 μg/ml) or ExsH (20 μg/ml) caused the conversion of 31% or 22% of the total succinoglycan to LMW forms, respectively. We determined that extracts of soluble proteins derived from the strain containing only the pET5a vector caused no cleavage of any form of succinoglycan. This control indicates that the cleavage of succinoglycan is due to ExoK or ExsH, respectively, and not due to any contaminating *E. coli* proteins present in the preparations. Furthermore, by purifying samples of depolymerized acid/heat-treated succinoglycan and subjecting them to sodium borohydride reduction, acid hydrolysis, and high performance anion exchange chromatography coupled with pulsed amperometric detection, we determined that both ExoK and ExsH cleave succinoglycan to yield galactose at the reducing end of the cleaved product (data not shown). Thus, we have demonstrated that ExoK and ExsH are endo-1,3-1,4-β-glycanases, as predicted by nucleotide sequence (10, 68, 162).

*R. meliloti* expresses and secretes ExoK and ExsH. The observation that native succinoglycan is highly refractory to cleavage by ExoK and ExsH was somewhat surprising, since our previous genetic analyses had implied that ExoK and ExsH cleave succinoglycan in culture supernatants (162). To help resolve this apparent contradiction, we raised polyclonal antibodies against ExoK and ExsH purified from the strains carrying pEXOK and pEXSH and used the antibodies to measure expression and secretion of ExoK and ExsH by *R. meliloti*. For these assays we tested *R. meliloti* strains cultivated in two types of growth media, MGS, in which the *exoK* gene is the major determinant of Calcofluor halo production by *R. meliloti* colonies (103, 162), and GMS, in which the *exsH/prsD/prsE* genes are the major determinants of production of LMW succinoglycan by *R. meliloti* cells (162).

We determined that *R. meliloti* expresses ExoK. Antibodies against ExoK protein bind
specifically to a 31 kDa protein (ExoK) that is expressed by *R. meliloti* cells with the *exoK* gene but that is not expressed by *exoK* mutants (Fig. 3). While ExoK accumulates to approximately the same levels in cells cultivated in either MGS or GMS medium (data not shown), ExoK accumulates to about 25-fold higher levels in culture supernatants in MGS medium (approximately 160 ng ExoK/ml) than it does in GMS medium (approximately 6.4 ng ExoK/ml) (Fig. 4).

We also determined that *R. meliloti* expresses ExsH. Antibodies raised against ExsH bind specifically to a 51 kDa protein (ExsH) that is present at high levels in culture supernatants of the wild-type strain but not in culture supernatants of *exsH*, *prsD*, or *prsE* mutants (Fig. 3). Whether strains are cultivated in MGS or GMS, ExsH accumulates in supernatants to approximately the same levels (approximately 160 ng ExsH/ml) (Fig. 4). ExsH also can be detected in *R. meliloti* cells of the wild-type strain but not in cells of *exsH* mutants (Fig. 3). The *prsD* and *prsE* mutants exhibit a substantial defect in the intracellular accumulation of ExsH but, in contrast to the case for *exsH* mutants, ExsH can be detected inside *prsD* and *prsE* mutants upon prolonged exposure of Western blots to film (data not shown). Here we have directly demonstrated that 1) *R. meliloti* expresses ExoK and ExsH, 2) ExoK and ExsH accumulate in culture supernatants early during the growth phase of cultures, indicating that they are likely being secreted rather than diffusing from lysed cells, and 3) secretion of ExsH but not ExoK is dependent upon the *prsD+/prsE+* genes (162). Our analyses also indicate that the concentrations of ExoK and ExsH in culture supernatants are at least 125-fold less than the highest concentrations of ExoK and ExsH we tested in the *in vitro* depolymerization assays. Thus, we have ruled out the possibility that expression of extremely high levels of ExoK and ExsH by *R. meliloti* might account for the contribution of these enzymes to production of LMW succinoglycan *in vivo*.

By directly testing *R. meliloti* cultures for glycanase activity by use of a Congo Red dye assay, we determined that an *exoK*+-dependent glycanase activity can be detected for strains cultivated in MGS but not GMS medium and that an *exsH*+-dependent glycanase activity can be detected for strains cultivated in GMS but not MGS medium (unpublished results). The *exoK*
result is consistent with our Western blot analyses (Fig. 4); ExoK is secreted to a much higher extent in MGS than in GMS cultures. The \textit{exsH} result is consistent with the observation that purified ExsH is much more active in GMS than in MGS medium (data not shown). Our results indicate that, while growth conditions have a dramatic effect on the accumulation and activity of ExoK and ExsH in \textit{R. meliloti} culture supernatants, the activities of ExoK and ExsH purified from \textit{E. coli} are comparable to the activities of ExoK and ExsH expressed by \textit{R. meliloti}.

**ExoK and ExsH efficiently cleave succinoglycan of cells actively synthesizing succinoglycan.**

To explain the apparent contradiction between 1) the large contribution of the \textit{exoK}+ gene and the \textit{exsH}+/\textit{prsD}+/\textit{prsE}+ genes to production of LMW succinoglycan in \textit{R. meliloti} cultures, and 2) the low efficiency with which ExoK and ExsH cleave native succinoglycan, we reasoned that, \textit{in vivo}, only nascent succinoglycan chains may be susceptible to cleavage and that they then become refractory to cleavage after a short time. To test this hypothesis, we treated a GMS medium culture of the \textit{R. meliloti exoK exsH} strain with 1) ExoK, 2) ExsH, 3) succinoglycan depolymerase of \textit{C. arvensicola}, 4) soluble proteins derived from the strain carrying pET5a, or 5) water, for a total of 24 hours. Treatments were conducted either i) in the presence of \textit{exoK exsH} cells actively producing succinoglycan, by treating day 3 cultures, or ii) in the absence of \textit{exoK exsH} cells, by removing cells from day 4 cultures and then treating the cell-free culture supernatants. Thus, for both sets of treatments the cultures were incubating and producing succinoglycan for the same total amount of time.

We observed that the addition to cultures of ExoK or ExsH protein caused 30% or 23%, respectively, of the total extracellular carbohydrate to accumulate as LMW forms (as determined by Biogel A5-m gel filtration chromatography), but only when the enzymes were added to cultures containing cells actively producing succinoglycan (Fig. 5). Addition of ExoK or ExsH to cell-free supernatants of day 3 \textit{exoK exsH} cultures (data not shown) or cell-free supernatants of day 4 \textit{exoK exsH} cultures caused no accumulation of LMW succinoglycan (Fig. 5). As expected, succinoglycan depolymerase from \textit{C. arvensicola} caused the accumulation of LMW
succinoglycan whether or not cells were present, and treatment with soluble proteins derived from the strain carrying pET5a or with water did not cause the accumulation of LMW succinoglycan in either case (Fig. 5). As a control, we confirmed that the LMW carbohydrate that accumulated in cultures treated with ExoK, ExsH, and succinoglycan depolymerase of *C. arvensicola* was actually succinoglycan rather than some other extracellular carbohydrate released by cells (data not shown). Our results directly demonstrate that ExoK and ExsH can contribute to production of LMW succinoglycan in *R. meliloti* cultures, presumably by depolymerizing HMW succinoglycan. Furthermore, the observation that cells must be present for efficient cleavage of succinoglycan by ExoK and ExsH in cultures, along with the results of our analyses of the activities of ExoK and ExsH produced by *R. meliloti* cultures, strongly suggests that ExoK and ExsH efficiently cleave succinoglycan chains only during a limited period after the newly synthesized succinoglycan chains emerge from the cell.
DISCUSSION

In the course of our experiments to test whether ExoK and ExsH are indeed succinoglycan depolymerases, we made an unexpected discovery. When added exogenously to cultures, both ExoK and ExsH are capable of efficiently cleaving succinoglycan from *R. meliloti* cells that are actively synthesizing the polysaccharide, but neither ExoK nor ExsH efficiently cleaves succinoglycan that is present in cell-free culture supernatants. The sum of our results leads to a striking inference; the physiologically relevant role of ExoK and ExsH in generating LMW succinoglycan is based on the enzymes specifically cleaving nascent succinoglycan rather than cleaving HMW succinoglycan that accumulates in the extracellular environment. Furthermore, ExoK and ExsH are clearly different from the succinoglycan depolymerase from *C. arvensicola*, which cleaves native succinoglycan extensively and which thus plays an important role in utilization of succinoglycan as a carbon source. The strikingly different properties of the *R. meliloti* glycanases suggest a different biological role.

The change that nascent succinoglycan undergoes that renders it insensitive to cleavage may be a time-dependent change in its conformation or aggregation state. Previous studies of the physical properties of succinoglycan may provide a useful framework for interpreting results from the depolymerization assays. Solutions of native succinoglycan are distinctive for their high viscosity, which may be attributed to non-uniform helicity of chains and/or lateral aggregation of chains (particularly at high succinoglycan concentrations) (26, 56, 71, 72). Neutral/heat-treatment of succinoglycan causes the succinoglycan to undergo a conformational transition resulting in an irreversible decrease in the viscosity of solutions upon cooling (56, 71, 72); this decrease correlates with conversion of succinoglycan to aggregates of uniformly helical chains (26). Acid/heat-treatment of succinoglycan causes an even greater decrease in viscosity, which has been attributed to shortening of chains (due to acid hydrolysis) as well as to a decreased tendency of chains to aggregate (56). Since our depolymerization results clearly establish that treatments that cause a decrease in viscosity of succinoglycan solutions correlate with an increase in susceptibility of succinoglycan to cleavage by ExoK and ExsH, we speculate that the extent of
helicity and/or aggregation of succinoglycan chains determine the extent to which succinoglycan can be cleaved by ExoK and ExsH.

The physical properties of succinoglycan may also have important biological consequences regarding the production of LMW succinoglycan in *R. meliloti* cultures. In the absence of salt, aggregation of succinoglycan occurs only at high succinoglycan concentrations (>1 mg/ml), but in the presence of even low concentrations of salt (10 mM sodium chloride) aggregation can occur at much lower succinoglycan concentrations (26). Based on the concentrations of salts in MGS and GMS media, succinoglycan would be predicted to aggregate in *R. meliloti* culture supernatants. One possible explanation for the high efficiency with which ExoK and ExsH cleave succinoglycan of cells actively synthesizing the polysaccharide is that nascent succinoglycan chains are highly susceptible to cleavage by ExoK and ExsH prior to aggregation of the chains. An endochitinase has been demonstrated to exhibit at least 80-fold greater activity and to yield a different set of cleaved oligosaccharide products when cleaving nascent chitin chains versus chitin chains present in insoluble aggregates (112). However, the situation must be different with respect to succinoglycan; since all forms of succinoglycan are soluble, the change in the conformation or state of aggregation must be more subtle.

Our results indicate that the type of growth medium used can exert dramatic post-translational effects on the accumulation and activity of the *R. meliloti* glycanases. ExoK, which may be secreted by the Type II secretion pathway (162), accumulates to approximately 25-fold greater amounts in *R. meliloti* culture supernatants in MGS versus GMS medium. This observation raises the possibility that *R. meliloti* may regulate the secretion of certain proteins, such as ExoK, in response to environmental conditions. Such an ability to regulate the nature of proteins it secretes could be an important factor underlying the various bacterial-plant interactions that occur during the development of a productive symbiosis. ExsH, which is secreted by a Type I secretion system (162), accumulates to approximately the same level in either medium, but the enzyme is much more active in GMS versus MGS. The decreased activity of ExsH in MGS may be due to effects of growth medium components on the enzyme itself or on
the physical properties of the substrate, succinoglycan.

Why does *R. meliloti* produce a polysaccharide and also express glycanases that depolymerize it? Perhaps this is a reflection of the importance of LMW succinoglycan in establishment of symbiosis. Although mutant analyses indicate that the *exoK, exsH, prsD, and prsE* genes are not required for establishment of symbiosis in an otherwise wild-type background (103, 162), it is possible that in the absence of ExoK and ExsH other mechanisms, such as direct synthesis of LMW succinoglycan, provide sufficient LMW succinoglycan for establishment of symbiosis. Alternatively, the production of a polysaccharide and a corresponding glycanase may provide a more general selective advantage to bacteria. Several other strains of bacteria, such as *R. leguminosarum* bv. *viciae* (58), *Bradyrhizobium japonicum* (49), and *Pseudomonas marginalis* (116), have been found to exhibit this trait, indicating that the phenomenon may be widespread.
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Fig. 1. SDS-PAGE analysis and Coomassie blue staining of proteins expressed by (1) BL21(DE3)/pEXOK, (2) BL21(DE3)/pEXSH, or (3) BL21(DE3)/pET5a. Approximately 2 μg of protein was loaded per lane. Treatments: (a) insoluble protein remaining after sonication of cells, (b) protein recovered after denaturation/renaturation procedure, (c) soluble fraction of renatured protein. Lines indicate positions of molecular weight markers (kDa). Arrows indicate position of ExoK (*) and ExsH (**).
Fig. 2. Plot of OD$_{410}$ versus depolymerase concentration for samples of succinoglycan treated for 24 hours with either ExoK (squares) or ExsH (triangles), then subjected to the Lever reducing end assay. We tested a series of five-fold dilutions of the two proteins, starting at a concentration of 20 μg/ml. Substrates: (A) 0.38 mg/ml native succinoglycan, (B) 0.27 mg/ml neutral/heat-treated succinoglycan, (C) 0.20 mg/ml acid/heat-treated succinoglycan. Arrows indicate OD$_{410}$ for untreated succinoglycan (*) or succinoglycan depolymerized completely to octasaccharide by succinoglycan depolymerase of *Cytophaga arvensicola**( )*. Each data point represents the average of three samples (standard deviations < 10%).
Fig. 3. Western blot analyses of expression of ExoK (A, B) and ExsH (C, D) by *Rhizobium meliloti* strains. Lanes contain either the cells (without supernatant) from the equivalent of 10 µl of culture (A, C) or 5 µl of cell-free supernatant (B, D). Strains were cultured either in MGS medium (A, B) or GMS medium (C, D). We used *exoY* mutant strains, which fail to produce succinoglycan, to enable the high-efficiency removal of cells from culture supernatants by centrifugation. Strains: 1) *exoY*, 2) *exoY exoK*, 3) *exoY exsH*, 4) *exoY exoK exsH*, 5) *exoY prsD*, 6) *exoY prsE*. Lines indicate positions of molecular weight markers (kDa). Arrow for (C) indicates position of protein band corresponding to ExsH, which is poorly resolved from another *R. meliloti* protein.
Fig. 4. Western blot analyses that compare the extent to which ExoK (A) and ExsH (B) accumulate in the culture supernatants of *exoY* strains cultivated in MGS versus GMS medium. Lanes 1 through 8 contain 5 μl of cell-free culture supernatant from an *exoY* strain, grown in GMS (lanes 1-4) or MGS (lanes 5-8). Supernatants were collected on day 1 (lanes 1, 5), day 2 (lanes 2, 6), day 3 (lanes 3, 7) and day 5 (lanes 4, 8) of incubation of cultures. We also loaded 5 μl aliquots of a five-fold dilution series of the soluble protein fraction of BL21(DE3)/pEXOK (A) or BL21(DE3)/pEXSH (B), corresponding to concentrations of 1.3 ng/ml (lane 9), 6.4 ng/ml (lane 10), 32 ng/ml (lane 11), and 160 ng/ml (lane 12). For both blots we also loaded 5 μl of 160 ng/ml of soluble protein from BL21(DE3)/pET5a (lane 13), and confirmed that the antibodies used here bind specifically to ExoK and ExsH rather than endogenous *Escherichia coli* proteins.
Fig. 5. Plot of OD$_{620}$ versus column fraction numbers, for cell-free culture supernatants of the $exoK$ $exsH$ strain cultivated in GMS, subjected to various treatments, and passed over a Biogel A5-m gel filtration column. Plots represent cultures from which cells were removed by centrifugation at day 4 prior to treatment (dashed line) or cultures that were treated at day 3 with cells still present (solid line). In both cases treatments were conducted for 24 hours. In the latter case cells were removed by centrifugation after the 24 hour treatment. Treatments: (A) ExoK (20μg/ml), (B) ExsH (20 μg/ml), (C) succinoglycan depolymerase of *Cytophaga arvensicola*, (D) soluble protein from BL21(DE3)/pET5a, and (E) water.
Bio-gel A-5m column fraction number

Optical density 620
Chapter 4

The Succinyl and Acetyl Modifications of Succinoglycan Influence the Susceptibility of Succinoglycan to Cleavage by the *Rhizobium meliloti* Glycanases ExoK and ExsH*

ABSTRACT

In *Rhizobium meliloti* cultures, the endo-1,3-1,4-β-glycanases ExoK and ExsH depolymerize nascent high-molecular-weight (HMW) succinoglycan to yield low-molecular-weight (LMW) succinoglycan. We report here that the succinyl and acetyl modifications of succinoglycan influence the susceptibility of succinoglycan to cleavage by these glycanases. It was previously shown that *exoH* mutants, which are blocked in succinylation of succinoglycan, exhibit a defect in production of LMW succinoglycan. We have determined that *exoZ* mutants, which are blocked in acetylation of succinoglycan, exhibit an increase in production of LMW succinoglycan. For both wild-type and *exoZ* strains, production of LMW succinoglycan is dependent on the *exoK*<sup>+</sup> and *exsH*<sup>+</sup> genes, implying that the ExoK and ExsH glycanases cleave HMW succinoglycan to yield LMW succinoglycan. By supplementing cultures of glycanase-deficient strains with exogenously added ExoK or ExsH, we have demonstrated directly that the absence of the acetyl group increases the susceptibility of succinoglycan to cleavage by ExoK and ExsH, that the absence of the succinyl group decreases the susceptibility of succinoglycan to cleavage, and that the succinyl effect outweighs the acetyl effect for succinoglycan lacking both modifications. Strikingly, non-succinylated succinoglycan actually can be cleaved by ExoK and ExsH to yield LMW succinoglycan, but only when the glycanases are added to cultures at greater than physiologically relevant concentrations. Thus, we conclude that the molecular weight distribution of succinoglycan in *R. meliloti* cultures is determined by both the levels of ExoK and ExsH glycanase expression and the susceptibility of succinoglycan to cleavage.
INTRODUCTION

Bacterial polysaccharides are crucial for establishment of the nitrogen fixing symbiosis between the soil bacterium *Rhizobium meliloti* and its host plant alfalfa (57, 94). *R. meliloti* has the capacity to produce two exopolysaccharides (EPSs), succinoglycan and EPS II, as well as a capsular polysaccharide, KPS (79, 123, 128). Each polysaccharide can be produced in symbiotically-active or inactive forms, and the bacterium must produce at least one of these polysaccharides in a symbiotically-active form in order to invade root nodules successfully (65, 69, 93, 94, 129). For example, the wild-type strain Rm1021, which fails to produce EPS II and which produces KPS in a symbiotically-inactive form, depends on production of succinoglycan in a symbiotically-active form for successful root nodule invasion. The symbiotically-active forms of these polysaccharides may function as signals to facilitate the nodule invasion process (8, 69).

