

Exopolysaccharides from *Sinorhizobium meliloti* Can Protect against H₂O₂-Dependent Damage

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Sinorhizobium meliloti requires exopolysaccharides in order to form a successful nitrogen-fixing symbiosis with *Medicago* species. Additionally, during early stages of symbiosis, *S. meliloti* is presented with an oxidative burst that must be overcome. Levels of production of the exopolysaccharides succinoglycan (EPS-I) and galactoglucan (EPS-II) were found to correlate positively with survival in hydrogen peroxide (H₂O₂). H₂O₂ damage is dependent on the presence of iron and is mitigated when EPS-I and EPS-II mutants are cocultured with cells expressing either exopolysaccharide. Purified EPS-I is able to decrease *in vitro* levels of H₂O₂, and this activity is specific to the symbiotically active low-molecular-weight form of EPS-I. This suggests a potential protective function of exopolysaccharides against H₂O₂ during early symbiosis.

All aerobically growing organisms are exposed to reactive oxygen species (ROS) produced by univalent reduction of oxygen within the cell. Autoxidation of enzymes and leakage from the electron transport chain are two sources of internally produced ROS (1). Extracellular sources of ROS that may enter bacterial cells include the oxidation of extracellular compounds and the secretion of redox-cycling compounds by neighboring organisms (2). In particular, hydrogen peroxide (H₂O₂) has high membrane permeation; this means that the intracellular level of H₂O₂ in *Escherichia coli* is equivalent to the environmental level under most culture conditions (1).

ROS damage major biomolecules in cells: superoxide damages iron-sulfur clusters, inactivating the corresponding proteins; hydroxyl radicals damage DNA, causing cell death; and H₂O₂, at high concentrations, may damage lipid membranes (1). Natural production of ROS in aerobically growing organisms and environmental exposure to ROS necessitate the production of antioxidants. Superoxide dismutases, which convert superoxide to H₂O₂, and catalases, which convert H₂O₂ to water and oxygen, are enzymatic examples of various antioxidants. Additionally, small-molecule antioxidants, such as ascorbate and glutathione, can scavenge ROS (3).

As an obligate aerobe, the nitrogen-fixing plant symbiont *Sinorhizobium meliloti* must encode mechanisms for preventing ROS-related damage. These mechanisms include 3 catalases and 2 superoxide dismutases as enzymatic protection, as well as small molecules (4).

S. meliloti cells encounter new ROS not only in the soil but also during the establishment of symbiosis with their plant hosts, such as *Medicago sativa* and *Medicago truncatula*. This symbiosis begins with complex signal exchanges between plant and bacterial partners. Plant-produced flavonoids initiate the bacterial transcription of *nod* genes and the consequent bacterial production of Nod factor, which stimulates root nodule morphogenesis. In addition to Nod factor, *S. meliloti* produces an exopolysaccharide, succinoglycan (EPS-I), that is required for successful bacterial invasion of host tissue through plant-derived infection threads. These are invaginations of the plant cell wall within which bacteria replicate and penetrate into deeper plant cell layers. As the infection threads reach newly divided plant cells in the emerging nodule, bacteria are released from infection threads into the plant cytoplasm,

where they terminally differentiate and fix nitrogen (5). The nodule provides the proper environment for nitrogen fixation, including low free-oxygen levels to protect the oxygen-sensitive nitrogenase.

Superoxide and H₂O₂ are present in infection threads and in fully developed 6-week-old nodules (6). These ROS are likely formed primarily by plant NADPH oxidase (7). While H₂O₂ can be damaging, it appears to be required for successful infection: reduction of H₂O₂ levels by overexpression of bacterial catalase results in decreased efficiency of symbiosis and has negative effects on the formation of infection threads (8).

ROS may act in more than one way during nodulation. In the first 2 min of bacterium-host interaction, the levels of ROS in the plant increase rapidly and transiently (9). However, after 5 min, the presence of bacteria or Nod factor has an inhibitory effect on ROS flux (10). Transient changes in the levels of ROS (induced by chemical inhibitors of plant NADPH oxidase) are able to mimic the initial loss and subsequent reinitiation of root hair polar growth that characterizes early symbiosis (11). All of this evidence points to some positive roles for ROS in the *S. meliloti*-*M. truncatula* symbiosis.

Exopolysaccharides have been associated with protection against H₂O₂. *Pseudomonas syringae* cells devoid of exopolysaccharides are sensitive to ROS (12). In a study of *S. meliloti*, Davies and Walker (13) carried out a two-part screen for mutants sensitive to H₂O₂ that were also defective in forming productive nitrogen-fixing nodules on alfalfa (*Medicago sativa*). Among the mutants that were both symbiotically deficient and sensitive to H₂O₂, one-third of the mutated genes (3 out of 9) were involved in the production of EPS-I; additionally, a mutant defective in EPS-I production (the *exoY* mutant) was sensitive to H₂O₂ (13). These

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studies point to a possible connection between ROS and EPS-I in *S. meliloti*.

The structure of *S. meliloti* EPS-I consists of repeating units of octasaccharides, each carrying three nonsugar modifications (succinyl, acetyl, and pyruvyl). This EPS-I is synthesized in both a high-molecular-weight (HMW) (hundreds of octasaccharide subunits) and a low-molecular-weight (LMW) (octasaccharide monomers, dimers, and trimers) form (14). The production of these two forms appears to be specified by separate biosynthesis genes (*exoQ* for the HMW form and *exoT* for the LMW form), each of which acts in conjunction with an additional gene, *exoP* (15). Additionally, the LMW form of EPS-I can be produced from the HMW form by the glycanase ExoK (16).

