

## Characterization of a *Sinorhizobium* Isolate and Its Extracellular Polymer Implicated in Pollutant Transport in Soil

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**A bacterium isolated from soil (designated 9702-M4) synthesizes an extracellular polymer that facilitates the transport of such hydrophobic pollutants as polynuclear aromatic hydrocarbons, as well as the toxic metals lead and cadmium in soil. Biolog analysis, growth rate determinations, and percent G+C content identify 9702-M4 as a strain of *Sinorhizobium meliloti*. Sequence analysis of a 16S rDNA fragment gives 9702-M4 a phylogenetic designation most closely related to *Sinorhizobium fredii*. The extracellular polymer of isolate 9702-M4 is composed of both an extracellular polysaccharide (EPS) and a rough lipopolysaccharide. The EPS component is composed mainly of 4-glucose linkages with monomers of galactose, mannose, and glucuronic acid and has pyruval and acetyl constituents. The lipid fraction and the negative charge associated with carbonyl groups of the exopolymer are thought to account for the binding of polynuclear aromatic hydrocarbons and cationic metals.**

The concept of facilitated transport (29) is based on the observations that hydrophobic organic chemicals and toxic metals can be transported greater distances and at higher concentrations in porous media than their sorptive distribution coefficients would predict (2, 11, 12, 21, 39). One mechanism for enhanced contaminant transport is the binding to mobile dissolved macromolecules that act as carriers of pollutants (26, 29). Extracellular polymers of bacterial origin have been identified as having potential for facilitating the transport of polynuclear aromatic hydrocarbons (PAHs) (10), as well as toxic metals, such as cadmium and lead (6, 9).

Several extracellular and capsular polymers from various soil bacteria were identified that reduced the retardation coefficients of phenanthrene (10) and cadmium and lead (6) in a low-carbon aquifer sand. The extracellular polymer from an isolate designated 9702-M4 (25) reduced the retardation of phenanthrene by 39% (10) and cadmium and lead by 87 and 60%, respectively (6). In this study, we report the chemical analysis of the polymer and the taxonomic identity of the bacterium that synthesizes it.

**Bacteria and culture conditions.** Isolate 9702-M4, *Sinorhizobium meliloti* strain 1021 (obtained from S. Winans, Department of Microbiology, Cornell University), and *S. meliloti* ATCC 9930 were grown and maintained as previously described (10, 19, 38). For Biolog analysis, bacteria were cultured on Bacto R2A agar (BD Biosciences, Franklin Lakes, N.J.).

**Phenotypic analysis of strain 9702-M4.** Smears of 9702-M4 were stained with Nile blue and toluidine blue and were observed by phase-contrast microscopy. 9702-M4 cells were negatively stained with 2% uranyl acetate for electron microscopy. Generation time was determined as previously described (19).

The Biolog GN MicroPlate system was used as described by the manufacturer (Biolog, Inc., Hayward, Calif.). *Medicago sativa* cv. Oneida seeds were used for nodulation experiments as previously described (38).

**Percent G+C determination.** DNA extraction and purification was performed as described (20). Determining percent G+C content was performed as previously described (28). Percent G+C was determined using the following equation: %G+C =  $(T_m - 53.9) \cdot 2.22$ , where  $T_m$  is the midpoint of the melting curve.

**PCR amplification of 16S rRNA gene.** DNA was extracted as previously described (5). The PCR procedure was as previously described (17). Sequencing was performed by the BioResource Center, Cornell University, on a Perkin-Elmer/Applied Biosystems Division Automated DNA sequencer.

**DNA sequence analysis.** Sequence construction was performed with SegMan (Lasergene by DNASTAR, Inc., 1999). Ribosomal Database Project II Sequence Match version 2.7 (27) was used to find similar 16S rRNA sequences for consensus tree construction. Sequence alignment was done with ClustalX (Lasergene by DNASTAR, Inc.). PHYLIP version 3.572 software was used to analyze aligned sequences (J. Felsenstein, Department of Genetics, University of Washington, Seattle). Kimura distances (22) were calculated using DNADIST (J. Felsenstein). An unrooted tree was constructed using NEIGHBOR (J. Felsenstein). Bootstrap confidence values were calculated with 100 replicates by using SEQBOOT; a consensus tree was created with CONSENSE (13). Branch lengths were calculated with FITCH (J. Felsenstein) and TreeViewPPC version 1.5.3 (34).