Succinoglycan is a polymer of an octasaccharide repeating unit consisting of one galactose and seven glucose units and modified with approximately one acetyl, succinyl, and pyruvyl modification per repeating unit (1, 83, 123). Wild-type *R. meliloti* produces succinoglycan in low-molecular-weight (LMW) forms, consisting of short oligomers of the octasaccharide repeating unit, and in high-molecular-weight (HMW) forms, consisting of hundreds to thousands of repeating units (8, 92). The molecular weight distribution of succinoglycan (as well as EPS II) seems to be relevant to symbiotic activity (8, 69); specific LMW forms of succinoglycan, highly charged tetramers of the octasaccharide repeating unit, have been proposed to be active in mediating nodule invasion (8).

The steps of octasaccharide synthesis and modification are well-defined genetically (10, 11, 12, 67, 68, 94, 103, 113). A series of genes (termed *exo*) involved in succinoglycan biosynthesis have been cloned and sequenced (10, 11, 12, 67, 68, 103, 113). Most of these genes have been assigned functions in synthesis and modification of the octasaccharide repeating unit, based on analyses of radiolabeled lipid-linked intermediates of succinoglycan biosynthesis that accumulate in various *exo* mutants and based on nucleotide sequence data (67, 68, 126).
How \textit{R. meliloti} accomplishes the production of two distinct size classes of succinoglycan (LMW and HMW) from pools of lipid-linked octasaccharide repeating units is less clear. Becker \textit{et al.} (14, 15) have proposed that ExoP regulates the extent of succinoglycan polymerization, and González \textit{et al.} (70) have reported that ExoT and ExoQ are required for the direct synthesis of LMW and HMW succinoglycan, respectively. Thus, \textit{R. meliloti} apparently can produce LMW and HMW succinoglycan by conducting limited polymerization as well as extensive polymerization of octasaccharide repeating units.

In addition, we have obtained genetic and biochemical evidence that \textit{R. meliloti} can produce LMW succinoglycan by a second mechanism, depolymerization of HMW succinoglycan (162, 163). The \textit{R. meliloti} \textit{exoK} and \textit{exsH} genes encode endo-1,3-1,4-β-glycanases (10, 68, 162) that can depolymerize succinoglycan to yield monomers or multimers of the octasaccharide repeating unit (163). ExoK and ExsH have been implicated in production of LMW succinoglycan in cultures, based on our observations that \textit{exoK exsH} double mutants exhibit a dramatic defect in production of LMW (162) and that the exogenous addition of ExoK or ExsH to cultures of the \textit{exoK exsH} strain restores production of LMW succinoglycan (163). Curiously though, we observed that neither ExoK nor ExsH can efficiently cleave HMW succinoglycan \textit{in vitro} (163). We resolved this apparent contradiction by determining that ExoK and ExsH specifically depolymerize nascent succinoglycan, but not succinoglycan that has accumulated in culture supernatants, to yield LMW succinoglycan (163). Apparently succinoglycan undergoes a transition from a glycanase-susceptible form to a glycanase-refractory form in cultures. This transition may correspond to a change in the physical structure of succinoglycan molecules, such as a change from random coils to helices or from individual molecules to aggregates (26), for example.

We were interested in determining whether the succinyl and acetyl modifications of succinoglycan might affect the molecular weight distribution of succinoglycan by influencing the susceptibility of succinoglycan to cleavage by ExoK and ExsH. The fact that \textit{exoH} mutants, which synthesize non-succinylated succinoglycan (93), and \textit{exoK exsH} mutants, which fail to
express the ExoK and ExsH glycanases (162), produce almost exclusively HMW succinoglycan had suggested that the absence of the succinyl modification from succinoglycan inhibits glycanase-mediated cleavage of the polysaccharide. The molecular weight distribution of the succinoglycan produced by exoZ mutants, which synthesize non-acetylated succinoglycan (125), had not been examined. However, Hollingsworth et al. (80) had demonstrated that deacetylation of surface polysaccharides produced by another rhizobial strain, *Rhizobium leguminosarum* bv. *trifolii*, increases the susceptibility of these polysaccharides to cleavage by a bacteriophage-induced lyase.

Here we report that the acetyl and succinyl modifications of succinoglycan do indeed influence the susceptibility of succinoglycan to cleavage by the ExoK and ExsH glycanases. By integrating the analyses of mutants blocked in modification of succinoglycan and/or expression of glycanases with the reconstitution of glycanase activity in cultures, we have determined that non-acetylated succinoglycan has a high susceptibility to cleavage by ExoK and ExsH, that normally-modified succinoglycan has an intermediate susceptibility to cleavage, and that non-succinylated succinoglycan has a low susceptibility to cleavage. We have also determined that, like normally-modified succinoglycan, non-acetylated and non-succinylated forms of succinoglycan undergo transitions from glycanase-susceptible to glycanase-refractory forms as they accumulate in cultures. Our results indicate that the molecular weight distribution of each of the variously-modified forms of succinoglycan can be manipulated with a high degree of control simply by culturing the various *R. meliloti* strains in the presence of varying amounts of exogenously-added glycanase. Furthermore, the cleavage of succinoglycan by glycanases may have important implications in biological phenomena, such as the establishment of nitrogen fixing symbiosis and biofilm formation by *R. meliloti*. 
MATERIALS AND METHODS

Bacterial strains, growth conditions, and Calcofluor halo assay. Strains used in this study are listed in Table 1. The following growth media were used: Luria Bertani (LB) (107), MGS (potassium phosphate (100 mM, pH 7.3 for liquid cultures or pH 6.8 for plates), mannitol (55 mM), monosodium glutamate (5 mM), and sodium chloride (8 mM)), and GMS medium (containing mannitol at a final concentration of 27.5 mM) (165). MGS and GMS were also supplemented with magnesium sulfate (1 mM), calcium chloride (0.25 mM), biotin (100 µg/l) and thiamine (100 µg/l for GMS) after autoclaving of media. Agar (1.5%) was included for solid media. For detection of Calcofluor binding to R. meliloti colonies, we used the Fluorescent Brightener 28 disodium salt (Sigma, St. Louis, MO) as previously described (94). The method for detection of diffusion of extracellular glycanases from colonies, based on a tester colony causing a Calcofluor halo to occur for an adjacent exoK exsH indicator colony, has been described (162).

Cultivation of Rhizobium meliloti strains and analyses of extracellular proteins. To measure and compare production of extracellular carbohydrate or expression of glycanases by various R. meliloti strains, we incubated LB cultures to saturation, determined the OD600 of cultures, washed cells in sterile 0.85% saline, inoculated 50 ml GMS or MGS cultures in 250 ml flasks with equal titers of cells, and incubated cultures at 30°C with aeration until a given time point or until cultures reached a given level of extracellular carbohydrate.

For experiments involving analyses of extracellular proteins, cells were removed from aliquots of culture supernatants by centrifugation (20800 × g, 5 min). The protein in 5 µl aliquots of cell-free supernatants were separated by discontinuous SDS-PAGE (10% polyacrylamide separating gels) and analyzed by use of Tropix Western Light protein detection kit (Bedford, Massachusetts). Polyclonal antibodies that recognize ExoK and ExsH have been described previously (163).
Analyses of extracellular carbohydrate in culture supernatants. Strains were cultivated in GMS as described above. Cells were removed from culture supernatants by centrifugation (27000 × g, 20 min); samples which appeared viscous were diluted in 5 volumes of distilled water and were subjected to further centrifugation (20000 × g, 20 min). We used previously described approaches for Biogel A-5 chromatographic analyses (92). For A5 column chromatography, samples consisting of up to 2 mg of carbohydrate from lyophilized culture supernatants dissolved in a final volume of 20 ml of column buffer (sodium phosphate (50 mM, pH 7.0), sodium chloride (100 mM)) were applied to the column. Succinoglycan consistently elutes in distinct HMW (excluded volume) and LMW (included volume) fractions.

Carbohydrate concentrations and relative reducing end concentrations were determined by the anthrone-sulfuric acid method (102) and the Lever method (100), respectively. It is important to note that the Lever assay overestimates the actual concentration of reducing ends for a given polysaccharide sample, presumably due to alkaline hydrolysis of polysaccharide chains during the course of the assay (74). We use the Lever assay here not to measure the precise degree of polymerization of polysaccharides but to provide a relative measure of degree of polymerization between samples.

Depolymerization of succinoglycan by ExoK and ExsH added exogenously to culture supernatants. Expression and purification of ExoK and ExsH, by use of the pET5a vector (Promega, Madison, WI) in Escherichia coli, has been described (163). For experiments involving supplementation of cultures with exogenously added ExoK or ExsH, 2 ml aliquots of culture were transferred from 50 ml cultures (in 250 ml flasks) to test tubes at the time of addition of enzyme (or addition of water as a control), and these cultures were further incubated with aeration. For experiments in which glycanases were added to growth medium at the time of inoculation of growth medium with bacteria, 2 ml aliquots of culture were incubated in test tubes with aeration for the entire cultivation period. For experiments in which glycanases were added to cultures at physiologically relevant levels (200 ng/ml for ExsH) over the time course of culture
incubation, glycanases were added to cultures at the following time points to the cumulative final concentrations that are listed: 18 hours, to 40 ng/ml; 42 hours, to 160 ng/ml; and 66 hours, to 200 ng/ml). For experiments in which much higher levels of glycanases (10 μg/ml final concentration) were added over the time course of culture incubation, glycanases were added to cultures in eight equal aliquots at approximately 12 hour intervals, beginning at the time of inoculation of the cultures with bacteria. Otherwise, glycanases were added to cultures at the time points and to the final concentrations indicated in the Results section. As a control we had previously determined that addition of purified ExoK and ExsH to *R. meliloti* cultures does not cause the bacteria to produce any extracellular carbohydrate in addition to the succinoglycan that is normally produced (163).
RESULTS

*exoH* mutations decrease production of low-molecular-weight succinoglycan, and *exoZ* mutations increase production of low-molecular-weight succinoglycan. To establish whether the succinyl and acetyl modifications of succinoglycan may influence the susceptibility of succinoglycan to cleavage by glycanases, we determined the ratio of LMW succinoglycan versus HMW succinoglycan produced by strains defective in expression of glycanases and/or defective in modification of succinoglycan. Specifically, we tested *R. meliloti* strains representing each combination of the wild-type or mutant allele for the glycanase genes *exoK* and *exsH*, the succinyl transferase gene *exoH*, and the acetyl transferase gene *exoZ*. For these experiments, we cultivated strains for five days in GMS minimal medium, in which succinoglycan comprises approximately 97% of the extracellular carbohydrate produced by *R. meliloti* (92, 162), and then separated the succinoglycan into HMW and LMW fractions by Biogel A-5 gel filtration chromatography.

Leigh and Lee (92) had previously determined that the wild-type strain, when grown under these conditions, produces approximately half of its succinoglycan in HMW forms and half in LMW forms (Table 2). We subsequently demonstrated that this production of wild-type LMW succinoglycan is almost entirely dependent on expression of the glycanases ExoK and ExsH (162). Thus, *exoK* mutants exhibit a slight defect in production of LMW succinoglycan, *exsH* mutants exhibit a more dramatic defect, and *exoK exsH* mutants exhibit the most severe defect, producing approximately 3% of their total extracellular carbohydrate in LMW forms (Table 2) (162). The phenotypes of these mutants reflect a decrease in conversion of HMW succinoglycan to LMW succinoglycan by glycanases (162).

As reported by Leigh and Lee (92), *exoH* mutants, which are defective in succinylation of succinoglycan, exhibit a severe defect in production of LMW succinoglycan (Table 2). The defect of *exoH* mutants is of approximately the same magnitude as that of *exoK exsH* mutants, and is only minimally affected by the additional mutation of both the *exoK* and *exsH* genes (Table 2), suggesting that under these cultivation conditions non-succinylated succinoglycan is a
poor substrate for cleavage by ExoK and ExsH.

Strikingly, we determined that \textit{exoZ} mutants, which are defective in acetylation of succinoglycan, exhibit a phenotype opposite to that of \textit{exoH} and \textit{exoK exsH} mutants in terms of the molecular weight distribution of succinoglycan (Table 2). The \textit{exoZ} mutant exhibits an increase in the proportion of its succinoglycan that is present in LMW forms. To determine which mutations would be epistatic in multiply-mutant strains, we tested \textit{exoZ exoK}, \textit{exoZ exsH}, and \textit{exoZ exoK exsH} mutants and determined that production of LMW succinoglycan by \textit{exoZ} mutants is almost entirely dependent on the \textit{exoK}+ and \textit{exsH}+ genes. This result implies that in cultures of \textit{exoZ} mutants, as in the wild-type strain, ExoK and ExsH cleave HMW succinoglycan to yield LMW succinoglycan (Table 2) and suggests that non-acetylated succinoglycan is a better substrate for cleavage than is normally-modified succinoglycan. Analyses of \textit{exoH exoZ} mutants indicate that production of LMW succinoglycan by \textit{exoZ} mutants is also dependent on the \textit{exoH}+ gene, suggesting that the negative effect on production of LMW succinoglycan associated with the absence of the succinyl modification almost entirely outweighs the positive effect associated with the absence of the acetyl modification.

\textbf{Changes in levels of succinoglycan production or glycanase expression are not sufficient to account for the effects of the \textit{exoH} and \textit{exoZ} mutations on the molecular weight distribution of succinoglycan.} Perhaps the simplest hypothesis to explain our data is that the acetyl and succinyl modifications of succinoglycan influence the susceptibility of succinoglycan to cleavage by ExoK and ExsH. However, a plausible alternative hypothesis is that the \textit{exoH} and/or \textit{exoZ} mutations cause changes in levels of glycanase expression or succinoglycan production, resulting in shifts in the ratio of glycanase to succinoglycan that cause shifts in the molecular weight distribution of succinoglycan. For example, Becker \textit{et al.} (11) have reported that \textit{exoH::Tn5} mutations exhibit a highly polar effect on transcription of the downstream \textit{exoK} gene, raising the possibility that \textit{exoH} mutants are defective in production of LMW succinoglycan due to decreased glycanase expression. Furthermore, Buendia \textit{et al.} (25) have
reported that when colonies are cultivated on growth medium containing the succinoglycan-binding dye Calcofluor and are visualized under UV-light, \textit{exoZ} mutant colonies exhibit a delay in the onset of fluorescence relative to colonies of the wild-type strain. This suggests the possibility that \textit{exoZ} mutants exhibit a decreased rate of production of succinoglycan.

To test this alternative hypothesis, we first employed a simple plate assay that is based on the use of Calcofluor to detect diffusion of glycanase activity from colonies (162). It had been observed previously that wild-type colonies not only fluoresce brightly when cultivated on medium containing Calcofluor, but that the colonies also gradually produce fluorescent halos (Calcofluor halos) (93). In contrast, colonies of the glycanase-deficient strain \textit{exoK exsH} fail to produce Calcofluor halos (162). Calcofluor halos are presumed to correspond to forms of succinoglycan that are sufficiently short to diffuse from colonies, and ExoK and ExsH apparently are crucial for production of these diffusible forms of succinoglycan. The plate assay for detection of glycanase activity entails spotting colonies of tester strains adjacent to colonies of the haloless, glycanase-deficient indicator strain \textit{exoK exsH}. Over the time course of approximately one week, the ability of tester strains to produce active extracellular glycanases is revealed by the onset of Calcofluor halos around colonies of the indicator strain; extracellular glycanases diffuse from the tester colony and degrade the succinoglycan at the surface of the indicator colony, causing a halo to form. For this assay, we use a growth medium termed MGS (which contains higher levels of phosphate and mannitol than does GMS) because Calcofluor phenotypes of \textit{R. meliloti} colonies are easier to discern by use of MGS than GMS medium (162). It is important to note that ExoK accumulates extracellularly to much higher levels in MGS than in GMS, and that ExsH exhibits much lower activity in MGS than in GMS (163). Thus, ExoK makes a greater contribution than ExsH in determining the ability of colonies of a given tester strain to cause the appearance of a Calcofluor halo around colonies of an adjacent indicator strain on MGS medium, whereas ExsH makes a greater contribution than ExoK in mediating production of LMW succinoglycan in GMS cultures (162).