EPS-I production is controlled by noncarbon nutrient limitation (e.g., limitation of nitrogen or phosphorus) (17) and some environmental stresses (18–20). Transcriptional regulators of EPS-I biosynthetic genes include the two-component system ExoS/ChvI (21) and the regulators SyrA and SyrM (22). Little is known about how environmental cues influence the action of these or other EPS-I regulators.

S. meliloti EPS-I mutants cannot form nitrogen-fixing nodules. Cheng and Walker (21) observed that these mutants fail to initiate and elongate infection threads on alfalfa (*Medicago sativa*). The symbiotic defect of EPS-I mutants can be reversed by the addition of exogenous EPS-I. Specifically, the LMW fraction is reported to be the active fraction necessary for symbiosis (23, 24).

S. meliloti also has the cryptic ability to produce a second exopolysaccharide, galactoglucan (EPS-II). EPS-II is a polymer of repeating galactose and glucose disaccharides with pyruvyl and acetyl modifications (25). Some commonly used lab strains do not produce EPS-II, due to disruption by a native insertion element in the regulatory gene *expR* (26). Biofilm formation and alfalfa root surface colonization are improved with the production of EPS-II (27). In the absence of EPS-I, EPS-II is sufficient for the nodulation of alfalfa but not for that of other host plants tested (26). Purified LMW EPS-II has also been reported to suppress the nodulation defects of a completely exopolysaccharide deficient *S. meliloti* strain, similarly to EPS-I (25).

In this work, we aimed to investigate the roles of EPS-I and EPS-II in the protection of *S. meliloti* against H₂O₂. We evaluated the ability of exopolysaccharides to provide protection against ROS-dependent death and the mechanism of that protection.

MATERIALS AND METHODS

Growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. Luria broth (LB) medium was used for bacterial growth (28), and 1.5% agar was added for solid media. Antibiotics were used at the following concentrations: streptomycin (Sm), 500 µg/ml; neomycin (Nm), 50 µg/ml; spectinomycin (Sp), 50 µg/ml; tetracycline (Tc), 10 µg/ml; hygromycin (Hy), 50 µg/ml. Nm^r of Rm7210 (*exoY*) was replaced with Sp^r as described previously (29).

H₂O₂ sensitivity assay. *S. meliloti* strains were inoculated into 3 ml LB with appropriate antibiotics and were incubated at 30°C overnight. Cultures were diluted to an optical density at 600 nm (OD₆₀₀) of 0.1 in LB medium without antibiotics and were grown to mid-exponential phase (OD₆₀₀, 0.3 to 0.4) at 30°C. Each culture was then diluted 1:100 in LB and was split in half; H₂O₂ was added, to a final concentration of 1 mM, to one-half of the cultures. H₂O₂-treated and untreated cultures were grown for 30 additional minutes at 30°C and were diluted to 1 × 10⁻⁵ to 2.5 × 10⁻⁶ of the starting volume. A 100-µl aliquot of each dilution was plated onto LB plates containing selective antibiotics. Plates were incubated at

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
<i>S. meliloti</i> strains		
Rm1021	Wild type (Sm ^r)	46
Rm7017	<i>exoD17::Tn5</i> (Sm ^r Nm ^r)	17
Rm7031	<i>exoA31::Tn5</i> (Sm ^r Nm ^r)	17
Rm7210	<i>exoY210::Tn5</i> (Sm ^r Nm ^r)	17
Rm7210Sp	<i>exoY210::Tn5-233</i> (Sm ^r Sp ^r Gm ^r)	This study
Rm8332	<i>exoQ332::Tn5</i> (Sm ^r Sp ^r)	47
Rm8530	<i>expR101</i> (Sm ^r)	48
Rm8532	<i>expR101 exoA31::Tn5</i> (Sm ^r Sp ^r)	48
MB801	<i>exoX::Tn5</i> (Sm ^r Nm ^r)	M. Barnett
APL41	<i>katA::uidA</i> pAPL10 integrated into Rm1021	This study
APL42	<i>katA::uidA</i> pAPL10 integrated into Rm7210	This study
APL43	<i>katA::uidA</i> pAPL10 integrated into MB801	This study
Plasmids		
pRK607	pRK2013Ω::Tn5-233 (Sp ^r Gm ^r)	29
pRF771	P _{trp} overexpression vector, pTE3 with extended polylinker (Tc ^r)	49
pE65	<i>nodD3</i> in pTE3 (Tc ^r)	50
pMB89	<i>syrA</i> in pTE3 (Tc ^r)	51
pS73	<i>syrM</i> in pTE3 (Tc ^r)	32
pDW33	Terminator and polylinker preceding <i>uidA</i> (Ap ^r Hy ^r)	52
pAPL10	<i>P_{katA}</i> in pDW33	This study

30°C. Colonies were counted after 3 to 4 days, and CFU counts per ml were back-calculated to determine the percentage of survival of treated versus untreated cultures.

H₂O₂ at a range of concentrations (0.3 to 10 mM) was tested on wild-type Rm1021. At 1 mM, H₂O₂ gave a midrange response allowing for the detection of both increased and decreased survival relative to that of the wild type. Similarly, we tested a range of time points (10 to 120 min) and chose 30 min as optimal. For coculture experiments, the strains were mixed in a 1:1 ratio and were grown together for 3 h prior to the addition of 1 mM H₂O₂.