**Analysis of crude exopolymer.** Polymer extraction was as previously described (8, 33). Protein content was determined as previously described (24), as well as acetylation content (30).

**TLC analysis.** Exopolymer was hydrolyzed as previously described (32). Monosaccharides and carboxylic acids were used as references. Precoated silica gel 60 thin-layer chromatography (TLC) plates (E. Merck, Darmstadt, Germany) were pre-

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pared as previously described (14). Plates were spotted with 2 mg of reference compounds ml<sup>-1</sup> and 20 mg of hydrosylate ml<sup>-1</sup>, were developed in a solution of isopropanol, acetone, and 0.1 M lactic acid (2:2:1), and were visualized as previously described (37).

**High-performance liquid chromatography (HPLC) analysis.** Exopolymer hydrosylate (10 mg ml<sup>-1</sup>) was analyzed as previously described (32).

**Purification of crude exopolymer.** Crude exopolymer (1.5 to 2.5 mg ml<sup>-1</sup>) was redissolved in 20 ml of aqueous 10 mM MgCl<sub>2</sub>, to which was added 0.005 mg of DNase ml<sup>-1</sup> and 0.1 mg of RNase (Sigma Chemical Co.) ml<sup>-1</sup>, and incubated at 37°C for 4 h. Protease Type IX from *Streptomyces griseus* (Sigma Chemical Co.) at 0.1 mg ml<sup>-1</sup> was added and incubated at 37°C for 24 h. The mixture was heated at 80°C for 30 min and centrifuged at 17,000 × g for 20 min. The supernatant was dialyzed.

Partially purified exopolymer was further purified by deoxycholate precipitation. It was dissolved in 0.05 M Tris base–0.1 M NaCl, to which 0.75 M deoxycholic acid (Sigma Chemical Co.) was added to obtain a critical micellization concentration of 0.75. The mixture was incubated at 65°C for 15 min and cooled on ice; 20% acetic acid was added to a final concentration of 1%, and the mixture was centrifuged at 17,000 × g for 5 min. The supernatant, purified extracellular polysaccharide (EPS), was separated from the precipitate, the lipopolysaccharide (LPS) fraction, which was dissolved in distilled water. The purified EPS and LPS were dialyzed and lyophilized. Crude and partially purified exopolymer, purified EPS, and LPS were assayed for the presence of 2-keto-3-deoxyoctanate (KDO) as previously described (40) except that periodic acid was used.

**DOC-PAGE analysis.** Deoxycholic-polyacrylamide gel electrophoresis (DOC-PAGE) analysis was performed as previously described (36). Two positive controls were used: phenol-extracted LPS from *Salmonella enterica* serovar Typhimurium (Sigma Chemical Co.) and LPS extracted from *S. meliloti* ATCC 9930. One gel was stained with alcian blue-silver stain to visualize both EPS and LPS and another with silver stain only to visualize LPS (35).

**Composition and linkage analysis of EPS.** The composition of purified EPS was determined as previously described (41). For linkage analysis, the sample was methylated as previously described (7). The methylated sample was analyzed as previously described (1, 41). For nuclear magnetic resonance (NMR) analysis, a sample was first dissolved in D<sub>2</sub>O, lyophilized, and dissolved again in D<sub>2</sub>O. A proton NMR spectrum was obtained using a Varian 300 MHz instrument at 28°C.

**Phylogenetic identification of strain 9702-M4.** Strain 9702-M4 is a gram-negative rod (19) with one to three lateral flagella and dimensions of 2.1 by 0.84 μm; it contains granules of poly-β-hydroxybutyrate but no metachromatic granules. Biolog analysis identified the isolate as *S. meliloti* B and thus put it in the *Rhizobiaceae*. The similarity value of 0.579 is considered a positive identification. The isolate's generation time of 2.8 h and percent G+C determination of 61.2 are both salient characteristics of *S. meliloti* (18). Biolog analyses have, however, shown both agreement and discrepancies between rhizobial strains when compared to 16S rRNA sequences (31).