For our experiments here, we used as tester colonies wild-type, \textit{exoH}, and \textit{exoZ} strains,
with each possible combination of wild-type or mutant alleles for the \(\text{exoK}\) and \(\text{exsH}\) genes. We also conducted a parallel set of analyses using this set of tester strains but with the \(\text{exoY}\) mutation also present in each strain. This enabled us to determine unambiguously whether halos occurring around the \(\text{exoK exsH}\) indicator strain were actually derived from succinoglycan produced by the indicator strain and not the tester strain, since the \(\text{exoY}\) mutation (present in the tester strain) blocks the first step in succinoglycan biosynthesis. We observed that the \(\text{exoZ}\) mutation had no detectable effect on levels of \(\text{ExoK}\) or \(\text{ExsH}\) activity (Fig. 1). The \(\text{exoH}\) mutation had no detectable effect on levels of \(\text{ExsH}\) activity, but \(\text{exoH::Tn5}\) did cause a decrease in levels of \(\text{ExoK}\) activity (Fig. 1), presumably because of the previously mentioned polar effect of the \(\text{exoH::Tn5}\) mutation on transcription of the downstream \(\text{exoK}\) gene (11).

Western blot analyses provided independent corroboration of the Calcofluor halo results. Using polyclonal antibodies that recognize \(\text{ExoK}\) or \(\text{ExsH}\) (163), we tested the strains \(\text{exoY, exoY exoH, exoY exoZ, and exoY exoH exoZ}\) to measure extracellular levels of \(\text{ExsH}\) (as expressed in GMS cultures) and \(\text{ExoK}\) (as expressed in GMS and MGS cultures) (Fig. 2). Our results confirm that the \(\text{exoH}\) mutation causes a decrease in extracellular accumulation of \(\text{ExoK}\) in MGS medium, though none of the strains produce detectable amounts of extracellular \(\text{ExoK}\) in GMS medium. The \(\text{exoH}\) mutation has no effect on extracellular levels of \(\text{ExsH}\). Given that \(\text{ExsH}\) makes a far greater contribution than does \(\text{ExoK}\) in terms of production of LMW succinoglycan by strains grown in GMS (162), the effect of the \(\text{exoH}\) mutation on expression of \(\text{ExoK}\) is not sufficient to explain the severe defect in production of LMW succinoglycan associated with the \(\text{exoH}\) strain. Furthermore, our results indicate that the \(\text{exoZ}\) mutation does not cause an increase in production of extracellular \(\text{ExoK}\) or \(\text{ExsH}\). Thus, our results rule out the possibility that the differences in production of LMW succinoglycan associated with the \(\text{exoH}\) and \(\text{exoZ}\) mutants are caused by the effects of the \(\text{exoH}\) and \(\text{exoZ}\) mutations on glycanase expression.

To test the possibility that the \(\text{exoH}\) and/or \(\text{exoZ}\) mutations cause changes in levels of total succinoglycan production, we cultivated wild-type, \(\text{exoH}\), and \(\text{exoZ}\) mutants in parallel and compared levels of succinoglycan production over an incubation period of five days. The \(\text{exoH}\)
mutant exhibited no change in levels of succinoglycan production in comparison to the wild-type strain (data not shown). The exoZ mutant exhibits an approximately 25% decrease in succinoglycan production relative to the wild-type strain, consistent with the delay in the appearance of Calcofluor fluorescence associated with colonies of this strain. However, we have determined directly that this difference is too subtle to account for differences in the molecular weight distribution of succinoglycan in wild-type versus exoZ cultures (see below).

The absence of the acetyl modification of succinoglycan increases the susceptibility of succinoglycan to cleavage by ExoK and ExsH. To directly test whether the acetyl and succinyl modifications of succinoglycan influence cleavage of the polysaccharide by glycanases, we proceeded to reconstitute normal levels of extracellular glycanase in cultures of exoK exsH glycanase-deficient strains, to see whether this would restore production of LMW succinoglycan to levels typical of the corresponding exoK⁺ exsH⁺ glycanase-producing strains. Given that ExsH makes a greater contribution to production of LMW succinoglycan than does ExoK (for glycanase-producing strains cultivated in GMS medium), we focused first on ExsH. To cultures of the glycanase-deficient strains exoK exsH, exoZ exoK exsH, exoH exoK exsH, and exoH exoZ exoK exsH, we added physiologically relevant amounts of ExsH (163) gradually to a cumulative final concentration of 200 ng/ml, over a time course of four days. We then separated the succinoglycan present in cultures into HMW and LMW fractions by Biogel A-5 column chromatography. As expected, we observed a close match between levels of LMW succinoglycan in cultures of the various glycanase-deficient strains to which ExsH had been added exogenously (Table 3), in comparison to cultures of the corresponding glycanase-producing strains to which no exogenous glycanase had been added (Table 2). Clearly, non-succinylated succinoglycan is cleaved to little or no extent, normally-modified succinoglycan is cleaved to a moderate extent, and non-acetylated succinoglycan is cleaved to a large extent.

We proceeded to test directly whether subtle differences in total succinoglycan production might account for the large differences in production of LMW succinoglycan for
exoK exsH versus exoZ exoK exsH cultures. We inoculated a series of cultures with either of the two strains, varying the titers of inocula over a wide range, added identical, physiologically relevant levels of ExsH to each culture, and then compared the ratio of HMW to LMW succinoglycan present in cultures of the two strains in which varying total amounts of succinoglycan had been produced over the course of culture incubation. The difference in production of LMW succinoglycan in exoK exsH versus exoZ exoK exsH cultures is apparent across a wide range of levels of total succinoglycan production (Fig. 3). Apparently, the absence of the acetyl group from succinoglycan greatly increases the susceptibility of succinoglycan to cleavage by ExsH.

We extended these analyses by adding ExsH to a final concentration of 200 ng/ml entirely at the time of inoculation of cultures, to test whether the timing of addition of ExsH would affect the yield of LMW succinoglycan. Although there was no effect on exoH exoK exsH and exoH exoZ exoK exsH cultures, exoK exsH and exoZ exoK exsH cultures both exhibited a dramatic increase in the conversion of HMW succinoglycan to LMW succinoglycan (Table 3). Thus, it is not the case that some fixed proportion of wild-type and exoZ mutant succinoglycan are produced in glycanase-refractory forms. Instead, the timing of glycanase addition as well as glycanase concentration determines how much HMW succinoglycan is converted to LMW succinoglycan.

We extended these analyses further by testing ExoK (at a final concentration of 200 ng/ml), added either throughout the course of culture incubations or at the time of inoculation of cultures. Interestingly, although ExoK is less effective than ExsH in producing LMW succinoglycan under these conditions, addition of ExoK at the onset of culture inoculations did result in a detectable increase in production of LMW succinoglycan (Table 3). Apparently, under these conditions ExoK has a similar substrate preference, but is less active than ExsH.

The absence of the succinyl modification from succinoglycan dramatically decreases but does not absolutely block cleavage of succinoglycan by ExoK and ExsH. At this point
we wanted to determine whether non-succinylated succinoglycan is absolutely refractory to cleavage by the glycanases ExoK and ExsH, or whether it can be cleaved but at a much lower efficiency than normally-modified succinoglycan. To distinguish between these two possibilities, we tested the effect of supplementing cultures of glycanase-deficient strains with levels of the glycanases that are greatly in excess of those found physiologically. We added either ExoK or ExsH to cultures to a final concentration of 10 μg/ml, which is approximately 50-fold higher than the physiological level of ExsH in GMS cultures of the wild-type strain. When the enzymes are added gradually throughout the period of culture incubation, ExsH, and to a lesser extent ExoK, partially convert the non-succinylated HMW succinoglycan normally produced by \( \text{exoH} \ \text{exoK} \ \text{exoH} \) and \( \text{exoH} \ \text{exoZ} \ \text{exoK} \ \text{exoH} \) strains to LMW succinoglycan (Table 4). Thus, nonsuccinylated succinoglycan has a low susceptibility to cleavage by ExoK and ExsH, but it can be cleaved extensively when these enzymes are added to cultures at sufficiently high concentrations. In addition, virtually all of the normally-modified and non-acetylated succinoglycan produced by \( \text{exoK} \ \text{exoH} \) and \( \text{exoZ} \ \text{exoK} \ \text{exoH} \) strains is converted to LMW succinoglycan under these conditions (Table 4), consistent with our results involving lower concentrations of glycanase.

**Each of the variously-modified forms of succinoglycan becomes refractory to cleavage as it accumulates in cultures.** We previously determined that normally-modified succinoglycan becomes refractory to cleavage as it accumulates in cultures. We proceeded to test whether the other variously-modified forms of succinoglycan exhibit the same property. We determined that addition of glycanase to cultures at a point about halfway through the period of culture incubations resulted in a decrease in yield of LMW succinoglycan (Table 4), relative to cases for which glycanase was added throughout the period of culture incubation. This is consistent with our previously reported observation that succinoglycan becomes refractory to cleavage by glycanases as it accumulates in cultures (163). We then determined that addition of the entire glycanase sample at the end of the period of culture incubation (after removal of cells
by centrifugation), followed by a 24-hour period of incubation, resulted in little or no production of LMW succinoglycan for any of the strains (Table 4). These results demonstrate that all of the variously-modified forms of succinoglycan share the common property that, as they accumulate in culture supernatants, they become refractory to cleavage by the ExoK and ExsH glycanases.

**Increasing conversion of high-molecular-weight succinoglycan to low-molecular-weight succinoglycan by glycanases correlates with a decreasing degree of polymerization of the remaining high-molecular-weight succinoglycan in cultures.** The observation that ExoK and ExsH cleave HMW succinoglycan in cultures to yield LMW succinoglycan suggests that increasing production of LMW succinoglycan across a series of strains would likely correlate with a decreasing degree of polymerization of the remaining HMW succinoglycan in cultures of these strains, particularly if depolymerization is neither an extremely rapid nor strictly processive process. To test this idea, we measured the relative degree of polymerization (concentration of carbohydrate per concentration of reducing ends) of HMW succinoglycan recovered from cultures of the wild-type strain and from cultures of various strains defective in expression of glycanases and/or acetylation and succinylation of succinoglycan (Table 5), and we observed the expected correlation (Fig. 4). Our results indicate that glycanase activity influences the molecular weight distribution of succinoglycan not just in terms of generating LMW succinoglycan, but also in terms of controlling the average degree of polymerization of the remaining pool of HMW succinoglycan. Importantly, the average degree of polymerization of HMW succinoglycan produced by *exoH* mutants is similar to that of *exoK exsH* mutants, again implying that the non-succinylated succinoglycan produced by the *exoH* strain is a poor substrate for cleavage by physiologically relevant levels of ExoK and ExsH.
DISCUSSION

Our results indicate that the acetyl and succinyl modifications of succinoglycan dramatically affect the molecular weight distribution of succinoglycan in *R. meliloti* cultures, apparently by influencing the susceptibility of the polysaccharide to cleavage by the ExoK and ExsH glycanases. In particular, the non-acetylated succinoglycan produced by *exoZ* mutants has a high susceptibility to cleavage, the normally-modified succinoglycan produced by the wild-type strain has an intermediate susceptibility to cleavage, and the non-succinylated succinoglycan produced by *exoH* mutants has a low susceptibility to cleavage. These characteristics pertain specifically to succinoglycan as it is actively being produced by *R. meliloti* strains; each of the variously-modified forms of succinoglycan undergoes a transition from a glycanase-susceptible to a glycanase-refractory form as it accumulates in cultures. Thus, the actual molecular weight distribution of succinoglycan in a given *R. meliloti* culture is determined by both the susceptibility of the polysaccharide to cleavage, and by the timing and levels of endogenous expression or exogenous addition of glycanase to the cultures.

Our results are consistent with a simple model, whereby *R. meliloti* strains cultivated in GMS medium synthesize almost all of their succinoglycan in long chain, HMW forms. Then, in proportion to the extent that a given strain expresses the ExoK and ExsH glycanases and to the extent that the succinoglycan produced by this given strain is susceptible to cleavage, the HMW succinoglycan is cleaved by these glycanases to yield LMW succinoglycan and residual, shorter chain forms of HMW succinoglycan.

How do the acetyl and succinyl modifications of succinoglycan affect the susceptibility of succinoglycan to cleavage by glycanases? One possibility is that the modifications influence the conformation of succinoglycan prior to its transition from glycanase-susceptible to glycanase-refractory forms. For example, Gravanis *et al.* (73) have proposed that the precise conformation of individual, non-succinylated succinoglycan chains may differ from that of normally-modified succinoglycan chains. Thus, the different susceptibilities to cleavage associated with the variously-modified forms of succinoglycan may reflect a different fit for each
substrate in the active sites of ExoK and ExsH.

A second possibility is that the acetyl and succinyl modifications of succinoglycan affect the rate of transition of succinoglycan from glycanase-susceptible to glycanase-refractory forms. Although we have determined that all of the variously-modified forms of succinoglycan undergo a transition to glycanase-refractory forms in culture, the nature of the transition itself is not known. Previous analyses of the physical properties of succinoglycan imply that purified succinoglycan samples can undergo disorder-order conformational transitions in solution, consisting of random coil to helix transitions and aggregation (26). Either or both might account for the transition of succinoglycan from glycanase-susceptible to glycanase-refractory forms. Interestingly, analyses of non-succinylated succinoglycan, as recovered from exoH mutant cultures or as generated by chemical desuccinylation of wild-type succinoglycan, indicate that the absence of the succinyl group results in an increase in the order-disorder transition temperature of the polysaccharide (56, 130). Thus the absence of the succinyl modification seems to increase the stability of ordered forms of succinoglycan. Also, increasing salt concentrations in succinoglycan solutions promote the disorder-order transition (26), and increasing salt concentrations in R. meliloti cultures promote a shift toward accumulation of more HMW and less LMW succinoglycan (15, 22). Whether this latter effect is due to decreased depolymerization of succinoglycan, or whether it is a function of regulation of the extent of polymerization of succinoglycan, remains to be determined.

The dramatic differences in generation of LMW succinoglycan, associated with adding glycanase to cultures early versus late in the course of cultivation, suggest that shifts in the timing and levels of glycanase expression, relative to the timing and levels of succinoglycan production, could have dramatic effects on the molecular weight distribution of the succinoglycan produced by R. meliloti strains. Several mutants have been described that exhibit increased succinoglycan production (exoR95, exoS96, exoX363, and exsB), decreased succinoglycan production (mucR and exoX319), or decreased expression of the exoK gene (mucR) (13, 47, 86, 122). Components of growth media can also affect levels of succinoglycan
production (47) and the extracellular accumulation of ExoK (163). Identification and characterization of additional genetic and environmental factors that cause changes in glycanase expression or succinoglycan production should enable further refinement of the evolving model for how *R. meliloti* controls the molecular weight distribution of succinoglycan.

Our findings may have implications beyond understanding succinoglycan production by cultures of *R. meliloti*. Glycanases may prove useful as tools in characterizing the differences in the physical structures of normally-modified versus non-succinylated succinoglycan, which in turn may provide new insights into why *exoH* mutants, but not *exoK exsH* mutants, exhibit a defect in invasion of alfalfa root nodules during establishment of symbiosis (93, 162). Fluctuations in the rates of transition of succinoglycan to glycanase-refractory forms and in the levels of active, extracellular glycanases may cause spatial and temporal heterogeneity of succinoglycan, in terms of the molecular weight distribution of the polysaccharide as it is being produced by *R. meliloti* in cultures or in natural habitats. In general such heterogeneity might have important consequences in development of bacterial-polysaccharide biofilms (117). Finally, our results may serve to bridge research on polysaccharide physical properties and polysaccharide molecular weight control, such that results from both fields can provide context and relevance for each other. Increased understanding of polysaccharide physical properties should help to further elucidate how glycanases control polysaccharide molecular weight, and the ability to engineer polysaccharides within particular molecular weight ranges should enable testing of assumptions about how physical properties of polysaccharides are influenced by polysaccharide molecular weight.
ACKNOWLEDGMENTS

We thank Latoya Maynard, who carried out research as part of the Undergraduate Research Opportunities Program at the Massachusetts Institute of Technology, for construction of the $exoK$ $exoH$ strain. This work was supported by Public Health Service grant GM31030 from the National Institutes of Health.
Fig 1. Calcofluor plate assay for measurement of the effects of the *exoZ* and *exoH* mutations on expression of extracellular glycanase activity by *Rhizobium meliloti* colonies. The individual colonies are tester strains, and the long strips of bacteria are the indicator strain. Genotypes of strains are indicated in the figure. Diffusion of glycanase activity from the various *exoY* tester colonies (which themselves are blocked in succinoglycan production) is detected based on formation of a fluorescent halo around the normally haloless *exoK exsH* indicator strain.
Fig. 2. Western blots measuring the effect of the \textit{exoZ} and \textit{exoH} mutations on extracellular accumulation of (A) ExsH (by use of anti-ExsH polyclonal antibodies) and (B, C) ExoK (by use of anti-ExoK polyclonal antibodies). The strains \textit{exoY, exoY exoZ, exoY exoH}, and \textit{exoY exoH exoZ} were cultivated in GMS medium (A, B) or in MGS medium (C) for a total of 5 days. Each lane contains a 5 \textmu{l} aliquot of cell-free culture supernatant from day 1, day 3, or day 5 cultures. For blots A and C, the control lane corresponds to the negative control strain \textit{exoY exoK exsH}. For blot B, the control lane corresponds to the \textit{exoY} strain cultivated in MGS medium, which serves as a positive control for detection of extracellular ExoK. Each control sample corresponds to cell-free supernatants of a day 5 culture. Arrows indicate expected positions of ExsH and ExoK. Lines indicate positions of molecular weight markers (kD).
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<th>exoY</th>
<th>exoY exoZ</th>
<th>exoY exoH</th>
<th>exoY exoH exoZ</th>
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<tr>
<td>Day:</td>
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<td>1 3 5</td>
<td>1 3 5</td>
<td>1 3 5</td>
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</tr>
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<td>A</td>
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<tr>
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<td>39</td>
<td></td>
<td></td>
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<tr>
<td>B</td>
<td>39</td>
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<td>C</td>
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<td>ExoK</td>
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<td>26</td>
<td>21</td>
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**Fig. 3.** Graph of low-molecular-weight carbohydrate (expressed as percentage of total extracellular carbohydrate in culture) generated by the addition of ExsH to cultures of glycanase-deficient strains versus the total amount of extracellular carbohydrate that had accumulated in cultures of these strains after four days of incubation. Note that approximately 97% of total extracellular carbohydrate is succinoglycan. Strains: *exoK exsH* (open squares) and *exoZ exoK exsH* (closed squares). ExsH was added to cultures gradually over the course of four days to the final, physiologically relevant concentration of 200 ng/ml.
Total extracellular carbohydrate in cultures (µg/ml)

Percentage of total extracellular carbohydrate in LMW forms

100 200 300 400

0 20 40 60 80 100
Fig. 4. Degree of polymerization of high-molecular-weight succinoglycan samples, expressed relative to degree of polymerization of wild-type high-molecular-weight succinoglycan (data from Table 5) plotted versus the percentage of total extracellular carbohydrate in cultures that is in high-molecular-weight forms (data from Table 2). Note that approximately 97% of total extracellular carbohydrate in cultures is succinoglycan.
Relative degree of polymerization of HMW succinoglycan

Percentage of total extracellular carbohydrate in HMW forms
### TABLE 1. Bacterial strains

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<th>Genotype</th>
<th>Source</th>
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<td>exoY210::Tn5</td>
<td>J. Leigh</td>
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TABLE 2. Percentage of total extracellular carbohydrate in culture supernatants that is in low-molecular-weight forms

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<th>Glycanase genotype</th>
<th>Succinylation/ acetylation genotype</th>
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<td></td>
<td>wild-type</td>
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<tr>
<td>wild-type</td>
<td></td>
</tr>
<tr>
<td>exoK</td>
<td></td>
</tr>
<tr>
<td>exoK exsH</td>
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</table>

\(^a\) Under the incubation conditions used here, approximately 97% of total extracellular carbohydrate is succinoglycan. Data represent average of two cultures. SD \(\leq 3.5\%\) except where indicated.