For assays of iron-dependent ROS damage, the membrane-permeant iron chelator 2,2'-dipyridyl (30) (final concentration, 1 mM; dissolved in ethanol) or an equal volume of ethanol was added to 1:100 dilutions of mid-exponential-phase cultures. Cells were grown at 30°C for 15 min before splitting and H₂O₂ addition. Survival was determined as described above.

Assays were repeated at least three times for each strain. The average survival of the *exoY* strain was 56% that of the wild type, while the survival of the *exoX* strain was 163% that of the wild type. Significance within any one experiment was determined by Student's *t* test.

Zone-of-inhibition assay. The zone-of-inhibition assay was performed as described previously (31). Overnight cultures were diluted to an OD₆₀₀ of 0.2, and 100 µl was added to 3 ml soft LB agar (0.7% agar) and was poured onto plain LB agar plates. After the soft agar had solidified, a filter paper disk (diameter, 5 mm) was placed on the center of the plate, and 5 µl 30% H₂O₂ was added to the disk. Plates were incubated at 30°C for 2 days, and the diameter of the zone of clearing was measured. Assays were performed at least in triplicate.

Total-catalase-activity assay. Overnight cultures of *S. meliloti* were diluted to an OD₆₀₀ of 0.1 in LB medium without antibiotics. Cultures were grown at 30°C to mid-exponential phase (OD₆₀₀, 0.3 to 0.4); then they were split, and 1 mM H₂O₂ was added to one set. Cultures were returned to 30°C for 30 min. Portions (1 ml each) of treated and untreated cultures were harvested by centrifugation for 1 min. Cell pellets were resuspended in 1 ml lysis buffer (50 mM sodium phosphate [pH 7] and

0.1% Triton X-100) and were diluted 5- to 10-fold in 1× Amplex Red reaction buffer.

Twenty-five microliters of diluted cell lysates was incubated with 20 μM (final concentration) H₂O₂ in a 96-well microtiter plate (Microfluor 2 Black; Thermo Labsystems) in a final volume of 50 μl. Plates were incubated for 30 min at room temperature. Amplex Red reagent (final concentration, 50 μM) and horseradish peroxidase (final concentration, 0.2 U/ml) in 1× reaction buffer were added to cell lysates to a final volume of 100 μl, and the mixture was incubated at 37°C for 30 min in the dark. Plates were read as described previously (10). A standard curve of catalase was used to determine equivalent catalase units in each *S. meliloti* sample. Catalase activity was normalized to total-cell protein as determined by a modified Bradford assay (Bio-Rad protein assay). All assays were performed at least in triplicate.

Construction of *uidA* transcriptional fusion and GUS assay. A *katA*-β-glucuronidase (GUS) transcriptional fusion plasmid was constructed by PCR amplifying the region from 22 bp upstream through the first 281 bp of the *katA* open reading frame (ORF) by using primers with SpeI/XhoI sites. Ligation of the fragment into pDW33 generated pAPL10. Conjugation into Rm1021, Rm7210, or MB801 resulted in the integration of the fusion plasmid into the *S. meliloti* genome via a single-crossover event and duplicated the region of the *katA* ORF. GUS assays were performed in triplicate as described previously (32).

EPS purification. *S. meliloti* exopolysaccharides were recovered from culture supernatants by cetrinide precipitation as described previously (33), followed by precipitation with 3 volumes of acetone. Pellets were resuspended in water and were incubated at 37°C for 1 h. Exopolysaccharide fractions were dialyzed against water for final purification. Levels of reducing sugars were calculated by using the neocuproine assay as described previously (34). The total carbohydrate content was determined by the anthrone-sulfuric acid method as described previously (33).

EPS fractionation. EPS-I preparations from culture supernatants of wild type or *exoX* (EPS-I-overproducing) strains were purified as described above and were separated on a Sephadex G-75 column (2.5 by 40 cm) equilibrated with a solution of 50 mM sodium phosphate (pH 7) and 100 mM sodium chloride (14). Eighty 3.4-ml fractions were eluted with the same sodium phosphate-sodium chloride buffer. Low-molecular-weight fractions were concentrated 5-fold in a SpeedVac concentrator prior to use. The total carbohydrate contents of fractions were determined by the anthrone-sulfuric acid method (33).

Measurement of iron concentrations. Fifty micrograms of purified EPS-I was added to 2-fold dilutions (ranging from 0 to 100 μM) of ferrous sulfate (FeSO₄) or ferric chloride (FeCl₃) dissolved in 0.5 M hydrochloric acid, and the mixtures were incubated at either room temperature or 30°C for 10 to 20 min. The iron concentration was measured using a modified (35) ferrozine assay (36). Assays of EPS-I-treated and untreated samples were performed in triplicate.

Measurement of hydrogen peroxide concentrations. Dilutions of H₂O₂ were added to purified exopolysaccharides, dialyzed *exoY* cell culture supernatants, or water in a 96-well microtiter plate (Microfluor 2 Black; Thermo Labsystems) in a final volume of 50 μl. Plates were incubated for 15 min at room temperature. The Amplex Red assay was performed as described previously (10). All assays were performed at least in triplicate.