The sequence of the 1,442-bp 16S rDNA fragment of strain 9702-M4 (GenBank accession No. AF357225) was aligned and

compared to members of the *Rhizobiaceae* (data not shown) and appeared to be most closely related to the genus *Sinorhizobium*. It formed an outgroup on the *Sinorhizobium* node and showed a relatedness of 97.8% with *Sinorhizobium fredii* and a relatedness of 97.6% with *Sinorhizobium xinjiangensis*.

9702-M4 did not nodulate *M. sativa* cv. Oneida, whereas *S. meliloti* strain 1021 did. Kuykendall et al. (23) have demonstrated that some *S. fredii* strains nodulate some cultivars of *M. sativa* and some do not; 9702-M4 may be a strain of *S. fredii* that does not nodulate *M. sativa* cv. Oneida. The symbiotic genes of *Sinorhizobia* spp. are known to be on large plasmids that can be transferred between species (15). The plasmids that carry nodulation genes can be lost resulting in the inability of the organism to nodulate roots of a compatible host (3). Although symbiotic plasmids may confer saprophytic competence (3), pressures of a subsurface soil environment may have cured 9702-M4 of its plasmid.

**Characteristics of extracellular polymer from 9702-M4.** Preliminary TLC and HPLC analyses of crude polymer indicated that it was comprised of glucose, galactose, and glucuronic acid at mole percentages of 40.4, 34.6, and 25.0, respectively. Ninhydrin did not reveal amino sugars. Crude polymer contained 0.08% protein and was 3.9% acetylated.

The compositional analysis of the purified EPS by gas chromatography-mass spectrometry (GC-MS), in contrast to the HPLC analysis, showed that it was composed of the following carbohydrate residues (with mole percent in parentheses): arabinose (1.7%), rhamnose (0.8%), glucuronic acid (13.8%), mannose (10.5%), galactose (21.1%), glucose (51.8%), and *N*-acetyl glucosamine (0.3%). The GC-MS analysis of this polymer detected small amounts of arabinose, rhamnose, and mannose, and thus it appeared to be more sensitive than the HPLC analysis that indicated a greater percent composition of glucuronic acid and galactose. The small percentage of *N*-acetylglucosamine detected by GC-MS may account for the inability of ninhydrin to detect it. The most abundant neutral sugars of 9402-M4's exopolymer, glucose, galactose, mannose, and glucuronic acid, and both acetyl and pyruval components make it unique compared to the EPS produced by many rhizosphere bacteria (16). Unlike exopolysaccharides that are associated with many members of the *Rhizobiaceae*, it appears to be neither a succinoglycan nor a galactoglycan (42).

The linkage analysis of neutral sugars of the purified EPS revealed two terminal residues and eight different linkages between the glucose, mannose, and galactose residues. Mannose and glucose were the two terminal residues and were present at 5.2 and 2.8%, respectively. Based on the percent distribution of the linkages, the ratio of linkages for 3-mannose (5.5%), 2-mannose (3.7%), 3-glucose (15.2%), 6-glucose (10.5%), 4-glucose (33.3%), 4,6-mannose (2.9%), 4,6-galactose (13.7%), and 4,6-glucose (7.2%) was 1:1:2:4:1:2:1, respectively, and indicated that 4-glucose was the main component of the polymer. The NMR spectrum revealed the presence of pyruvyl and acetyl groups. The electron-rich carbonyl groups of the pyruvyl, acetyl, and glucuronic acid residues are likely to provide binding sites for cationic metals lead and cadmium.