\(^b\) SD = 6%
\(^c\) Not determined
\(^d\) SD = 12.5\%
TABLE 3. Low-molecular-weight extracellular carbohydrate (expressed as percentage of total extracellular carbohydrate) generated by addition of glycanase (final concn of 200 ng/ml) to cultures<sup>a</sup>

<table>
<thead>
<tr>
<th>Strains</th>
<th>Enzyme added throughout course of culture incubation</th>
<th>Enzyme added entirely upon inoculation of cultures</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>+ ExsH</td>
<td>+ ExoK</td>
</tr>
<tr>
<td>&lt;i&gt;exoK exsH&lt;/i&gt;</td>
<td>54</td>
<td>9</td>
</tr>
<tr>
<td>&lt;i&gt;exoZ exoK exsH&lt;/i&gt;</td>
<td>90</td>
<td>35</td>
</tr>
<tr>
<td>&lt;i&gt;exoH exoK exsH&lt;/i&gt;</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>&lt;i&gt;exoH exoZ exoK exsH&lt;/i&gt;</td>
<td>13</td>
<td>7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Under the incubation conditions used here, approximately 97% of total extracellular carbohydrate is succinoglycan.
<table>
<thead>
<tr>
<th>Strains</th>
<th>+ ExsH</th>
<th>+ ExoK</th>
<th>+ ExsH</th>
<th>+ ExoK</th>
<th>+ExsH</th>
<th>+ ExoK</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>exoK exsH</em></td>
<td>100</td>
<td>93</td>
<td>43</td>
<td>54</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>exoZ exoK exsH</em></td>
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<td>100</td>
<td>46</td>
<td>51</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td><em>exoH exoK exsH</em></td>
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<td>21</td>
<td>9</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>exoH exoZ exoK exsH</em></td>
<td>61</td>
<td>39</td>
<td>9</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Under the incubation conditions used here, approximately 97% of total extracellular carbohydrate is succinoglycan.
TABLE 5. Degree of polymerization of high-molecular-weight succinoglycan samples, expressed relative to degree of polymerization of wild-type high-molecular-weight succinoglycan

<table>
<thead>
<tr>
<th>Succinylation/ acetylation genotype</th>
<th>Glycanase genotype</th>
<th>wild-type</th>
<th>exoZ</th>
<th>exoH</th>
<th>exoH exoZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>exoH exoZ</td>
<td>wild-type</td>
<td>1.0b</td>
<td>0.52</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>exoK</td>
<td>wild-type</td>
<td>2.2b</td>
<td>0.57</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>exsH</td>
<td>wild-type</td>
<td>9.6b</td>
<td>2.0b</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>exoK exsH</td>
<td>wild-type</td>
<td>29</td>
<td>28</td>
<td>27</td>
<td>29</td>
</tr>
</tbody>
</table>

aData represent averages for succinoglycan from two cultures. SD < 20% of avg degree of polymerization for a given sample, except as indicated.

b SD ≤ 30% of avg degree of polymerization for a given sample.

c Not determined
Chapter 5

Development of an Assay for Detection of Enzymatic Depolymerization of Succinoglycan,
Based on the Use of Congo Red Dye*

* Manuscript in preparation.
We have developed an assay for detection of glycanase-mediated depolymerization of the \textit{Rhizobium meliloti} exopolysaccharide succinoglycan in solution, based on the use of Congo Red dye. We developed this assay as part of our ongoing effort to characterize the activities of the \textit{R. meliloti} endo-1,3-1,4-\(\beta\)-glycanases ExoK and ExsH, which influence the molecular weight distribution of this symbiotically-important and industrially-relevant polysaccharide. We observed that native succinoglycan, prepared from cultures of a wild-type \textit{R. meliloti} strain, is refractory to binding by Congo Red, but that heat treatment of succinoglycan and the removal of the acetyl and succinyl modifications from succinoglycan convert succinoglycan to forms which can be bound rapidly by Congo Red. We determined that depolymerization of succinoglycan, as mediated by purified endo-1,3-1,4-\(\beta\)-glycanases, can be measured based on a decrease in the rate and extent to which Congo Red binds depolymerized succinoglycan in solution. Our results indicate that Congo Red is more sensitive in the detection of depolymerization mediated by certain glycanases, such as the \textit{R. meliloti} ExoK and ExsH proteins, than in the detection of depolymerization mediated by other glycanases, such as the succinoglycan depolymerase of \textit{Cytophaga arvensicola}. The differences in sensitivity may reflect differences in the process by which these glycanases cleave succinoglycan chains. We have also determined that Congo Red can be used to measure depolymerization of succinoglycan as mediated by ExoK and ExsH expressed endogenously in \textit{R. meliloti} cultures. Thus, Congo Red can be used to compare directly the activities of purified versus endogenously expressed forms of glycanases.
INTRODUCTION

Congo Red dye has proven useful in analyses of the physical properties and physical structures of a wide range of macromolecules, based on the ability of Congo Red to bind these macromolecules. For example, Congo Red is used for detection of β-amyloid plaques associated with Alzheimer's disease (146), for analyzing the basis of prion protein aggregation in prion diseases (30), and for visualization of a recently reported, novel class of fibrous polymers composed of non covalently bound oligopeptide repeating units (167). Congo Red is also used for the detection of many structurally diverse polysaccharides, such as chitin (5), barley glucan (159), xylan (159), and galactomannan (48). Congo Red has been used in analyses of aggregation of chitin (5) and may potentially be useful for detecting aggregation of other polysaccharides. Congo Red is also commonly used in the detection of enzymatic depolymerization of polysaccharides (159).

Polysaccharide depolymerization assays based on the use of Congo Red serve as an excellent complement to the standard approach of measuring increases in the concentration of reducing ends. Reducing end assays are particularly useful for estimating the relative number of cleavage events that have occurred during depolymerization of polysaccharide samples, regardless of the distribution of cleavage sites along a polysaccharide chain (100). Assays based on the use of Congo Red are particularly useful for discriminating between two extreme mechanisms of cleavage, processive exo-type cleavage, proceeding from the ends of polysaccharide chains, versus nonprocessive endo-type cleavage, occurring at sites internal to polysaccharide chains (48, 153). Congo Red assays are more sensitive in detection of endo-type versus exo-type cleavage because endo-type cleavage causes a more rapid decrease in the average molecular weight of the remaining polysaccharide substrate per cleavage event, in comparison to exo-type cleavage (48). Based on these and other useful characteristics of Congo Red, we became interested in applying Congo Red to the measurement of glycanase-mediated depolymerization of a particular polysaccharide, succinoglycan.

Succinoglycan is an exopolysaccharide produced by several strains of soil bacteria,
including *Rhizobium meliloti*, *Agrobacterium radiobacter*, and *Pseudomonas* strains (36, 76). Succinoglycan is a polymer of an octasaccharide repeating unit, consisting of galactose, glucose, acetate, succinate, and pyruvate, in a ratio of 1:7:1:1:1 as produced by *R. meliloti* (1). The extent to which succinoglycan is succinylated and acetylated varies between bacterial strains (36). In cultures, *R. meliloti* produces succinoglycan in low molecular weight (LMW) forms, reported to consist of monomers, trimers, and tetramers of octasaccharide repeating units (8), and in high molecular weight (HMW) forms, consisting of polymers of much higher degrees of polymerization (92).

The molecular weight of the polysaccharide affects its function in both biological and industrial processes. Succinoglycan plays a crucial role in the establishment of the *R. meliloti*-alfalfa nitrogen fixing symbiosis, and LMW forms in particular are proposed to be the symbiotically active forms of the polysaccharide (8, 92, 93). Succinoglycan (as produced by a *Pseudomonas* strain) is used industrially for enhanced recovery of petroleum from oil fields, and in this application HMW forms of the polysaccharide are crucial for generation of highly viscous solutions of succinoglycan (36).

For *R. meliloti*, the steps in the biosynthesis of succinoglycan repeating units are well defined, both genetically (25, 67, 68, 103) and biochemically (126, 148), but the mechanisms for regulation of polysaccharide molecular weight are less clear. Given the importance of molecular weight in mediating the biological and industrial functions of succinoglycan, and given the absence of generalizable models for bacterial control of exopolysaccharide molecular weight, recently we have focused our efforts in understanding how *R. meliloti* regulates the molecular weight distribution of the succinoglycan that it produces.

Bacteria can potentially control the molecular weight distribution of polysaccharides by polymerization mechanisms and/or by depolymerization mechanisms: genetic and biochemical evidence indicates that *R. meliloti* actually does both for succinoglycan (14, 70, 162, 163). With regard to polymerization, the ExoP protein has been proposed to modulate the molecular weight distribution of succinoglycan, based on the observation that a particular truncation of ExoP is
associated with a shift toward production of LMW succinoglycan (14, 15). Also, the *exoQ* and *exoT* genes have been implicated in direct synthesis of HMW succinoglycan and oligomers of LMW succinoglycan, respectively, and the *exoP* gene has been implicated in production of both of these forms of succinoglycan (70). With regard to depolymerization, *R. meliloti* expresses two endo-1,3-1,4-β-glycanases, ExoK (10, 68) and ExsH (162), that depolymerize nascent succinoglycan, but not succinoglycan that has accumulated in culture supernatants, to yield LMW succinoglycan (163). Interestingly, the absence of the acetyl modification from succinoglycan dramatically increases the susceptibility of the polysaccharide to cleavage, and the absence of the succinyl modification dramatically decreases the susceptibility of succinoglycan to cleavage (164). Thus, the level of glycanase expression and the susceptibility of succinoglycan to cleavage together determine the contribution of glycanases in controlling the molecular weight distribution of succinoglycan.

During the course of our analyses of the activities of the glycanases ExoK and ExsH, we developed a simple assay for the rapid detection of enzymatic depolymerization of succinoglycan in solution, by use of Congo Red dye. We determined that native succinoglycan prepared from cultures of a wild-type *R. meliloti* strain is actually a poor substrate for binding by Congo Red, but that heat treatments and the removal of the succinyl and acetyl modifications from succinoglycan convert succinoglycan to forms that Congo Red can bind rapidly. We determined that enzymatic depolymerization of native and acid/heat-treated succinoglycan can be detected based on decreased binding of Congo Red to polysaccharide. Our results suggest several potential applications for Congo Red in further analyses of the molecular weight control of succinoglycan, such as monitoring thermal transitions of succinoglycan samples, testing components of growth media for their effects on the activities of the ExoK and ExsH glycanases, and characterizing the process by which these glycanases cleave succinoglycan molecules.
MATERIALS AND METHODS

Bacterial strains and growth conditions. *R. meliloti* strains used in this study are listed in Table 1. The following growth media were used: Luria Bertani (LB) (107), MGS (potassium phosphate (100 mM, pH 7.3 for liquid cultures or pH 6.8 for plates), mannitol (55 mM), monosodium glutamate (5 mM), and sodium chloride (8 mM)), and GMS medium (containing mannitol at a final concentration of 27.5 mM) (165). MGS and GMS were also supplemented with magnesium sulfate (1 mM), calcium chloride (0.25 mM), biotin (100 µg/l) and thiamine (100 µg/l for GMS) after autoclaving of media.

Preparation and heat treatment of succinoglycan. Succinoglycan was prepared from *R. meliloti* cultures by the following procedure. *R. meliloti* was cultured overnight in LB liquid medium. Cells were washed one time in sterile 0.85% sodium chloride solution and then introduced at a 1:100 dilution into one liter of MGS liquid medium in a two liter flask. MGS cultures were cultivated for seven days at 30°C with aeration. Then cells were removed from cultures by centrifugation (typically 12000 × G, 20 minutes). Cell-free supernatants were lyophilized, and the freeze-dried material was resuspended in 1/3 the original culture volume. HMW succinoglycan was precipitated from these solutions by the addition of three vols of ethanol. Samples were subjected to centrifugation (12000 × G, 20 minutes), the ethanolic supernatants were discarded, and the precipitated HMW succinoglycan was dissolved in distilled water. Succinoglycan solutions were dialyzed against distilled water for four days with at least two changes of water per day, lyophilized, and stored at -20°C.

Neutral/heat-treatment of succinoglycan entailed heating a solution of succinoglycan (typically in vols ≤ 1 ml at concentrations ≤ 1 mg/ml) in a boiling water bath for 15 min, then allowing the sample to cool to room temperature. Acid/heat-treatment of succinoglycan entailed dissolving succinoglycan in 50 mM oxalic acid, heating the solution in a boiling water bath for 90 min, allowing the sample to cool to room temperature, neutralizing the sample by addition of sodium hydroxide, and then dialyzing the sample against distilled water for one day with at least
four changes of water. This procedure has been used previously in the depyruvylation and desuccinylation of a galactoglucon (115).

**Quantitation of concentrations of carbohydrate and reducing ends.** Carbohydrate concentrations and relative reducing end concentrations were determined by the anthrone-sulfuric acid method (102) and the Lever method (100), respectively. It is important to note that the Lever assay overestimates the actual concentration of reducing ends for a given polysaccharide sample, presumably due to alkaline hydrolysis of polysaccharide chains during the course of the assay (74). We use the Lever assay here not to measure the precise degree of polymerization of polysaccharides but to provide a relative measure of degree of polymerization between samples.

**Binding of Congo Red dye to succinoglycan.** We used the following method to assay for binding of Congo Red dye to succinoglycan in solution. We dissolved succinoglycan to a final concentration of 25 µg/ml in 1 ml of reaction buffer (final concentration of 100 mM potassium phosphate, pH 6.8; 1 M sodium chloride). We transferred samples to cuvettes, and measured the OD$_{525}$ of samples prior to addition of Congo Red. For samples that exhibited a detectable absorbance (in practice, only $exoH$ and $exoH$ $exoZ$ succinoglycan), we recorded the OD$_{525}$ reading and adjusted subsequent readings by subtracting this absorbance. We prepared a stock of Congo Red dye (0.5 mg/ml) and removed undissolved dye by centrifugation. We then added Congo Red to the succinoglycan samples (to a final concentration of 10 µg/ml), mixed the dye thoroughly with samples, and measured the OD$_{525}$ of these samples at the following times after addition of Congo Red: 1 min, 5 min, 10 min, 15 min, 30 min, 60 min, 120 min, and 180 min.

**Preparation of glycanases.** The succinoglycan depolymerase of *C. arvensicola* was prepared by a variation on a previously described method (75). The *R. meliloti* glycanases ExoK and ExsH were expressed in *Escherichia coli* by use of a T7 expression system and were purified as
previously described (163).