RESULTS

EPS production improves survival in H₂O₂. To determine whether EPS-I has an effect on the survival of *S. meliloti* in H₂O₂, we challenged three strains expressing different levels of EPS-I (wild-type, *exoY*, and *exoX* strains) with 1 mM H₂O₂ for various times and determined the CFU/ml (Fig. 1A). Preliminary experiments with different concentrations of H₂O₂ showed that 1 mM provided a midrange response in the wild-type strain (Rm1021), allowing us to distinguish both increased and decreased survival (data not shown). As little as 10 min of exposure to H₂O₂ de-

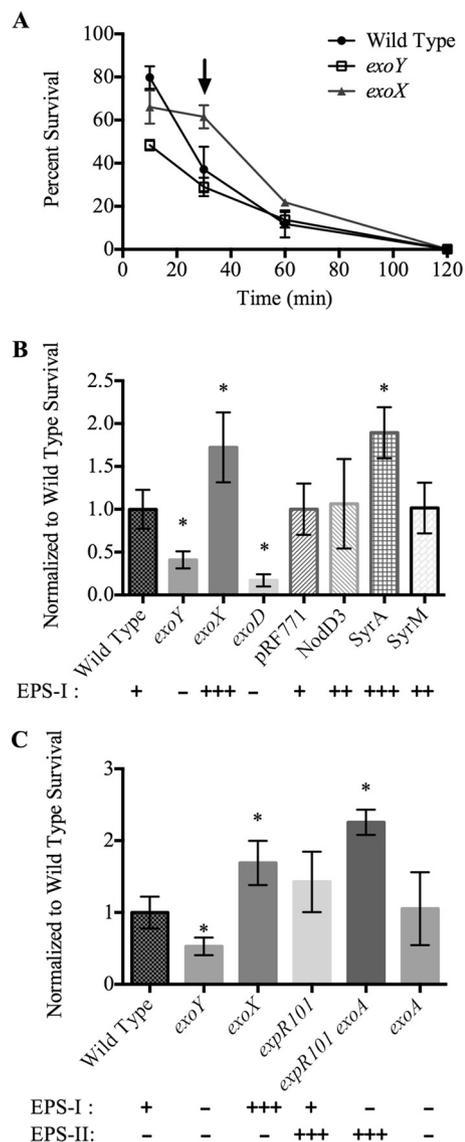


FIG 1 Effects of H₂O₂ on the survival of *S. meliloti* strains. (A) *S. meliloti* strains were tested for survival in 1 mM H₂O₂ at various time points. The arrow indicates the 30-min time point chosen for subsequent assays. (B) Survival of several *S. meliloti* strains expressing different levels of EPS-I after treatment with 1 mM H₂O₂. Survival was normalized to that of the wild-type strain Rm1021 (33% ± 7.4%) or to that of pRF771 (37% ± 11%). (C) Survival of *S. meliloti* strains expressing different levels of EPS-I or EPS-II after treatment with H₂O₂. Survival was normalized to the average survival of the wild type (23% ± 5.3%). Qualitative levels of EPS-I or EPS-II expression are denoted by -, +, ++, and +++. Error bars indicate standard deviations. Asterisks indicate significant differences (*P* < 0.05) from the wild type.

creased viability in all strains (ranging from approximately 50% to 80% survival). After 30 min in H₂O₂, significant differences among EPS-I⁻ (*exoY*), EPS-I-overexpressing (*exoX*), and wild-type strains were consistently seen (Fig. 1A). Based on these results, we chose the 30-min time point for further experiments. The EPS-I-overexpressing (*exoX*) strain showed no significant decrease in survival between 10 and 30 min in H₂O₂. This indicates that increased EPS-I production correlates with increased *S. meliloti* survival in H₂O₂.

S. meliloti strains expressing different levels of EPS-I due to Tn5

TABLE 2 Zones of inhibition of EPS-I mutants

Strain	Zone of inhibition by H ₂ O ₂ (cm)
Wild type	4.7 ± 0.1
<i>exoY</i> mutant	4.9 ± 0
<i>exoX</i> mutant	4.1 ± 0.2
<i>exoA</i> mutant	4.9 ± 0.1

insertion mutations (*exoY*, *exoX*, *exoD*) or to overexpression of EPS-I and *nod* gene regulators (*nodD3*, *syra*, *syrm*) were tested for survival in 1 mM H₂O₂ for 30 min (Fig. 1B). Given the day-to-day variability of the overall percentage of survival, we normalized the data for each strain within an experiment to the average for the wild type. Relative survival was the same if strains were normalized to a strain carrying neutral Tn5 or to the wild-type strain (data not shown). Strains overexpressing EPS-I (e.g., the *exoX* strain or the *syra*-overexpressing strain) consistently showed levels of survival higher than that of the wild type, while strains with decreased EPS-I expression (e.g., the *exoY* and *exoD* strains) showed lower levels of survival in H₂O₂.

S. meliloti can also produce a second acidic extracellular polysaccharide: EPS-II (26). To determine whether EPS-II can protect cells against H₂O₂ damage, we tested strains producing EPS-I, EPS-II, or both for viability in 1 mM H₂O₂ (Fig. 1C). The relative level of survival of a strain producing both EPS-I and EPS-II (*expR101*) was higher than that of the wild type. The *expR101* *exoA* double mutant produces only EPS-II. Comparison of the relative survival levels of *expR101* *exoA* and *exoA* strains showed that EPS-II alone improved *S. meliloti* survival in H₂O₂ almost 2-fold. The increase in survival seen in EPS-II-producing strains seems to be similar to the increase seen with EPS-I.