DOC-PAGE analysis showed that the exopolymer had both EPS and LPS constituents (Fig. 1). The KDO assay on the crude, partially purified, and purified exopolymer and the LPS pellet showed that the greatest concentration of KDO, a sig-

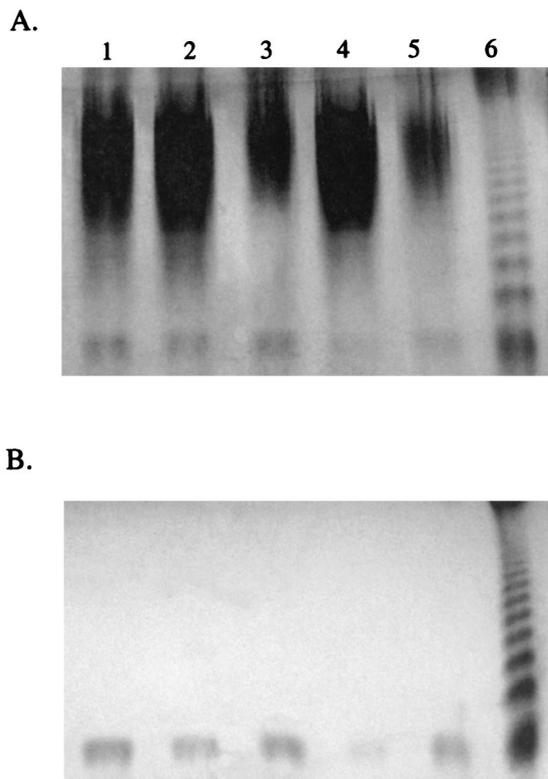


FIG. 1. DOC-PAGE analysis of EPS extracted from isolate 9702-M4. (A) Gel was prestained with alcian blue and then silver stain to visualize both EPS and LPS. (B) Gel was silver stained to visualize LPS only. Lanes: 1, 10  $\mu\text{g}$  of crude exopolymer; 2, 10  $\mu\text{g}$  of purified EPS; 3, 10  $\mu\text{g}$  of LPS pellet; 4, 5  $\mu\text{g}$  of partially purified exopolymer; 5, 5  $\mu\text{g}$  of LPS pellet; 6, 5  $\mu\text{g}$  of *S. enterica* serovar Typhimurium LPS.

nature component of LPS, was associated with the LPS fraction and in decreasing concentration with, respectively, crude, partially purified, and purified EPS and thus demonstrated that the LPS component could be separated, but not completely, from the EPS. Further purification of the purified EPS with deoxycholic acid did not remove any more LPS (data not shown). The silver staining of the LPS showed that the LPS of 9702-M4 appeared to correspond to the high-mobility rough LPS (RLPS) like *S. fredii* HH303 and *S. meliloti* NR3133 (36) but showed no signs of low-mobility smooth LPS (SLPS), as seen in extracts of *S. enterica* serovar Typhimurium (Fig. 1) and *S. meliloti* ATCC 9930 (data not shown) and other strains of *S. fredii* and *S. meliloti* (36). Thus, the RLPS component of 9702-M4 exopolymer makes it similar to those strains of *S. fredii* and *S. meliloti* that synthesize RLPS only (36) and further supports its phylogenetic designation based on the sequence of the 16S rDNA fragment.

The present characterization of the exopolymer of 9702-M4 may account for its ability to act as a carrier of both hydrophobic polynuclear aromatic compounds and toxic heavy metals. It is mobile in porous media under saturated conditions (9, 10) and relatively resistant to biodegradation (9). The integrated LPS fraction of the polymer would have an affinity for hydrophobic hydrocarbons, and the negatively charged EPS fraction would have an affinity for cationic metal ions. Czajka et al. (9) reported that crude exopolymer from strain 9702-M4

could be mineralized at rates between 0.00057 and 0.0048  $\text{min}^{-1}$ , but that polymer mineralization was significantly reduced when lead was adsorbed to it. This reduction in mineralization suggests that the lead molecules may be attracted to the negative charge of the carbonyl groups and, once bound to the polymer, would inhibit its microbial degradation. Cabral (4) showed that the crude polymer's binding affinity for PAHs was not altered by binding to cadmium and the cadmium binding was not changed by bound PAHs. Thus, the two types of contaminant binding appear to result from different polymer components. Because isolate 9702-M4 appeared to be a *Sinorhizobium*, and most likely *S. fredii*, exopolymers from other strains of *S. fredii* or *S. meliloti* may also facilitate the transport of polyaromatic hydrocarbons and toxic metal contaminants of groundwater and soil and may be used in engineered remediation of contaminated aquifers.

**Nucleotide sequence accession number.** The GenBank accession number for the partial sequence of the 16S rRNA gene for isolate 9704-M2 is AF357225.

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