**Glycanase assays.** To detect glycanase-mediated depolymerization of succinoglycan *in vitro*, succinoglycan samples were dissolved in a buffer similar to GMS medium (6 mM potassium phosphate, pH 7.0, 1 mM magnesium sulfate, 0.25 mM calcium chloride), and aliquots of glycanase were added to the desired final concentration. Samples were incubated at 30°C for 24 hours. Then aliquots of reactions were tested to determine the relative concentration of reducing ends and to measure the rate of binding of Congo Red to succinoglycan in the samples. To detect glycanase-mediated depolymerization of succinoglycan in cultures, *exoY* strains of *R. meliloti* were cultivated in 10 to 50 ml vols of GMS or MGS liquid media for 3 days. Aliquots of 2 ml of cultures were transferred to test tubes. Succinoglycan was added to these cultures to a final vol of 100 µg/ml. For certain treatments, aliquots of glycanase were also added at this point. Cultures were incubated for one additional day. Cells were removed by centrifugation, and cell-free culture supernatants were tested for the rate and extent to which Congo Red could bind the HMW succinoglycan remaining in the cultures. For all glycanase reactions involving native succinoglycan, samples were subjected to neutral/heat-treatment after completion of glycanase treatment but prior to testing of binding of Congo Red to samples. In these cases, samples were allowed to cool to room temperature for approximately one hour prior to testing for binding by Congo Red.
RESULTS

**Congo Red dye fails to bind native succinoglycan.** In order to develop an assay to detect depolymerization of succinoglycan based on the use of Congo Red dye, we first aimed to identify conditions under which Congo Red can bind to succinoglycan prepared from cultures of a wild-type *R. meliloti* strain. Although it has been reported that Congo Red can bind to a wide variety of polysaccharides dissolved in dilute buffer at room temperature (159), we observed no apparent binding of Congo Red to succinoglycan under these conditions (data not shown). It has also been reported that supplementation of dilute buffer with sodium chloride can improve the binding of Congo Red to certain polyanionic polysaccharides (159), but we observed no such effect on the binding of Congo Red to succinoglycan (Fig. 1). These results were somewhat surprising to us, given that we had previously observed that Congo Red can bind to succinoglycan under another specific set of conditions (162). In an assay to detect diffusion of glycanases from bacterial colonies, we had observed that Congo Red can bind wild-type succinoglycan that has been incorporated into top agar (162). In this assay, the succinoglycan is dissolved in top agar solutions prior to the step at which the top agar solutions are heated at $\geq 100^\circ$C to accomplish melting of the agarose. Thus, the succinoglycan undergoes a heat treatment prior to the step at which binding by Congo Red can be detected. These observations suggested to us the possibility that heat treatment may convert succinoglycan to a form that is susceptible to binding by Congo Red.

This possibility seemed particularly intriguing, given that we had also previously observed that heat treatment converts succinoglycan to forms that are more susceptible to cleavage by the *R. meliloti* endo-1,3-1,4-β-glycanases ExoK and ExsH (163). Succinoglycan prepared from *R. meliloti* cultures is a relatively poor substrate for cleavage by ExoK and ExsH (163). Each enzyme cleaves $\leq 3\%$ of HMW succinoglycan to LMW forms upon prolonged incubations (163). However, neutral-heat treatment (heating at 100°C for 15 minutes, followed by cooling to room temperature) converts succinoglycan to a form that is moderately susceptible to cleavage by ExoK and ExsH (163). Acid/heat-treatment (heating at 100°C for 90 minutes in
50 mM oxalic acid, followed by cooling, neutralization, and dialysis) converts succinoglycan to a form that is even more highly susceptible to cleavage by ExoK and ExsH (163). Each enzyme can cleave approximately 25% of HMW acid/heat-treated succinoglycan samples to LMW forms (163).

Neutral/heat-treatment and acid/heat-treatment of succinoglycan solutions have also been demonstrated to cause hysteresis, an irreversible decrease in the viscosity of a polysaccharide solution at room temperature following heat treatment (26, 56). For succinoglycan, hysteresis is hypothesized to reflect a change in the conformation or aggregation state of succinoglycan molecules in solution (26, 56).

**Congo Red dye binds heat treated succinoglycan.** To test the possibility that heat treatments convert succinoglycan to forms that can be bound by Congo Red, we dissolved samples of native succinoglycan, neutral/heat-treated succinoglycan, and acid/heat-treated succinoglycan at identical concentrations (25 μg/ml) in a reaction buffer (100 mM potassium phosphate (pH 6.8), 1 M sodium chloride), added Congo Red dye (to 10 μg/ml final concentration), and measured the change in OD525 of samples over time. Our results indicate that Congo Red accomplishes little or no binding to native succinoglycan under these conditions, even after 24 hours (Fig. 1). In contrast, Congo Red can bind neutral/heat-treated succinoglycan (Fig. 1); half maximal binding occurs within 15 minutes and maximal binding occurs within 60 minutes. Congo Red can also bind acid/heat-treated succinoglycan, and this binding occurs even more rapidly than is the case for neutral/heat-treated succinoglycan (Fig. 1); half maximal binding occurs within 1 minute, and maximal binding occurs within 30 minutes. Despite the great difference in the kinetics of Congo Red binding to the two heat-treated forms of succinoglycan, Congo Red binds these two forms of succinoglycan to approximately the same final extent (Fig. 1).

To determine whether the effect of neutral/heat-treatment of succinoglycan on binding by Congo Red is irreversible, we heated a sample of succinoglycan at 100°C for 15 minutes,
removed the sample from heat, and tested aliquots of the sample over a time course of seven days for binding by Congo Red. Our results indicate that, over a time course of two days, neutral/heat-treated succinoglycan exhibits a relatively small gradual decrease in terms of the rate and extent of binding by Congo Red (Fig. 2). We observed little or no additional decrease in the rate and extent of binding by Congo Red between the second and seventh days (data not shown). Thus, at least over the time course of one week, the rate and extent of binding of Congo Red to neutral/heat-treated succinoglycan gradually decrease but then stabilize, and neutral/heat-treated succinoglycan remains highly susceptible to binding by Congo Red. The small gradual decrease in the rate of Congo Red binding that is associated with the first two days of incubation of neutral/heat-treated succinoglycan at room temperature suggests that neutral/heat-treated succinoglycan may continue to undergo a gradual change in its conformation or aggregation state even after having cooled to room temperature (Fig. 2).

Congo Red dye also binds to forms of succinoglycan that lack the acetyl and/or succinyl modifications. Clearly, acid/heat-treatment of succinoglycan has a more dramatic effect than neutral/heat-treatment, in terms of increasing the rate at which Congo Red binds (Fig. 1). In general, acid/heat-treatments remove ketal modifications from polysaccharides, and the particular method that we used here has been shown previously to remove both ketal and acyl modifications from polysaccharide (115). Thus, we considered the possibility that removal of the pyruvyl, succinyl, and/or acetyl modifications might account at least partially for the increase in the rate of Congo Red binding to acid/heat-treated succinoglycan.

Mutants of *R. meliloti* that produce non-succinylated succinoglycan (*exoH*), non-acetylated succinoglycan (*exoZ*), or non-succinylated non-acetylated succinoglycan (*exoH exoZ*) have been isolated previously (93, 125). We tested the rate of binding of Congo Red to both native and neutral/heat-treated forms of succinoglycan produced by these strains. Our results indicate that, for native forms of succinoglycan, the absence of the acetyl modification causes a slight increase in the rate of Congo Red binding, the absence of the succinyl modification causes
a greater increase, and the absence of both modifications causes an even greater increase (Fig. 3A).

To confirm that the rapid rate of binding of Congo Red to \textit{exoH exoZ} succinoglycan is associated specifically with the absence of the acetyl and succinyl modifications, we used an independent approach to generate non-acetylated non-succinylated succinoglycan. We conducted base treatment of wild-type succinoglycan, which removes the acetyl and succinyl modifications, and then used this polysaccharide as a substrate to test the rate of binding by Congo Red. We determined that base treatment is sufficient to increase the rate of Congo Red binding to succinoglycan to the same level as that associated with \textit{exoH exoZ} succinoglycan (Fig. 3A).

The acetyl and succinyl modifications of succinoglycan and the thermal history of succinoglycan solutions independently influence the rate at which Congo Red can bind succinoglycan. The observation that Congo Red binds more rapidly to acid/heat-treated succinoglycan than to \textit{exoH exoZ} succinoglycan (Fig. 3A) implied that removal of the acetyl and succinyl modifications by acid/heat-treatment could only partially account for the dramatic effect of this treatment on binding of Congo Red to succinoglycan. To test whether heat treatment may account for the rest of this effect, we conducted neutral/heat-treatment of \textit{exoH}, \textit{exoZ}, \textit{exoH exoZ}, and base treated succinoglycan, and tested the rate of binding by Congo Red to these forms of succinoglycan. For these neutral/heat-treated forms of succinoglycan, we observed the same trend that we had seen previously, in terms of the effects of the acetyl and succinyl modifications on the rate of Congo Red binding (Fig. 3B). However, neutral/heat-treatment also independently increases the rate of Congo Red binding to each form of succinoglycan (Fig. 3B). Furthermore, the additive effects of neutral/heat-treatment and of the absence or removal of the acetyl and succinyl modifications are equivalent to the effect of acid/heat-treatment, in terms of increasing the rate of binding of Congo Red to succinoglycan (Fig. 3B). Our results indicate that the succinyl and acetyl modifications and the thermal history of a succinoglycan sample
Congo Red dye is useful for detection of glycanase-mediated depolymerization of succinoglycan in solution. We proceeded to design an assay to detect glycanase-mediated depolymerization of succinoglycan in solution, by use of Congo Red. The assay entails treating succinoglycan in solutions with glycanase and then measuring the rate of Congo Red binding to samples of glycanase-treated succinoglycan, in comparison to samples of non-treated succinoglycan. We focused our efforts on testing two substrates, native wild-type succinoglycan and acid/heat-treated succinoglycan. Although Congo Red fails to bind to native wild-type succinoglycan, we devised a simple method that enabled us to detect depolymerization of native succinoglycan by use of Congo Red; we conducted neutral/heat-treatment of native succinoglycan samples after treatment of the polysaccharide with glycanase but prior to testing the polysaccharide for binding by Congo Red. Since Congo Red binds acid/heat-treated succinoglycan rapidly, we did not include this extra heat treatment step for measurement of the depolymerization of acid/heat-treated succinoglycan.

In our first set of analyses, we treated succinoglycan samples with a succinoglycan depolymerase purified from the soil bacterium *Cytophaga arvensicola* (75). This endo-1,3-1,4-β-glycanase is capable of depolymerizing all forms of succinoglycan completely to monomers of the octasaccharide repeating unit (75, 125). Thus, we used this depolymerase as a control to confirm that Congo Red cannot bind to succinoglycan that has been completely depolymerized. We incubated succinoglycan samples in the presence of a range of concentrations of this depolymerase (0.2 μl stock to 5 μl stock per ml of glycanase reaction) for a total of 24 hours. We then measured the rate at which Congo Red dye binds the succinoglycan in these samples, and, as an independent measure of glycanase activity, we measured the relative concentration of reducing ends in the samples. We had previously determined that, at the highest concentration of depolymerase used, the depolymerase depolymerizes both native and neutral/heat-treated succinoglycan completely to octasaccharide subunits within 24 hours (163). As expected, we
observed that Congo Red fails to bind native succinoglycan (Fig. 4A) and acid/heat-treated succinoglycan (Fig. 5A) that have been depolymerized completely. We also determined that the Congo Red assay can be used to detect the partial depolymerization of native succinoglycan (Fig. 4A) and acid/heat-treated succinoglycan (Fig. 5A) mediated by lower concentrations of depolymerase, based on a slower rate of binding and a decreased maximal extent of binding of Congo Red to partially depolymerized succinoglycan.

In our next set of analyses, we tested whether the same approach could be used to detect depolymerization of native and acid/heat-treated succinoglycan as mediated by the *R. meliloti* glycanases ExoK and ExsH. For these experiments, we used preparations of ExoK and ExsH that had been expressed and purified from *Escherichia coli* (163). In contrast to the case for succinoglycan depolymerase purified from *C. arvensicola*, ExoK (Fig. 4B, Fig. 5B) and ExsH (Fig. 4C, Fig. 5C) can accomplish only partial depolymerization of succinoglycan under the reaction conditions used here. Nonetheless, this partial depolymerization can be detected based on a slower rate of binding and a decreased maximal extent of binding of Congo Red dye to succinoglycan (Fig. 4, Fig. 5).

**Congo Red dye is more sensitive in the detection of depolymerization of native succinoglycan than acid/heat-treated succinoglycan.** Our data indicate that the Congo Red assay is more sensitive in the detection of partial depolymerization of native succinoglycan than in detection of partial depolymerization of acid/heat-treated succinoglycan. For equal increases in the concentration of reducing ends in samples of the two types of succinoglycan treated with the same glycanase (Fig. 6), the rate and extent of Congo Red binding to succinoglycan decreases much more rapidly for neutral/heat-treated succinoglycan (which in this assay corresponds native succinoglycan that was incubated in the presence of glycanase, and then heated prior to testing of the rate of binding by Congo Red) (Fig. 4) than for acid/heat-treated succinoglycan (Fig. 5). One possible explanation is that molecules of acid/heat-treated succinoglycan exhibit a lower minimal degree of polymerization necessary for binding of Congo
Red, than do molecules of neutral/heat-treated succinoglycan.

**Congo Red dye is more sensitive in the detection of depolymerization mediated by ExsH and ExoK than by the C. arvensicola succinoglycan depolymerase.** Our data also indicate that Congo Red exhibits different degrees of sensitivity in the detection of depolymerization of succinoglycan as mediated by the three glycanases. For equal increases in the concentration of reducing ends of succinoglycan samples treated with each of the three enzymes (Fig. 6), the Congo Red assay exhibits relatively low sensitivity for detection of the activity of the C. arvensicola succinoglycan depolymerase (Fig. 4A, Fig. 5A), moderate sensitivity for detection of the activity of ExoK (Fig. 4B, Fig. 5B), and relatively high sensitivity for detection of the activity of ExsH (Fig. 4C, Fig. 5C). These differences in sensitivity may reflect differences in the distribution of cleavage sites along succinoglycan molecules. For a given number of cleavage events, as determined by quantitation of reducing ends, an increasingly broad distribution of cleavage sites should correlate with a more rapid decrease in the average molecular weight of the remaining HMW succinoglycan, and this should correlate with increasing sensitivity of Congo Red in detecting these cleavage events (48).

**Congo Red dye can be used to detect the activities of ExoK and ExsH in R. meliloti cultures.** We proceeded to test whether the Congo Red assay could also be used to detect the glycanase activities associated with ExoK and ExsH as expressed endogenously in R. meliloti cultures. We were particularly interested in testing R. meliloti strains cultivated in two types of growth media, GMS and MGS. Genetic analyses that we conducted previously imply that, for R. meliloti colonies cultivated on MGS medium, the exoK+ gene makes a greater contribution than the exsH+ gene in terms of production of glycanase activity, but that for R. meliloti strains cultivated in GMS cultures, the exsH+ gene makes a greater contribution than the exoK+ gene in terms of production of LMW succinoglycan (162). Western blot analyses that we conducted previously indicate that ExsH accumulates extracellularly to approximately the same levels in
MGS and GMS cultures (160 ng/ml), but that ExoK accumulates extracellularly to approximately 25 fold higher levels in MGS cultures (160 ng/ml) than in GMS cultures (6 ng/ml) (163). GMS and MGS media are actually quite similar; mannitol, glutamic acid, and potassium phosphate are major components of both media (162, 165). The main differences between the media are that 1) MGS contains higher concentrations of mannitol and potassium phosphate than does GMS, 2) MGS is supplemented with sodium chloride, and 3) GMS is supplemented with trace levels of a variety of metal salts (162, 165). Apparently one or more of these differences between the two media translate into effects on the extracellular accumulation and/or activities of ExoK and ExsH (162, 163).

We examined whether the Congo Red assay could be used to directly measure the activities of ExoK and ExsH present in *R. meliloti* cultures, and, if so, whether the levels of activities of these enzymes would be consistent with the results of our previous analyses. In order to prevent endogenously expressed succinoglycan from interfering with depolymerization of succinoglycan added exogenously to cultures, we used *exoY* mutant strains for these analyses. The *exoY* gene encodes the glycosyl transferase required for the first step in biosynthesis of the octasaccharide repeating unit of succinoglycan, and thus *exoY* mutants are blocked in production of succinoglycan. Furthermore, we determined that *exoY* strains do not produce any endogenous substrates to which Congo Red can bind in cultures. For the glycanase assay, we cultivated *R. meliloti* *exoY*, *exoY exoK*, *exoY exsH*, and *exoY exoK exsH* strains in GMS or MGS minimal media for three days, supplemented the cultures with succinoglycan, incubated the cultures for an additional day, and then measured the rate of Congo Red binding to the succinoglycan that had been added exogenously to the cultures.

In preliminary experiments we tested native, neutral/heat-treated, and acid/heat-treated succinoglycan as substrates for this assay. We determined that the Congo Red assay is most sensitive in detection of depolymerization of neutral/heat-treated succinoglycan in cultures (data not shown), consistent with the observations that neutral/heat-treated succinoglycan is more susceptible to cleavage than native succinoglycan (163) and that Congo Red is more sensitive in
the detection of partial depolymerization of neutral/heat-treated succinoglycan than of acid/heat-
treated succinoglycan. Therefore, we focused on using neutral/heat-treated succinoglycan in
subsequent experiments.

Using the approach described above, we determined that \( \text{exsH}^+ \)-dependent and \( \text{exoK}^+ \)-
dependent glycanase activities can be detected for \( R. \ meliloti \) strains cultivated in both GMS
(Fig. 7A) and MGS media (Fig. 7B). However, consistent with our previous observations (162),
the \( \text{exsH}^+ \)-dependent activity makes a greater contribution to glycanase activity in GMS cultures
(Fig. 7A), whereas the \( \text{exoK}^+ \)-dependent activity makes a greater contribution to glycanase
activity in MGS cultures (Fig. 7B).