The response of the *exoA* mutant compared to that of the wild type in liquid culture was surprising, considering that the *exoA* mutant should also be deficient in EPS-I production. To test the sensitivity of the *exoA* mutant in other ways, a zone-of-inhibition assay was performed (Table 2). This showed that the *exoA* mutant is indeed sensitive to H₂O₂, and the differences in phenotype between the *exoA* mutant and other EPS-I-deficient mutants may be due to the assay conditions.

Differences in H₂O₂ survival between EPS-I-overexpressing and EPS-I-deficient strains do not appear to arise from differences

in catalase response. While transcription of the bifunctional catalase/peroxidase *kataA* is induced by H₂O₂, we found that the degree of *kataA* induction in the *exoX* and *exoY* mutants did not differ significantly from that in the wild type (Fig. 2A). Additionally, the total equivalent catalase activities of these three strains were not significantly different (Fig. 2B).

EPS-I and EPS-II can protect neighboring cells from H₂O₂.

To determine if secreted EPS-I protects against H₂O₂ in *trans*, we cocultured *exoY*^{SP} cells (EPS-I⁻ strain carrying the *exoY* Tn5 insertion, with Nm^r replaced with Sp^r) with either *exoY* or *exoX* cells for 3 h prior to challenge with H₂O₂ (Fig. 3A). Cells were washed in order to remove antibiotics prior to coculture. The relative survival of the *exoY*^{SP} strain was similar to that of the *exoY* strain alone, and the *exoY*^{SP} strain cocultured with the *exoY* strain showed no significant increase in survival. However, coculture with the *exoX* strain increased the survival of the *exoY*^{SP} strain 4-fold. Similarly, coculture of the *exoY* strain with the EPS-II-producing strain (*expR101* *exoA*) showed a 50% increase in *exoY* strain survival (Fig. 3B).

To test if an exported factor is responsible for the increase in survival seen in the coculture of *exoY*^{SP} and *exoX* strains, we grew *exoY*^{SP} cells in heat-killed medium conditioned with either the *exoY* or the *exoX* strain. We saw no increase in the relative survival of the *exoY*^{SP} strain grown in *exoX* strain-conditioned medium over that of the *exoY*^{SP} strain alone (data not shown). We grew the *exoY* strain with increasing concentrations of purified EPS-I in order to determine if EPS-I alone can improve the survival of EPS-I⁻ strains (data not shown). No significant improvement in *exoY* survival was seen with the concentrations of EPS-I tested.

Iron is required for H₂O₂-dependent death, independently of EPS-I production. A mechanism for H₂O₂-dependent death is the generation of hydroxyl radicals through iron-requiring Fenton chemistry (37). To determine if iron is required for the H₂O₂-dependent death of *S. meliloti*, we added the cell-permeant iron chelator 2,2'-dipyridyl (final concentration, 1 mM) to cells 15 min prior to H₂O₂ addition (Fig. 4A). Dipyridyl increased the survival of the wild-type, *exoY*, and *exoX* strains to nearly the levels of non-H₂O₂-treated cells regardless of EPS-I production, indicating that iron is required for the H₂O₂-dependent death of *S. meliloti*. The concentrations (CFU/ml) of non-H₂O₂-treated wild-type cultures with and without dipyridyl were similar (1.60 × 10⁶ to

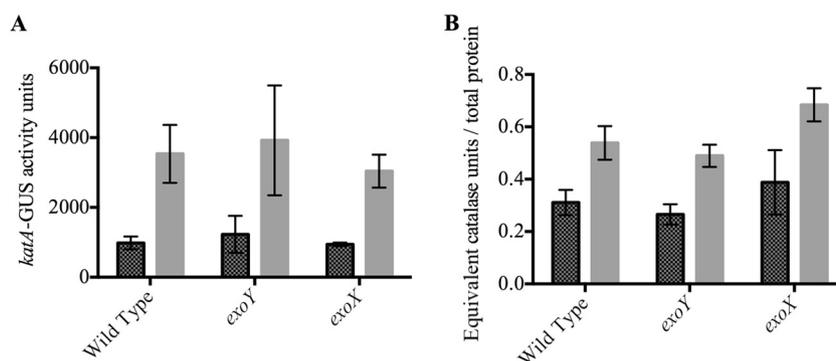


FIG 2 Activity of *kataA* in response to H₂O₂. (A) Expression of *kataA* as determined by *uidA* transcriptional fusion with (light shaded bars) or without (dark shaded bars) a 30-min induction with 1 mM H₂O₂. *kataA* expression is not significantly different in different *S. meliloti* strain backgrounds. (B) Total equivalent catalase activity from cell lysates left untreated (dark shaded bars) or treated with 1 mM H₂O₂ (light shaded bars), normalized to total cell protein. Error bars indicate standard deviations. Each strain was tested in triplicate.