The specific activities of purified ExoK and ExsH are approximately equal to the
specific activities of ExoK and ExsH as expressed endogenously in \( R. \ meliloti \) cultures.
Having determined that it is possible to use Congo Red for the measurement of activities of
ExoK and ExsH as expressed endogenously in \( R. \ meliloti \) cultures, we sought to compare the
activities of endogenously expressed ExoK and ExsH to the activities of ExoK and ExsH
expressed and purified from \( E. \ coli \). To accomplish this, we cultivated the \( \text{exoY} \ \text{exoK} \ \text{exsH} \) strain
in GMS and MGS media for three days. At this point, we added purified ExoK and/or ExsH to
the levels that these enzymes are normally present in cultures of the given media (163), and we
also added neutral/heat-treated succinoglycan. We cultivated the strains for an additional day
and then measured the binding of Congo Red to the exogenously added succinoglycan. In this
way, we reconstituted the levels of glycanase typically present in GMS and MGS cultures of
\( \text{exoY}, \ \text{exoY} \ \text{exoK}, \) and \( \text{exoY} \ \text{exsH} \) strains (163). For both GMS and MGS media, purified ExsH
added to cultures of the \( \text{exoY} \ \text{exoK} \ \text{exsH} \) strain exhibits approximately the same specific activity
as ExsH expressed endogenously by \( \text{exoY} \) and \( \text{exoY} \ \text{exoK} \) strains (Fig. 7). For both GMS and
MGS media, purified ExoK added to cultures of the \( \text{exoY} \ \text{exoK} \ \text{exsH} \) strain exhibits a somewhat
lower specific activity than ExoK expressed endogenously by \( \text{exoY} \) and \( \text{exoY} \ \text{exsH} \) strains
(Fig. 7). For MGS media in particular, this difference corresponds to an approximately 5-fold
decrease in activity (data not shown). These results imply that purification of ExsH and ExoK from *E. coli* causes little or no decrease in the specific activities of these glycanases.
DISCUSSION

We have described here the development of an assay for detection of glycanase-mediated depolymerization of succinoglycan in solution, based on the use of Congo Red dye. We have determined that Congo Red has the capacity to bind succinoglycan, but that the presence of acetyl and succinyl modifications on succinoglycan and the thermal history of succinoglycan samples dramatically affect the rate and extent to which Congo Red can bind. We have demonstrated that glycanase-mediated depolymerization of succinoglycan in vitro can be detected based on decreases in the rate and extent of binding of Congo Red to succinoglycan samples. Our data suggest that, when used in conjunction with a reducing end assay, the Congo Red assay may be particularly useful for comparing glycanases in terms of the distribution of the sites of cleavage that they generate along succinoglycan chains. The Congo Red assay can also be used to measure the activity of glycanases present in cultures, enabling comparisons of the specific activities of both purified and endogenously expressed preparations of glycanases.

Congo Red dye may prove useful as a tool for determining precisely how heat-treatment affect the physical properties of succinoglycan. Neutral/heat-treatment of succinoglycan has previously been shown to result in hysteresis of succinoglycan solutions, and this hysteresis has been proposed to reflect irreversible changes in the conformation or aggregation state of succinoglycan molecules (26, 56). We have previously shown that neutral/heat-treatment converts succinoglycan to a form that is more susceptible to cleavage by the R. meliloti glycanases ExoK and ExsH (163). And here we have demonstrated that neutral/heat-treatment also converts wild-type succinoglycan from a form that Congo Red fails to bind, to a form that Congo Red can bind rapidly and extensively. It is not yet clear how each of these observations is related, but it seems likely that changes in the conformation or aggregation state of succinoglycan associated with heat treatment are directly responsible for increasing the susceptibility of succinoglycan to cleavage by ExoK and ExsH and to binding by Congo Red. Further analyses aimed at determining whether each of these transitions of succinoglycan occurs at the same temperature and with the same kinetics would help in testing this idea. The
observation that neutral/heat-treated succinoglycan exhibits a small gradual decrease over time in the rate at which it is bound by Congo Red hints at the possibility that molecules of neutral/heat-treated succinoglycan continues to undergo gradual changes in their collective conformation or aggregation state after having cooled to room temperature. Testing for subtle changes in the viscosity and other physical properties of succinoglycan solutions over an extended time course following neutral/heat-treatment might help in distinguishing whether the effects of neutral/heat-treatment on the physical properties of succinoglycan are truly irreversible, or whether heat treated succinoglycan partially or completely reverts to the native form over time.

It is not clear why the absence or removal of the acetyl and succinyl modifications of succinoglycan increase the rate at which Congo Red binds to succinoglycan, but these effects are distinct from those associated with neutral/heat-treatment of succinoglycan. With regard to the succinyl modification, one possible explanation is that the decrease in negative charge density of succinoglycan associated with the removal or absence of the succinyl modification facilitates binding by Congo Red, which itself is negatively charged (159). The absence or removal of the acetyl modification may affect the conformation or aggregation state of succinoglycan molecules (130). Regardless of the precise explanation, the observation that Congo Red binds succinoglycan at different rates depending on the presence or absence of the acetyl and succinyl modifications suggests that Congo Red could be used in future genetic analyses aimed at isolation of mutants of *R. meliloti* or other succinoglycan-producing bacterial strains that exhibit defects in acetylation or succinylation of succinoglycan.

The Congo Red assay may prove useful in studying the process by which different endo-1,3-1,4-β-glycanases cleave succinoglycan. The *C. arvensicola* succinoglycan depolymerase has the capability to cleave succinoglycan prepared from *R. meliloti* cultures completely to monomers of the octasaccharide repeating unit (75). In contrast, the *R. meliloti* glycanases ExoK and ExsH can only partially depolymerize such succinoglycan samples and can accomplish the production of only tiny quantities of LMW succinoglycan (less than 3% of HMW succinoglycan is converted to LMW forms *in vitro*) (163). Here we have determined that, for a given increase
in reducing ends associated with glycanase treatment of succinoglycan, the Congo Red assay is more sensitive for the detection of glycanase activity associated with ExoK and ExsH than of glycanase activity associated with the *C. arvensicola* succinoglycan depolymerase. Our results imply that ExoK and ExsH cleave succinoglycan by a nonprocessive mechanism, such that cleavage sites are broadly distributed across succinoglycan molecules. In contrast, *C. arvensicola* succinoglycan depolymerase seems to cleave succinoglycan by a substantially different process. Given that every 1,3-1,4-β-linkage of succinoglycan molecules is actually susceptible to cleavage by the *C. arvensicola* succinoglycan depolymerase, and given that Congo Red is relatively insensitive in detecting depolymerization mediated by the *C. arvensicola* glycanase, it may be the case the *C. arvensicola* depolymerase cleaves succinoglycan chains by a processive mechanism, rather than by randomly cleaving the susceptible linkages distributed across succinoglycan chains. If this is the case, then ExoK/ExsH and the *C. arvensicola* succinoglycan depolymerase may represent distinct classes of endo-1,3-1,4-β-glycanases that function analogously to endo-type and exo-type glycanases, respectively (153).

Finally, the Congo Red assay may prove useful in analyzing how environmental conditions affect the activities of the ExoK and ExsH glycanases. Reducing end analyses are not particularly well suited to this task, given the relatively low sensitivity of the reducing end assay in detection of depolymerization of succinoglycan by ExoK and ExsH (163), and given that certain components of growth media may yield high background readings (G. M. Y. and G. C. W, unpublished results). We have demonstrated here that the Congo Red assay can be used for detection of depolymerization of neutral/heat-treated succinoglycan by ExoK and ExsH as expressed endogenously by *R. meliloti*. This type of analysis could be extended to test whether conditions that cause a decrease in production of LMW succinoglycan may exert their effects by causing a decrease in the activities of the ExoK and ExsH glycanases.
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FIG. 1. Congo Red dye fails to bind succinoglycan but does bind neutral/heat-treated and acid/heat-treated succinoglycan. Binding of Congo Red is indicated based on an increase in OD525. Graph indicates the rate of binding of Congo Red dye to succinoglycan in solution over a time course of three hours. Data represent the average of three samples (st dev ≤ 10% of avg). Inset: Measurements of the rate of binding of Congo Red dye to succinoglycan in solution over a time course of 24 hours. Symbols: Open squares, native succinoglycan; open triangles, neutral/heat-treated succinoglycan; open circles, acid/heat-treated succinoglycan; dashed line, no succinoglycan.
FIG. 2. Neutral/heat-treated succinoglycan exhibits a small gradual decrease over time in terms of the rate and extent to which Congo Red can bind to it. Wild-type succinoglycan was subjected to neutral/heat-treatment, removed from heat, and incubated at room temperature for the following lengths of time prior to Congo Red assay: open squares, 1 minute; open triangles, 30 minutes; open circles, 6 hours; closed squares, 24 hours; closed triangles, 48 hours. Data represent the average of three samples (st dev ≤ 5% of avg).
FIG. 3. Heat treatment and the absence/removal of acetyl and succinyl modifications independently increase the rate of binding of Congo Red dye to succinoglycan. Graph indicates the rate of binding of Congo Red to various forms of succinoglycan over a time course of 3 hours. Data represent the average of three samples (st dev $\leq 10\%$ of avg). Inset: Measurements of the rate of binding of Congo Red dye to various forms of succinoglycan over a time course of 24 hours. Symbols represent the following types of succinoglycan: open squares, wild-type; open triangles, $exoH$; open circles, $exoZ$; closed squares, $exoH$ $exoZ$; closed triangles, base treated; closed circles, acid/heat-treated. (A) native forms of succinoglycan (acid/heat-treated succinoglycan sample included for comparison). (B) neutral heat treated forms of succinoglycan (acid/heat-treated succinoglycan sample included for comparison).
FIG. 4. Glycanase-mediated depolymerization of native succinoglycan can be detected by use of Congo Red dye. Native succinoglycan samples were treated with glycanase for 24 hours, then were subjected to neutral/heat-treatment and were assayed for binding with Congo Red. The rate of binding of Congo Red to succinoglycan is plotted as OD<sub>525</sub> versus time. Data represent the average of three samples (st dev ≤ 10% of avg). Three dilutions of each glycanase were tested.

(A) *Cytophaga arvensicola* succinoglycan depolymerase. Symbols: open squares, 5 µl stock per ml reaction; open triangles, 1 µl stock per ml reaction; open circles, 0.2 µl stock per ml reaction.

(B) Purified ExoK. Symbols: open squares, 5 µg ExoK per ml reaction; open triangles, 1 µg ExoK per ml reaction; open circles, 0.2 µg ExoK per ml reaction. (C) Purified ExsH. Symbols: open squares, 5 µg ExsH per ml; open triangles, 1 µg ExsH per ml; open circles, 0.2 µg ExsH per ml. For each graph, dashed line indicates succinoglycan sample that was not treated with enzyme.
FIG. 5. Glycanase-mediated depolymerization of acid/heat-treated succinoglycan can be detected by use of Congo Red dye. Acid/heat-treated succinoglycan samples were treated with glycanase for 24 hours and then were assayed for binding with Congo Red. The rate of binding of Congo Red to succinoglycan is plotted as OD$_{525}$ versus time. Data represent the average of three samples (st dev $\leq$ 10% of avg). Three dilutions of each glycanase were tested. (A) *Cytophaga arvensicola* succinoglycan depolymerase. Symbols: open squares, 5 µl stock per ml reaction; open triangles, 1 µl stock per ml reaction; open circles, 0.2 µl stock per ml reaction. (B) Purified ExoK. Symbols: open squares, 5 µg ExoK per ml reaction; open triangles, 1 µg ExoK per ml reaction; open circles, 0.2 µg ExoK per ml reaction. (C) Purified ExsH. Symbols: open squares, 5 µg ExsH per ml; open triangles, 1 µg ExsH per ml; open circles, 0.2 µg ExsH per ml. For each graph, dashed line indicates succinoglycan sample that was not treated with enzyme.
FIG. 6. Plot of the relative concentration of reducing ends (as determined by Lever assay and expressed as OD$_{410}$) generated by glycanase-mediated depolymerization of succinoglycan as a function of glycanase concentration in depolymerization reactions. Succinoglycan samples were treated with glycanase for 24 hours and then were tested by the Lever assay. Data represent the average of three samples (st dev $\leq$ 10% of avg). Substrates: (A) native succinoglycan; (B) acid/heat-treated succinoglycan. Glycanase: open squares, *Cytophaga arvensicola* succinoglycan depolymerase; open triangles, ExoK; open circles, ExsH. Each glycanase was tested at three concentrations, representing a series of five fold dilutions. For the *C. arvensicola* succinoglycan depolymerase, the dilutions correspond to 5 $\mu$l, 1 $\mu$l, and 0.2 $\mu$l of stock per ml reaction. For ExoK and ExsH, the dilutions correspond to 5 $\mu$g/ml, 1 $\mu$g/ml, and 0.2 $\mu$g/ml glycanase per reaction. Arrows indicate absorbance associated with succinoglycan samples that have not been treated with glycanase: (*), native succinoglycan; (**), acid/heat-treated succinoglycan.
FIG. 7. Glycanase-mediated depolymerization of neutral/heat-treated succinoglycan can be detected in *Rhizobium meliloti* cultures. Data are plotted as OD$_{525}$ versus time. Data represent the average of three samples (st dev $\leq 10\%$ of avg). *exoY*, *exoY*, *exoY exsH*, and *exoY exoK exsH* mutant strains, all of which are blocked in production of succinoglycan, were cultivated for three days in minimal media. At this point, succinoglycan was added to cultures, and for certain treatments, purified ExoK and/or ExsH was also added to the levels that these enzymes normally accumulate in cultures. The cultures were incubated one additional day. Finally, the rate of binding of Congo Red to succinoglycan in the cultures was determined. Growth media: (A) GMS, (B) MGS. Strains/treatments: open squares, *exoY* strain; open triangles, *exoY exoK* strain; open circles, *exoY exsH* strain; no symbol, *exoY exoK exsH*; closed squares, *exoY exoK exsH* strain plus ExoK and ExsH protein; closed triangles, *exoY exoK exsH* strain plus ExsH protein; closed circles, *exoY exoK exsH* strain plus ExoK protein.
Chapter 6

Conclusions
My dissertation research has focused on the issue of whether endogenously expressed glycanases contribute to production of low molecular weight (LMW) succinoglycan by *Rhizobium meliloti*. I have determined that *R. meliloti* expresses two endo-1,3-1,4-β-glycanases, ExoK and ExsH, that both contribute to production of LMW succinoglycan by *R. meliloti* (162). I have determined that ExoK and ExsH specifically cleave nascent succinoglycan, but not succinoglycan that has accumulated in cultures, to yield LMW succinoglycan (163). I have also determined that the acetyl and succinyl modifications of succinoglycan influence the susceptibility of the polysaccharide to cleavage by these glycanases (164).

**Model for contribution of glycanases to production of low molecular weight succinoglycan**

Based on my results and the results of previously conducted genetic and biochemical analyses of succinoglycan biosynthesis, I propose the following model to explain the role of ExoK and ExsH in generating LMW succinoglycan in cultures (Fig. 1). *R. meliloti* synthesizes succinoglycan in HMW forms that initially are susceptible to cleavage by ExoK and ExsH but that, at some point, undergo a transition to forms that are refractory to cleavage. In the absence of ExoK and ExsH, the succinoglycan produced by *R. meliloti* accumulates in cultures in HMW forms. In the presence of ExoK and ExsH, some proportion of the glycanase-susceptible HMW succinoglycan is cleaved by the glycanases, to yield LMW succinoglycan and residual shorter chain forms of HMW succinoglycan. The extent of cleavage depends on the levels of expression and activity of the glycanases; increasing expression or activity of glycanases results in more extensive cleavage of the HMW succinoglycan. The extent of cleavage also depends on the acylation state of the succinoglycan; non-acetylated succinoglycan is more susceptible to cleavage by ExoK and ExsH, and non-succinylated succinoglycan is less susceptible to cleavage by ExoK and ExsH, in comparison to normally-modified succinoglycan. This model may provide a valuable framework for improving understanding of several issues, as described below.
How important are glycanases in the overall production of low molecular weight succinoglycan?

*R. meliloti* can apparently produce at least traces of LMW succinoglycan independent of the *exoK* and *exsH* genes (162), and it is conceivable that other mechanisms, such as the direct synthesis of LMW succinoglycan as mediated by ExoT (70), make a substantial contribution to the production of LMW succinoglycan under growth conditions different from those tested here. Comparison of the effects of variations in growth conditions (such as changes in osmolarity) on production of LMW succinoglycan by wild-type and *exoK exsH* strains should reveal whether ExoK and ExsH account for essentially all LMW succinoglycan produced under a wide range of growth conditions, or whether the contribution by ExoK and ExsH is substantial only under a limited set of growth conditions. Furthermore, epistasis analyses designed to test whether or not ExoK and ExsH contribute to the production of LMW succinoglycan during *in vitro* succinoglycan polymerization analyses should help to rigorously establish whether direct polymerization of LMW succinoglycan and glycanase-mediated production of LMW succinoglycan are actually entirely independent mechanisms.

What accounts for the transition of succinoglycan from glycanase-susceptible to glycanase-refractory forms?

The nature of the transition of succinoglycan from glycanase-susceptible forms to glycanase-refractory forms is unclear. One possible explanation, based on previously published analyses of the physical properties of succinoglycan, is that this transition corresponds to a change in the conformation (e.g. random coil to helix) or aggregation state (e.g. single chains to aggregates of chains) of polysaccharide molecules (26, 56). One approach that may prove useful in clarifying this issue would be to compare again the effects of variations in growth conditions on production of LMW succinoglycan by wild-type and *exoK exsH* strains, but to focus on the extent to which factors such as osmolarity affect ExoK and ExsH mediated cleavage of succinoglycan. For example, increasing the sodium chloride concentration in cultures causes a
shift toward production of more HMW and less LMW succinoglycan (22); increasing the sodium chloride concentration also causes an increase in the stability of aggregates of succinoglycan in succinoglycan solutions (26). Perhaps high salt concentrations in *R. meliloti* cultures promote the conversion of nascent, glycanase-susceptible forms of succinoglycan to glycanase-refractory forms, thus decreasing the conversion of HMW succinoglycan to LMW succinoglycan by ExoK and ExsH and causing a shift toward accumulation of HMW forms of succinoglycan in the cultures. In general, it may prove useful to identify growth conditions that affect the transition of succinoglycan from glycanase-susceptible to glycanase-refractory forms, and then to determine how these conditions affect the physical properties of succinoglycan, as a means of elucidating the nature of this transition. For this approach to be most useful, it would be crucial to develop techniques to distinguish between the effects of growth medium components on the activities of the ExoK and ExsH glycanases themselves versus on the susceptibility of succinoglycan to cleavage by these glycanases.

**How do environmental factors affect the extracellular accumulation of ExoK and ExsH?**

The extent to which growth medium components and genetic factors affect the expression and extracellular accumulation of ExoK and ExsH is not well understood. ExoK accumulates extracellularly to much a higher concentration in MGS medium (approximately 160 ng/ml) than in GMS medium (approximately 6 ng/ml), even though ExoK is present intracellularly at approximately the same levels in cells cultivated in either medium (163). Preliminary analyses suggest that a combination of low potassium phosphate levels and the presence of trace metals cause a decrease in the extracellular accumulation of ExoK in growth media (Louis LeCour, personal communication). Perhaps these factors affect the rate of secretion of ExoK from cells or the rate of degradation of extracellular ExoK. Further analyses may prove useful in terms of identifying proteins involved in secretion or degradation of ExoK, which themselves might turn out to be crucial for symbiosis even though ExoK apparently is not. Regardless of the precise explanation, the difference in the extracellular accumulation of ExoK underlines the importance
of measuring the levels of extracellular glycanases during the course of analyses aimed at determining the effects of growth medium components on the molecular weight distribution of succinoglycan.

**Which forms of succinoglycan are symbiotically-active?**

One rationale behind my experiments aimed at identifying and characterizing glycanases expressed by *R. meliloti* was to more rigorously test the hypothesis that LMW succinoglycan is crucial for symbiosis, but my analyses seem to have raised more questions than they have answered. Clearly the *exoK* gene and the *exsH/prsD/prsE* genes are not required for symbiosis in an otherwise wild-type background; each single and double mutant is proficient in the establishment of symbiosis (162). A recent analysis conducted by Cheng and Walker (35) indicates that certain symbiotically proficient *exo* mutant strains (*exoZ, exoS*) exhibit substantial defects in the initiation and extension of infection threads but nonetheless can establish productive symbioses. In light of their findings, it may prove worthwhile to reexamine the symbiotic phenotypes of *exoK exsH* mutants at a higher level of resolution to determine whether *exoK exsH* mutants also exhibit such symbiotic defects. Yet the fact that *exoK exsH* mutants can establish effective symbiosis implies that this strain can produce symbiotically active succinoglycan (162). Perhaps mechanisms that are independent of the *exoK* and *exsH* genes provide sufficient levels of LMW succinoglycan to mediate successful nodule invasion. However, an intriguing alternate hypothesis is that nascent HMW succinoglycan produced by the *exoK exsH* strain (and the wild-type strain) may be sufficient to mediate invasion, and that once succinoglycan undergoes its transition to glycanase-refractory forms it is no longer symbiotically active. If this is the case then purified HMW succinoglycan may be symbiotically inactive simply because it is in a non-nascent form. Furthermore, the symbiotic defect associated with *exoH* mutants (93) might be explained by non-succinylated succinoglycan undergoing an unusually rapid transition from a nascent, symbiotically-active form to a non-nascent, symbiotically-inactive form.
Unresolved issues regarding the PrsD/PrsE Type I secretion system

My analyses raise several questions about the expression of Type I protein secretion systems by *R. meliloti*. In general, Type I secretion systems consist of an ABC-type transporter (HlyB, PrtD), a membrane fusion protein (HlyD, PrtE), and an outer membrane component (TolC, PrtF) (55, 152). The genetic screen that I conducted, based on mutagenesis of *exoK* mutants followed by screening for colonies with severe defects in the production of Calcofluor halos, yielded a combined total of 12 independent mutant alleles of *prsD* and *prsE* (162). This implies that there is a high probability that my screen was saturated in terms of identifying genes that are involved in secretion of ExsH and that are 1) not transductionally linked to the *exo* region, 2) not lethal when mutated, and 3) not functionally redundant with other genes (162). The absence of mutations in an outer membrane component member of a Type I secretion system is conspicuous (162). It is possible that such a component is expressed by *R. meliloti* and is crucial for secretion of ExsH, but that the gene encoding this component fails to meet one or more of the criteria listed above. It is also technically possible that the *R. meliloti* PrsD/PrsE system is distinct from previously described Type I secretion systems in that the *R. meliloti* system simply does not require an outer membrane component for successful secretion of ExsH from cells. It is noteworthy that, for the strain *Rhizobium leguminosarum* bv. *viciae*, homologs of *prsD* and *prsE* have also been cloned and have been implicated in secretion of several proteins, including NodO and two polysaccharide lyases that degrade the exopolysaccharide produced by this strain (58, 59). Yet no outer membrane component has been reported for that system either.

The *R. meliloti* genome includes at least one other pair of genes, *expD1/expD2*, that based on nucleotide sequence are predicted to encode a Type I secretion system (16). Mutations in *expD1* or *expD2* block the production of EPS II, which in turn blocks the establishment of symbiosis for strains that are dependent upon biosynthesis of EPS II for root nodule invasion (65). Regarding strains that produce succinoglycan, I have determined that, like *prsD* single
mutants, *prsD expD1* double mutants are symbiotically proficient (G. M. Y., and G. C. W., unpublished results). This rules out the possibility that the symbiotic proficiency of *prsD/prsE* mutants is due to the functional replacement of PrsD/PrsE by ExpD1/ExpD2. Nonetheless, it is conceivable that *R. meliloti* expresses additional Type I secretion systems, and that certain individual systems or combinations of systems are required for establishment of symbiosis by *R. meliloti* strains that produce succinoglycan.

While *prsD* and *prsE* are not required for symbiosis by *R. meliloti*, these genes are required for symbiosis by *R. leguminosarum* bv. *viciae* (58). The symbiotic requirement for *prsD* and *prsE* is presumed to be associated with the secretion of a symbiotically crucial protein by PrsD/PrsE (58). The identity of such a protein has not yet been determined, but genetic analyses have ruled out the possibility that it might correspond to NodO or the two polysaccharide lyases (58, 59).

**Further applications and significance of research on *Rhizobium meliloti* glycanases**

The model proposed here implies that the molecular weight distribution of succinoglycan can be manipulated experimentally 1) by genetically blocking glycanase expression, 2) by adding exogenous glycanases to the precise levels desired, and 3) by genetically blocking acetylation and/or succinylation of succinoglycan. Improved understanding of techniques for the manipulation of the molecular weight distribution of succinoglycan could prove useful in terms of tailoring preparations of succinoglycan for specific industrial or commercial applications. For example, succinoglycan produced by a *Pseudomonas* strain is used in enhanced recovery of petroleum from oil fields in the North Atlantic, and exquisite control of the viscosity of succinoglycan solutions is desirable for this application (36). If glycanase-mediated control of polysaccharide molecular weight also applies to this *Pseudomonas* strain, then genetically altering glycanase production might enable the production of preparations of succinoglycan that yield optimal viscosities in solution. Regarding basic research issues, the development of an ability to produce preparations of HMW succinoglycan in which molecules fall within discrete
molecular weight ranges could prove useful in terms of testing assumptions about how the physical properties of succinoglycan vary with polysaccharide molecular weight. Also, supplementation of wild-type *R. meliloti* cultures with additional ExoK and/or ExsH glycanase may serve as a simple way to generate large quantities of LMW succinoglycan for use in analyses of the symbiotic activity of succinoglycan. Finally, the observation that at least several bacterial strains in addition to *R. meliloti* express glycanases that depolymerize the strains’ own polysaccharides hints that this phenomenon may have a broader biological relevance than we have previously considered.
Fig. 1. ExoK and ExsH cleave nascent succinoglycan, but not succinoglycan that has accumulated in cultures, to yield LMW succinoglycan. Nascent, non-acetylated succinoglycan is more susceptible to cleavage, and nascent, non-succinylated succinoglycan is less susceptible to cleavage, in comparison to normally-modified succinoglycan.
Appendix A

*Rhizobium meliloti* May Have the Capacity to Express a Third Succinoglycan Depolymerase
Introduction

We have previously shown that *Rhizobium meliloti* expresses two endo-1,3-1,4-β-glycanases, ExoK and ExsH, that depolymerize nascent high molecular weight (HMW) succinoglycan to yield low molecular weight (LMW) succinoglycan in cultures (162, 163). Here we report that *R. meliloti* may also have the capacity to express a third glycanase. We have cloned and sequenced an *R. meliloti* gene, provisionally designated *egl*, that is predicted to encode an endo-1,3-1,4-β-glycanase secreted by a Type I secretion system. Interestingly, despite a high degree of homology between the putative third glycanase and ExsH, the *egl* gene does not seem to influence the production of Calcofluor halos by *R. meliloti* colonies and purified Egl protein does not seem to exhibit succinoglycan depolymerase activity. It remains to be determined whether Egl exhibits glycanase activity under reaction conditions or with substrates other than those that we tested here, or whether Egl is a non-functional glycanase.

Discovery and cloning of *egl*, a gene that encodes a homolog of the ExsH glycanase

We discovered the *egl* gene serendipitously, while mapping the positions of several Tn5 insertions in the *exsH* gene (*hal13* (162) and insertions 308, 334, and 343 (103)) by Southern hybridization. For these Southern hybridization analyses, we used a 276 bp Xhol-BglII fragment of *exsH* (corresponding to nucleotides 1120 to 1395 of the 1395 bp *exsH* ORF) as a probe (162). With this probe, we determined the hybridization patterns of *EcoRI* and *EcoRI-BglII* digested genomic DNA that had been prepared from wild-type and *exsH* mutant strains (Fig. 1). As predicted based upon the nucleotide sequence of the *exsH* gene, the probe hybridized to a 3.0 kb *EcoRI* fragment and an internal 2.7 kb *EcoRI-BglII* fragment of wild-type genomic DNA (162). These restriction fragments contain the *exsH* gene. And as predicted based upon mutant analyses, the positions of these fragments were shifted for genomic DNA prepared from the various *exsH* mutant strains, reflecting the presence of Tn5 insertions in the *exsH* gene. Unexpectedly, we also observed that the probe hybridized to a 5.0 kb *EcoRI* fragment and an internal 3.2 kb *EcoRI-BglII* fragment of genomic DNA from the wild-type strain and from each
of the *exsH* mutants (Fig. 1). Based on the sizes of these fragments and based on the fact that these fragments were common to both wild-type and *exsH* mutants, we concluded that the fragments do not contain the *exsH* gene but instead contain a sequence that is highly homologous to the *exsH* probe. Our results suggested the possibility that these fragments may encode a glycanase homologous to ExsH.

We cloned and sequenced this region of DNA, and determined that it encodes a 1395 bp ORF homologous to *exsH*. Specifically, by using the 276 bp *XhoI*-BglII fragment of *exsH* as a probe in colony hybridization analyses, we isolated two cosmids, designated pG400 and pG401, from a cosmid library of *R. meliloti* genomic DNA. Restriction analyses imply that these cosmids contain partially overlapping segments of genomic DNA (specifically, a 5.0 kb *EcoRI* fragment and a 7.2 kb *EcoRI* fragment), and Southern hybridization analyses indicate that the *exsH* probe hybridizes to the 5.0 kb *EcoRI* fragment and the internal 3.2 kb *EcoRI*-BglII fragment, confirming that we had cloned the fragment homologous to the *exsH* probe (data not shown). We subcloned the 5.0 kb *EcoRI* fragment in both orientations into pUC19 to yield pG402 and pG403 and in both orientations into pSW213 to yield pG404 and pG407. We determined that the internal 3.2 kb *EcoRI*-BglII fragment actually corresponds to a 3.2 kb *BglII* fragment within the 5.0 kb *EcoRI* fragment. We subcloned this 3.2 kb *BglII* fragment in both orientations into the *BamHI* site of pBluescript II KS to yield pG405 and pG406. We generated a series of nested deletions across the 3.2 kb *BglII* fragment and determined the nucleotide sequence of 1.9 kb of this fragment. Nucleotide sequence analyses revealed the presence of a 1395 bp ORF which is 80% identical to *exsH* in terms of nucleotide sequence (data file is termed "gly3.seq"). We have provisionally designated this ORF *egl*. We determined that this striking conservation of nucleotide sequence between *exsH* and *egl* does not extend does not extend to regions upstream or downstream of the two ORFs (data not shown).

The deduced amino acid sequence of Egl protein indicates that Egl is also 80% identical to ExsH at the level of amino acid sequence (Fig. 2). Like ExsH (162), the Egl protein is composed of an N-terminal domain typical of proteins secreted by Type I secretion systems and
a C-terminal domain homologous to endo-1,3-1,4-β-glycanases (Fig. 3). Thus, Egl is a promising candidate for a glycanase expressed by *R. meliloti* that may be secreted by a Type I secretion system and that may mediate depolymerization of succinoglycan.

**Egl may be expressed by *R. meliloti* and secreted by the PrsD/PrsE Type I secretion system**

Preliminary analyses of proteins in *R. meliloti* culture supernatants hint at the possibility that Egl may be expressed by *R. meliloti* and that Egl may be secreted by the PrsD/PrsE Type I secretion system. By concentrating proteins present in the supernatants of *R. meliloti* cultures, separating them by SDS-PAGE, and visualizing proteins with Coomassie blue dye, we had previously determined that *prsD* and *prsE* mutants are blocked in the extracellular accumulation of several proteins (162) (Fig. 4). ExsH seemed to be an excellent candidate for a protein secreted by PrsD and PrsE, based on nucleotide sequence analyses, Calcofluor halo analyses, and the equivalent distributions of HMW and LMW succinoglycan in cultures of *exsH*, *prsD*, and *prsE* mutants (162). However, we observed no apparent difference between the profiles of extracellular proteins in supernatants of wild-type versus *exsH* mutants, as determined by Coomassie blue staining (162) (Fig. 4). We hypothesized that *R. meliloti* may secrete ExsH at such low levels that the protein cannot be detected by Coomassie blue staining, or that *R. meliloti* may secrete one or more additional proteins of the same molecular weight as ExsH, thus obscuring the absence of ExsH in *exsH* mutant supernatants (162). By raising polyclonal antibodies against ExsH and conducting Western blot analyses on *R. meliloti* culture supernatants, we were able to demonstrate directly that ExsH does accumulate extracellularly and that this accumulation is dependent upon the *prsD* and *prsE* genes (163). Returning to analyses of *R. meliloti* supernatant proteins by visualization with Coomassie blue, we observed that *egl* mutants seem to lack an extracellular protein of approximately 51 kDa, the size predicted for Egl (Fig. 4). Furthermore, *prsD* and *prsE* mutants lack a protein of the same size (Fig. 4). These observations suggest that, like ExsH, the Egl protein is expressed by *R. meliloti* and is secreted from cells by the PrsD/PrsE Type I secretion systems.
We have determined that anti-ExsH antibodies do not recognize the 51 kDa protein secreted by *R. meliloti* that may correspond to Egl (data not shown). Furthermore, anti-ExsH antibodies do not recognize Egl expressed and purified from *Escherichia coli* (data not shown). To more rigorously test whether Egl is actually expressed and secreted by *R. meliloti*, it may prove useful to generate antibodies that specifically recognize Egl.

**Egl may be a non-functional glycanase**

Preliminary analyses suggest that Egl may be a non-functional glycanase, or at least that Egl is not active under the same conditions for which ExsH is active. By transferring plasmids containing the *egl* gene into wild-type, *exoK, exsH*, and *exoK exsH* strains and observing Calcofluor halo phenotypes of the resulting strains, we determined that extra copies of the *egl* gene have no effect on the rate of Calcofluor halo formation by *R. meliloti* colonies (data not shown). By generating mini-Tn5-Km and Tn5 mutations in the cloned *egl* gene, homogenotizing these mutations, and transducing them into wild-type, *exoK, exsH*, and *exoK exsH* strains, we determined that mutations in *egl* cause no detectable defects in Calcofluor halo production (data not shown). By inoculating alfalfa seedlings with *egl, egl exoK, egl exsH, and egl exoK exsH* strains and scoring the strains for their ability to induce pink root nodules and to promote growth of plants in the absence exogenously-added fixed nitrogen, we determined that the *egl* gene is not required for establishment of symbiosis in wild-type, *exoK, exsH*, or *exoK exsH* backgrounds (data not shown). Finally, by expressing and purifying Egl from a heterologous system and testing the activity of Egl *in vitro*, we failed to observe any glycanase activity associated with the protein. For this final experiment, we cloned *egl* under the control of the T7 promoter in the vector pET5a, to yield the plasmid pG411. We expressed and purified Egl from *E. coli* using previously described techniques (163). And then we tested Egl for glycanase activity, using standard reaction conditions under which ExoK and ExsH exhibit glycanase activity (163). We used acid/heat-treated succinoglycan as a substrate (163). Under these conditions, we observed no glycanase activity associated with Egl (Table 1).
ExsH-Egl hybrid proteins yield insights into the differences in glycanase activity associated with ExsH and Egl

To gain insights into the apparent lack of activity associated with the Egl protein, we proceeded to construct a series of ExsH-Egl hybrid proteins and to test the activity of these proteins (Fig. 5). We were particularly interested in determining whether differences in amino acids localized within a single, short segment of the two glycanases might account for the differences in activity between ExsH and Egl, or whether differences in amino acids distributed across the length of the two proteins additively contribute to the differences in activity between the two proteins.