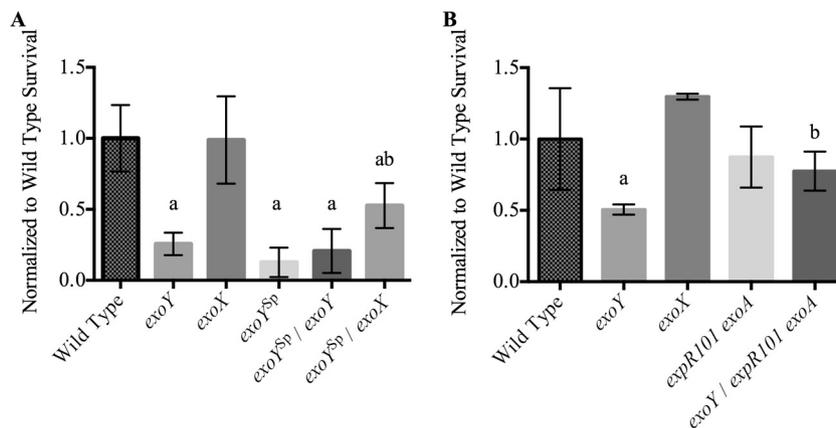


FIG 3 Effects of coculture on the survival of *S. meliloti* strains expressing different levels of EPS-I and EPS-II. The *exoY*^{Δp} or *exoY* exopolysaccharide-deficient strain, cocultured with a strain overproducing EPS-I (A) or with a strain expressing EPS-II (B), was challenged with H₂O₂. Survival was normalized to the average survival of the wild type. Error bars indicate standard deviations. The lowercase letter “a” above a bar indicates a significant difference from the wild type (P , <0.05); the letter “b” indicates a significant difference between coculture and culture of the *exoY* strain alone (P , <0.05).

1.78×10^6 CFU/ml without dipyrindyl compared to 2.00×10^6 to 2.12×10^6 CFU/ml with dipyrindyl).

To determine if EPS-I is capable of binding to iron, we incubated 50 μ g purified EPS-I with either FeSO₄ or FeCl₃ for 10 to 20 min. We determined the remaining amount of free Fe²⁺ by using ferrozine (FeCl₃-incubated samples were reduced prior to Fe²⁺ detection). There was no difference between the amounts of Fe²⁺ detected in the presence and absence of EPS-I (Fig. 4B). This held true even if EPS-I was removed by cetrinide precipitation prior to ferrozine detection (data not shown).

EPS is able to decrease H₂O₂ levels *in vitro*. We tested EPS-I and EPS-II for their abilities to decrease H₂O₂ levels *in vitro* (Fig. 5). Exopolysaccharides were purified by cetrinide and acetone precipitation, followed by dialysis against water. We incubated purified EPS-I or EPS-II with various concentrations of H₂O₂. The final concentrations of H₂O₂ were assayed using Amplex Red reagent. The addition of 100 μ g/ml to 400 μ g/ml exopolysaccharide was able to decrease detectable H₂O₂ levels in a concentration-dependent manner (Fig. 5A; see also Fig. S1 in the supplemental material).

An *exoY* cell culture supernatant purified in the same manner as EPS-I or EPS-II had no effect on Amplex Red fluorescence, indicating that the decrease in H₂O₂ detection is due to exopoly-

saccharides and is not a by-product of purification (Fig. 5B). In addition, equal amounts of glucose or glucose tetrasaccharide did not decrease H₂O₂ levels (data not shown). The fluorescence of resorufin (the fluorescent product produced by Amplex Red after H₂O₂ detection) was not affected by the addition of EPS-I (see Fig. S2 in the supplemental material). From these results, we conclude that both species of *S. meliloti* exopolysaccharide decrease H₂O₂ levels *in vitro*, while glucose monomers and oligomers do not.

Low-molecular-weight (LMW) EPS-I fractionated over a size exclusion column was able to decrease H₂O₂ levels (Fig. 5B). High-molecular-weight (HMW) EPS-I from size exclusion column fractionation was much less effective at decreasing H₂O₂ levels, indicating that LMW EPS-I is the active fraction responsible for eliminating H₂O₂. HMW EPS-I has a high ratio of total sugars to reducing sugars, while LMW EPS has a low ratio (Table 3).

DISCUSSION

Aerobic organisms such as *S. meliloti* are exposed to both internal and external sources of ROS. H₂O₂-dependent damage can be avoided if external H₂O₂ is prevented from entering the cell or if internal H₂O₂ is detoxified. Here we have shown that the levels of

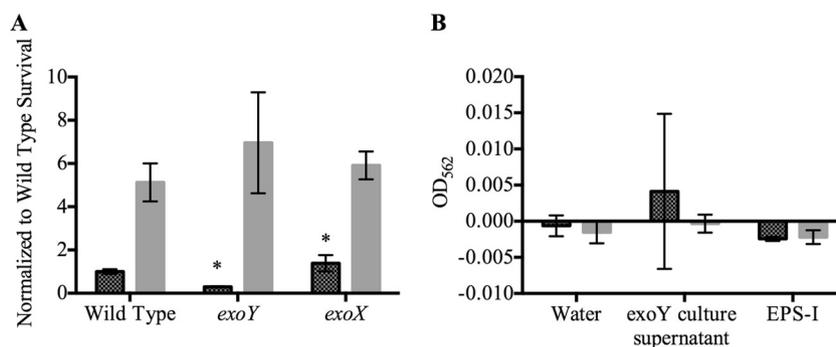


FIG 4 Chelation of iron with dipyrindyl enhances the survival of *S. meliloti* in H₂O₂. (A) Survival of *S. meliloti* strains in 1 mM H₂O₂ without (dark shaded bars) or with (light shaded bars) the addition of the iron chelator dipyrindyl. Survival is normalized to that of the wild-type strain not treated with dipyrindyl. Error bars indicate standard deviations. Asterisks indicate significant differences from the wild type without dipyrindyl (P , <0.05). (B) Absorbance of ferrozine reagent in response to amounts of Fe²⁺ (dark shaded bars) or Fe³⁺ and Fe²⁺ (light shaded bars) found in EPS-I or the *exoY* culture supernatant.

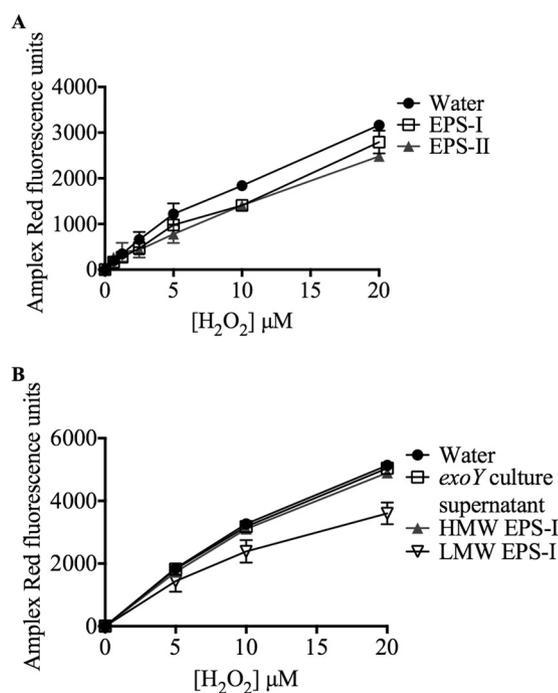


FIG 5 Exopolysaccharides decrease H₂O₂ concentrations *in vitro*. (A) Amount of H₂O₂ detected by Amplex Red when incubated with 7 μg EPS-I (open squares) or 7 μg EPS-II (filled triangles). (b) Amplex Red detection of H₂O₂ incubated with either the culture supernatant from the *exoY* strain (open squares), 25 μg HMW EPS-I (filled triangles), or 20 μg LMW EPS-I (open inverted triangles). Error bars indicate standard deviations (not visible in all data points due to small errors).

the external polysaccharides produced by *S. meliloti*, EPS-I and EPS-II, correlate with survival in H₂O₂.

The protection against H₂O₂ is independent of the genetic mechanism of altering the levels of exopolysaccharides. Strains that overproduce both Nod factor and EPS-I do not appear to have a survival advantage similar to that of strains that produce only EPS-I. This may be due to differing levels of EPS-I production, or possibly to excess stress on the cells due to the overproduction and export of Nod factor (22). The existing data do not distinguish between these two possibilities.

We have also shown that *S. meliloti* strains producing EPS-II have improved survival in H₂O₂ compared to wild-type strains that produce only EPS-I. To our knowledge, this is the first report of EPS-II protection against ROS-related damage. While EPS-II is not required for symbiosis with *Medicago* spp., production of EPS-II in the absence of EPS-I can allow productive symbiosis with *Medicago sativa*, though not with *M. truncatula* (26).

In coculture, *S. meliloti* mutants expressing increased levels of either EPS-I or EPS-II can protect exopolysaccharide-deficient cells against H₂O₂. This is interesting, given that coinoculation of *S. meliloti* Nod factor and EPS-I mutants allows for the formation of functional nodules that are not formed by either mutant alone (38, 39). This could result partly from the coprotection of cells against H₂O₂, but that explanation does not rule out the possibility of exopolysaccharides acting as specific signals.

While there was variability in survival from day to day, which makes comparisons across assays difficult, the correlation between the amount of exopolysaccharide produced and survival

was reproducible and consistent. There can be limitations to this assay if the strain tested has growth problems independent of H₂O₂ stress. An example of this type of problem was seen with the *exoA* mutant, which had longer doubling times and an apparently wild type level of survival after exposure to H₂O₂. However, an alternative assay (zone of inhibition) showed that the *exoA* mutant is indeed sensitive to H₂O₂. The technical differences between these two assays (different growth stages, levels of H₂O₂ exposure, and culture media) show that sensitivity to H₂O₂ can depend on other growth conditions. However, both of these assays showed that EPS-I-deficient mutants are sensitive to H₂O₂ while EPS-I-overproducing mutants are resistant.

We wondered whether the decrease in the survival of the *exoY* strain and other EPS-I-deficient strains was due to a general inability to handle environmental stresses. However, this would not explain why EPS-I-overproducing strains are resistant to H₂O₂ but not discernibly different in total catalase (anti-H₂O₂) activity. Additionally, the ability of a 3-h coculture to increase the survival of EPS-I-deficient strains in H₂O₂ indicates that the protective moiety in EPS-I-overproducing strains can act in *trans*, though this does not rule out the possibility that something coexpressed with EPS-I or EPS-II is responsible for protection against H₂O₂.

Heat-killed conditioned medium did not improve the survival of the *exoY* strain; likewise, addition of exogenous EPS-I to the culture medium did not appear to improve the survival of EPS-I-deficient strains under the conditions tested. These observations are at odds with our inference that exopolysaccharides are able to act in *trans*, which we based on the observation that *exoY* cells are protected in coculture. One possible reason for this lack of protection by medium or exogenous EPS-I is that living cells are required for coprotection. For example, perhaps the quantity of purified EPS-I, or that of EPS-I in the conditioned medium, is not sufficient, while living cells produce EPS-I continuously, such that levels are kept high. Another possibility is that the purified EPS-I may not be chemically identical to native EPS-I produced in culture. It is possible that heat treatment or purification changes the chemistry. On the other hand, we observe that purified *S. meliloti* exopolysaccharides, specifically the LMW EPS-I fraction, are capable of decreasing the detectable levels of H₂O₂ *in vitro*. For this reason, we favor the idea that the concentrations of EPS-I added to the culture medium do not accurately replicate the local concentrations needed to protect cells from H₂O₂ damage. Nonetheless, we keep open the possibility that another factor, coproduced with EPS-I in culture and disturbed by mutations of the *exo* genes, plays a larger role *in vivo* than EPS-I and is lost or changed in heat-treated culture and in EPS-I purification.