To construct the ExsH-Egl hybrid proteins, we identified several restriction sites (BsrI, BssHII, and BsmI) that are precisely conserved between the exsH and egl genes, conducted partial restriction digests of the cloned exsH and egl genes, and ligated DNA fragments to generate ORFs that represent hybrids of the two genes (Fig. 5). We expressed the hybrid proteins by use of the T7 expression system (Fig. 6). We then measured the glycanase activities associated with ExsH, Egl, and each hybrid protein (Table 1, Table 2).

Our results indicate that no single amino acid residue of Egl accounts for the inactivity of Egl protein (Table 1, Table 2). Any short segment of Egl can be swapped into the ExsH protein without completely eliminating glycanase activity (Table 1, Table 2). Longer segments of Egl, swapped into ExsH, cause increasingly dramatic defects in glycanase activity (Table 1, Table 2). Furthermore, any single short segment of ExsH can be swapped into the Egl protein without restoring glycanase activity (Table 1, Table 2). Longer segments of ExsH are required for restoration of glycanase activity (Table 1, Table 2).

Conclusions

We have identified a gene, designated egl, that encodes a protein homologous to the ExsH glycanase. Interestingly, purified Egl exhibits no detectable glycanase activity. Analyses
of the glycanase activities of ExsH-Egl hybrid proteins indicate that multiple differences in the amino acid sequences of the ExsH and Egl proteins, distributed across the length of the two proteins, additively account for the differences in the glycanase activities associated with the two proteins. It remains to be determined whether Egl is simply a non-functional homolog of endo-1,3-1,4-β-glycanases, or whether Egl is active but only under conditions other than those which we have tested here.
Fig. 1. Southern hybridization data reveal the presence of a sequence homologous to the *exsH* gene in the *Rhizobium meliloti* genome. For this experiment, a 276 bp *XhoI-BglII* fragment of the *exsH* gene (corresponding to nucleotides 1120 to 1395 of the 1395 bp *exsH* ORF) was used as a probe against *R. meliloti* genomic DNA. Lanes 1 and 7, wild-type; lanes 2 and 8, *exsH13ΩTn5*; lanes 3 and 9, insertion 308; lanes 4 and 10, insertion 334; lanes 5 and 11, insertion 343; lane 6, marker. Marker bands (kb) are indicated on the left side of blots. (A) Two hour exposure. (B) Overnight exposure.
Fig. 2. Comparison of the deduced amino acid sequence of ExsH and Egl. ExsH and Egl are 80% identical at the level of amino acid sequence. Amino acids that are identical between the two proteins are indicated in upper-case letters.
ExsH MSkTV1NAvG tPLyYsGSST AWFSAstGSGP tLhGTAGNDS MWgDSSVnVT MIGgrGDDIY YLYsINRAy EAageGVDTI
EgL MSrTVtNALG ePLsYgGSST AWFSAAsGSGP 1LyGTAGNDS MWaDSSVdVT MIGdsGDDIY YLYsGNRAs EApsaGVDTI

ExsH sTWMSytLPa NFENLTVTGs grFaFGNeAd NIIkGGSStQ TiGGrGNDV LiGAGGADTF vFaRGNGSDL IfDFngyDFdV
EgL nTWMSyLSPe NFENLTVTGv egFgFGNSAs NIIsGGSStQ TiGgaGNDV LtGAGGADTF aFkRGNGSDL IsDFgsDDvV

ExsH RLdGYGFtSF eqILsNVAQE GaDLrLhLAD GEslVFANTt ADeLqAhQFr LsLDRSVLsQ TFSDeFNTLQ LrnGTSgVWD
EgL RLsGYGFtSF dhILsNVAQE GLDLkLsLAD GEyLVFANTs ADgLhAnQFs LaLDRSVLtQ TFSDKFNTLQ LsdGTSgVWD

ExsH aKfWWAPEKG ATLssNgEqQ WYiNPsYePt ASvNPFSVnm GVltITAApa SeAIQAEnBG YDYTSGMLTT YSSFAYTGY
EgL pKyWWAPEKG ATLTgNdElQ WYvNPtYqPt ASaNPFSVtd GVLTITAKpa SgAIQAETNG YDYTSGMLTT YSSFAYTGY

ExsH FEMRADMPDD QGvWPAFWLL PaDGsWPPEL DVVErGQDs NTVIAVHSN ETGsrTSIen sVkVaDaSGF HtYGVLWTEE
EgL FEMRADMPDD QGaWPAFWLL PgDGtWPPEL DVVEMrGQDP NTVIAVHSN ETGsqTSIas aarVTdtSGF HkYGVLWTEE

ExsH EIVWFPPDAA IARADTPSDM HDPMYMLVNL AvGGlAGtPr DGLadGsEMK iDYiKAYSLD ADWqI*
EgL EIVWFPPDAA IARADTPSDM HDPMYMLVNL AiGGmAAGPt DGLmgGaEMK vDYvKAYSLD ADWHi*
**Fig. 3.** Comparison of the C-terminal half of Egl (amino acids 222 to 465) and the homologous domains of the endo-1,3-1,4-β-glycanases ExsH of *Rhizobium meliloti* (162), BglA of *Rhodothermus marinus* (143), and LicA of *Clostridium thermocellum* (136). Amino acids which are identical among at least two of the proteins are indicated in upper-case letters.
| 222 | Egl      | FSDdFNTLQL sDGtSGVWDp  KyWWAPEKGA TLtGNdELQW  YvNptYqPTA  SaNPSVtDG  VIITIATkPAS  qAIQAEtNGY |
|-----|---------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| 233 | ExsH    | FSDEFNtLQL rNGTsGVWDa  KlFWAPEKGA  TLssNGEqQW  YiNPsYePTA  SvNPFsVnnG  VIITIATaPAS  eAIQAEInGY |
| 244 | BglA    | WSDEFdySgL pDpek..... WdYDvGg  hGwGQnELQy  YTr..... A  rieNArVggG  VliIearhE.  py..... EGr |
| 254 | LicA    | WSDEFNgsSei .Nman..... WsYDdpt  nGrwNGEvQs  YTq..... NNAYikDG  aLvIeARkE.  ditepsgEtY |
| 302 | Egl      | DYTSGMLTTY SSFAQTYGYF EMRADMnPDDQ  GaWPAPWLLP  gDGtW.  ....... .PPELDVVE  MnGQDPNTVI  ATVHSNE... |
| 313 | ExsH    | DYTSGMLTTY SSFAQTYGYF EMRADMnPDDQ  GvWPAPWLLP  aDGsW.  ....... .PPELDVVE  MrGQDSNTVI  ATVHSNE... |
| 324 | BglA    | eYTSarLvTr GKaSWTYGrF EIRAr1PsGr  GtWPAPWMLP  drqTygsaYW  PdnGEIDIME  hvGfnPDvVh  GTVHtKaynh |
| 335 | LicA    | hYTSskLrTk GkkSWkYGkF  EIRAKMPqQ  GiWPAPWmMp  eDepfygt.W  PkcGEIDIME  llGhePDkiy  GTiHfgEph. |
| 382 | Egl      | ...TGSQTSI asaarVTdTs  GfHKyGvLWT  EEEIVwYFDD  AAIARAD ......... .TPSDMHDp  MYMLVNLAiG |
| 393 | ExsH    | ...TGSrTSI ensvkVaDAS  GfHtGvLWT  EEEIVwYFDD  AAIARAD ......... .TPSDMHDp  MYMLVNlAVG |
| 404 | BglA    | llgTqrgGSI  rvPTar.... DfHVYaIWt  PEEIRwFvDD  sLYy.... rf  pnerltdpea  dwrhwPFDqDp  FhLImNIAVG |
| 414 | LicA    | ...keSQGty tlPegqTfAd  DfHVYsIEWe  PgEIRwYIdg  LkHyAvAndwy  srdpyladdy  typa.PFDQn  FfLIIINIsVG |
| 461 | Egl      | Gm..AGPPTd GLmgGAEMKV  DYVKAYsLDA  DWHi*..... |
| 472 | ExsH    | G1..AGTPrD GLADGsEMKi  DYkAYsLDA  DWhi*..... |
| 501 | BglA    | GAwGgqgqVd peAFAQV1VV  DYVRVYrwe ......... |
| 512 | LicA    | GWPgGyPd.e ttvFPqQMVV  DYVRVYqkdk  ypHrekpake |
**Fig. 4.** SDS-PAGE separation and Coomassie brilliant blue staining of extracellular proteins prepared from *R. meliloti* culture supernatants. Lines indicate positions of molecular weight markers (kDa). Top two arrows (a, b) indicate proteins which are clearly present in *exoY*, *exoY exsH*, *exoY egl*, and *exoY exsH egl* supernatants but are absent from *exoY prsD* and *exoY prsE* supernatants. Lower two arrows indicate predicted positions of (c) Egl protein and (d) ExsH protein. The presence of additional proteins produced by *R. meliloti* that migrate similarly to ExsH may obscure detection of a band due to ExsH. Alternately, ExsH protein may be produced in quantities too low to be detected by this approach. Lanes: 1) *exoY* strain, 2) *exoY prsD* strain, 3) *exoY prsE* strain, 4) *exoY exsH* strain, 5) *exoY egl* strain, and 6) *exoY exsH egl* strain.
**Fig. 5.** Diagram of the ORFs of ExsH, Egl, and ExsH-Egl hybrid proteins cloned into pET5a expression vector. Hybrid ORFs were constructed by digesting the *exsH* and *egl* ORFs at restriction sites shared between the two ORFs and then ligating gene fragments to yield hybrid ORFs. Shaded boxes correspond to segments of the ExsH ORF. Open boxes correspond to segments of the Egl ORF. Restriction sites: *Bsr*I (nucleotide 621), *BssHII* (nucleotide 969), and *BsmI* (nucleotide 1101).
The diagram shows the positions of restriction sites (BsrI, BssHII, BsmI) and ORFs for several plasmids (pG315, pG411, pG315-2, pG315-3, pG315-4, pG411-2, pG411-3, pG411-4, pG315-5, pG411-5, pG315-6, pG411-6). The exsH ORF and egl ORF are indicated by different patterns. The scale is marked at 250 bp intervals.
Table 1. Glycanase activities of ExsH, Egl, and ExsH-Egl hybrid proteins. Acid/heat-treated succinoglycan was used as the substrate in these reactions. Glycanase activity is indicated by an increase in the OD410 reading.

<table>
<thead>
<tr>
<th>Expression plasmid</th>
<th>OD410 (time = 0)</th>
<th>OD410 (time = 12 hrs)</th>
<th>OD410 (time = 40 hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pG315</td>
<td>0.239</td>
<td>0.380</td>
<td>N.D.</td>
</tr>
<tr>
<td>pG411</td>
<td>0.205</td>
<td>0.201</td>
<td>N.D.</td>
</tr>
<tr>
<td>PG315-2</td>
<td>0.255</td>
<td>0.241</td>
<td>0.254</td>
</tr>
<tr>
<td>pG315-3</td>
<td>0.267</td>
<td>0.443</td>
<td>0.441</td>
</tr>
<tr>
<td>pG315-4</td>
<td>0.265</td>
<td>0.628</td>
<td>0.642</td>
</tr>
<tr>
<td>pG411-2</td>
<td>0.259</td>
<td>0.283</td>
<td>0.327</td>
</tr>
<tr>
<td>pG411-3</td>
<td>0.258</td>
<td>0.243</td>
<td>0.255</td>
</tr>
<tr>
<td>pG411-4</td>
<td>0.253</td>
<td>0.255</td>
<td>0.273</td>
</tr>
</tbody>
</table>

N.D. = not determined
Table 2. Glycanase activities of ExsH-Egl hybrid proteins. Acid/heat-treated succinoglycan was used as the substrate in these reactions. Glycanase activity is indicated by an increase in the OD\textsubscript{410} reading.

<table>
<thead>
<tr>
<th>Expression plasmid</th>
<th>OD\textsubscript{410} (time = 0)</th>
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<th>OD\textsubscript{410} (time = 24 hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pG315-5</td>
<td>0.234</td>
<td>0.262</td>
<td>0.326</td>
</tr>
<tr>
<td>pG411-5</td>
<td>0.222</td>
<td>0.217</td>
<td>0.205</td>
</tr>
<tr>
<td>pG315-6</td>
<td>0.227</td>
<td>0.244</td>
<td>0.262</td>
</tr>
<tr>
<td>pG411-6</td>
<td>0.214</td>
<td>0.218</td>
<td>0.202</td>
</tr>
</tbody>
</table>
Appendix B

The Acetyl and Succinyl Modifications of Succinoglycan Affect the Physical Properties of the Polysaccharide*

Introduction

As part of our ongoing effort to understand the role of the rhizobial polysaccharide succinoglycan in the establishment of the *R. meliloti*-alfalfa nitrogen fixing symbiosis, we initiated a collaboration with Vic Morris (Institute of Food Research, Norwich Laboratory, Norwich Research Park) to determine the extent to which succinyl and acetyl modifications influence the physical properties of succinoglycan. For this collaboration, I prepared samples of succinoglycan from cultures of wild-type, *exoZ*, *exoH*, and *exoH* *exoZ* strains, which correspond to normally-modified succinoglycan, non-acetylated succinoglycan, non-succinylated succinoglycan, and non-acetylated/non-succinylated succinoglycan, respectively. Mike Ridout (Institute of Food Research, Norwich Laboratory, Norwich Research Park) then analyzed physical properties of solutions of these polysaccharide samples. Here I summarize the results of these analyses and discuss these results in the context of our recent findings that succinoglycan undergoes a transition from glycanase-susceptible forms to glycanase-refractory forms in cultures (163) and that the acetyl and succinyl modifications of succinoglycan influence the susceptibility of succinoglycan to cleavage by the *R. meliloti* glycanases ExoK and ExsH (164).

Analyses of succinoglycan prepared from wild-type, *exoZ*, *exoH*, and *exoH* *exoZ* strains

Ridout *et al.* (130) determined that the succinyl and acetyl modifications of succinoglycan affect the physical properties of solutions of the polysaccharide. Measurements of the viscosities of succinoglycan solutions across a range of shear rates indicate that the absence of the succinyl modification results in substantially higher viscosity, relative to normally-modified succinoglycan (130). The absence of the succinyl modification also causes increased pseudoplasticity (decreasing viscosity with increasing shear rate) and increased cooperativity of the order-disorder transition of succinoglycan (130). The absence of the acetyl modification causes a decrease in the order-disorder transition temperature of succinoglycan and the absence of the succinyl modification causes an increase in the order-disorder transition temperature (130).
The study by Ridout et al. (130) has resulted in improved understanding of how the acetyl and succinyl modifications affect the physical properties of succinoglycan. Previous studies, based on analyses of the non-acetylated succinoglycan produced by a Pseudomonas strain, had indicated that chemical removal of the succinyl modifications of succinoglycan by base treatment causes an increase in the denaturation temperature of the polysaccharide (56) and causes a change in stiffness of the polymer (although seemingly contradictory results have been reported with regard to the direction of this effect) (71, 73). Since these previous studies were based on analyses of succinoglycan lacking the acetyl modification (56, 71, 73), they did not specifically address the effect of acetyl modifications on physical properties of succinoglycan. If the authors of the previous studies had tested acetylated/succinylated forms of succinoglycan (e.g. succinoglycan produced by wild-type R. meliloti), base treatment of succinoglycan still would not have been useful for analyzing the effect of the acetyl modification alone on the physical properties of succinoglycan. This is because base treatment removes both the succinyl and acetyl modifications from succinoglycan (125). The use of mutants specifically defective in acetylation and/or succinylation of succinoglycan (exoZ, exoH, and exoH exoZ strains) enabled Ridout et al. (130) to analyze the effects of each type of modification in the presence and the absence of the other modification.

Interpretation of analyses of the physical properties of succinoglycan

The results of the study by Ridout et al. (130) are particularly interesting in the context of several of our own recent findings. We have determined that succinoglycan undergoes a transition from glycanase-susceptible to glycanase-refractory forms as the polysaccharide accumulates in cultures (163). We have also determined that the absence of the acetyl modification increases the susceptibility of succinoglycan to cleavage by glycanases, and that the absence of succinyl modification decreases the susceptibility of succinoglycan to cleavage by glycanases (164).
The results of Ridout et al. (130) hint that differences in the physical properties of succinoglycan that are caused by the absence of the acetyl or succinyl modification may explain differences in the susceptibility of differently-modified forms of succinoglycan to cleavage by glycanases. For example, it may be the case that the acetyl and succinyl modifications affect the conformation of succinoglycan chains and/or the rate of transition of chains from glycanase-susceptible to glycanase-refractory forms. To address this issue, it may prove useful to analyze the physical properties of succinoglycan samples prior to and during the transition from glycanase-susceptible to glycanase-refractory forms.

Ridout et al. (130) observed that the HMW succinoglycan prepared from cultures of these four strains exhibited approximately equal average molecular weights (no more than approximately two-fold variation between strains) as determined by gel permeation chromatography. In contrast, we have observed that HMW succinoglycan prepared from cultures of exoZ and wild-type strains exhibits a lower average degree of polymerization in comparison to HMW succinoglycan prepared from cultures of exoH and exoH exoZ strains, as determined by reducing end analyses (164). We would like to resolve the apparent discrepancy in these two sets of results in order to determine whether differences in the degree of polymerization of the succinoglycan samples prepared from different cultures may be partially responsible for differences in the physical properties of these succinoglycan samples. It may prove useful to analyze the physical properties of high molecular weight succinoglycan prepared from cultures of exoZ, exoH, and exoH exoZ strains that are also defective in expression of the glycanases ExoK and ExsH in order to address this issue.
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