TABLE 3 Quantification of total and reducing sugars of HMW and LMW EPS-I

EPS-I form (source) ^a	Concn of sugar (μg/ml)		Total/reducing sugar ratio ^b
	Total ^c	Reducing ^d	
HMW (<i>exoX</i> strain)	487	1.4	348
LMW (Rm1021)	415	6.6	62

^a Both HMW EPS-I and LMW EPS-I were fractionated by size exclusion chromatography.

^b High ratios indicate high-molecular-weight EPS-I (longer sugar chains per reducing end). Conversely, low ratios indicate low-molecular-weight EPS-I.

^c As determined by an anthrone-sulfuric acid assay.

^d As determined by a neocuproine assay.

Both EPS-I and EPS-II appear to be competent to decrease H_2O_2 levels as detected by Amplex Red. The concentrations of EPS-I and EPS-II used in these assays are within the range needed to complement the symbiotic defect of EPS-I-defective cells (24). The detection of the fluorescent product of this assay, resorufin, is unchanged with the addition of EPS-I. An increase in the concentration of EPS-I leads to a greater decrease in H_2O_2 levels. We believe that these data, taken together, indicate that EPS-I and EPS-II can act as antioxidants, rather than interfering with the Amplex Red assay itself. These data do not rule out the possibility that something that copurifies with EPS-I and EPS-II, and is not present in the culture supernatants from EPS-I-deficient strains, is responsible for decreasing H_2O_2 levels. Also, these experiments do not show whether EPS-I acts as an antioxidant during symbiosis.

LMW EPS-I is reported to be the active fraction required for symbiosis (23, 24). The ability of LMW EPS-I to suppress the symbiotic defects of EPS-I-deficient strains seems to be inhibited by the presence of HMW EPS-I (24). The EPS-I added to cultures to test survival in H_2O_2 contained a mixture of both HMW and LMW forms. Perhaps the reason we do not see improved survival of EPS-I-deficient strains when purified EPS-I is added to the growth medium is that HMW EPS-I has an inhibitory effect. Alternatively, the addition of mixed-molecular-weight EPS-I may provide insufficient amounts of LMW EPS-I to be effective against H_2O_2 .

We have not investigated the chemical mechanisms by which exopolysaccharides could decrease H_2O_2 levels. Both exopolysaccharides are produced in HMW and LMW forms, and in both cases, the LMW form is reported to rescue symbiotic defects (24, 40). The improved activity of LMW EPS-I may be due to an increased number of reducing ends compared to total sugars (Table 3), or the improved mobility or diffusibility of the smaller EPS-I may lead to better antioxidant activity. LMW EPS-I also has a higher degree of succinylation than HMW EPS-I (14). These succinyl groups may also be possible targets of H_2O_2 .

We cannot rule out the possibility that the antioxidant activity of these exopolysaccharides is due to something, such as an ion or small molecule, that coprecipitates with EPS-I and EPS-II rather than to the exopolysaccharides themselves. While we did not see iron binding to EPS-I, likely due to a lower sensitivity of our assay, specific LMW EPS-I forms have recently been reported to have iron-chelating properties (41). It is not clear if the ability of LMW EPS-I to bind iron results in protection of that iron and, therefore, decreased production of hydroxyl radicals. If this is the case, it might explain the ability of exopolysaccharides to protect against H_2O_2 *in vivo* but would not necessarily explain the ability to decrease H_2O_2 levels *in vitro*. Calcium also binds EPS-I (42), raising the question of whether other ions might do so as well. One candidate is manganese, since Mn(II) is capable of scavenging superoxide (2, 43). The levels of manganese present during symbiosis have not been analyzed.

Earlier studies found that exogenous addition of exopolysaccharides from other rhizobial species or EPS-I lacking nonsugar modifications is not competent to rescue the symbiotic defects of *S. meliloti* EPS-I mutants (24). However, recent work in the *Rhizobium leguminosarum*–*Vicia sativa* system indicates that specific exopolysaccharides may not be required for early stages of symbiosis (44, 45). Perhaps heterologous exopolysaccharides capable of reducing H_2O_2 can reconstitute some of the functions of native exopolysaccharides.

The requirement for EPS-I during symbiosis may be due in part to its ability to protect the bacteria from ROS. This could explain how two structurally different exopolysaccharides can function to allow productive symbiosis (26), since they both have the ability to decrease H_2O_2 levels *in vitro*. Whether exopolysaccharides actually function during symbiosis to modulate H_2O_2 levels, and whether this is the only role for these polysaccharides, remains an open question. The fact that EPS-II cannot overcome the requirement for EPS-I in symbiosis with *M. truncatula* and several other host plants, despite its ability to decrease H_2O_2 levels, implies that exopolysaccharides have additional functions during symbiosis.

We have shown that both EPS-I and EPS-II are protective against H_2O_2 -dependent death in *S. meliloti*. Our work suggests that the exopolysaccharides may provide this protection by decreasing the amount of H_2O_2 in the surrounding environment. The LMW fraction of EPS-I is the effective form for decreasing H_2O_2 levels. Future studies are needed to address the symbiotic importance of decreased levels of H_2O_2 and the mechanism of such a decrease by exopolysaccharides.

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