Characterization of the Lipopolysaccharides and Capsules of *Shewanella* spp.

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Received 22 February 2002/Accepted 4 June 2002

Electron microscopy, sodium dodecyl sulfate-polyacrylamide gel electrophoresis with silver staining and 1H, 13C, and 31P-nuclear magnetic resonance (NMR) were used to detect and characterize the lipopolysaccharides (LPSs) of several *Shewanella* species. Many expressed only rough LPS; however, approximately one-half produced smooth LPS (and/or capsular polysaccharides). Some LPSs were affected by growth temperature with increased chain length observed below 25°C. Maximum LPS heterogeneity was found at 15 to 20°C. Thin sections of freeze-substituted cells revealed that *Shewanella oneidensis*, *S. algae*, *S. frigidimarina*, and *Shewanella* sp. strain MR-4 possessed either O-side chains or capsular fringes ranging from 20 to 130 nm in thickness depending on the species. NMR detected unusual sugars in *S. putrefaciens* CN32 and *S. algae* BrY. It is possible that the ability of *Shewanella* to adhere to solid mineral phases (such as iron oxides) could be affected by the composition and length of surface polysaccharide polymers. These same polymers in *S. algae* may also contribute to this opportunistic pathogen's ability to promote infection.

*Shewanella* organisms are generally associated with aquatic habitats and play important roles in the cycling of particulate iron and organic matter, but they can also be opportunistic pathogens (33, 45). Because of their environmental significance, they are presently under vigorous investigation and many new species were recently included in the genus (45). Most *Shewanella* organisms are capable of dissipative reduction of a wide range of electron acceptors, including metal oxides [e.g., those of Fe(III) and Mn(IV)]. Several reductive mechanisms are possible. Electron flux from the organism to the solid oxide may occur via (i) direct contact of bacterial outer membrane and oxide surface (2, 9, 21, 31), (ii) organic shuttles (such as humic acids or quinones to mediate electron flow (22, 32), or (iii) a combination of both processes. For the direct contact model, metal reductases (such as c-type cytochromes) seem to be embedded in the outer membrane of dissipatory metal-reducing bacteria to facilitate electron flow (13, 29, 30). Lipopolysaccharide (LPS) and outer membrane proteins could play principal roles in establishing and maintaining contact with oxide minerals so that electron transport to the terminal acceptor occurs. The junction between cell and mineral must be tight so as to ensure that the reductase functions effectively (23). However, the cell surface structure and LPS of *Shewanella*, which could affect the cell-mineral connection, are poorly understood (28, 39, 40, 48). In this present article we characterize those structural elements that can extend beyond the outer face of the outer membrane, i.e., the LPS O-side chains and capsular polymers.

**Bacterial strains and growth conditions.** The strains used in this study are shown in Table 1. Most of these strains were kindly provided by Doug Lies (Jet Propulsion Laboratory, Pasadena, Calif.). *S. algae* BrY was supplied by both D. Lies and F. Caccavo, Jr. (while he was at the Department of Microbiology, University of New Hampshire, Durham); the former is designated *S. algae* BrYHEL and the latter BrYFC. All were cultured on either tryptic soy broth or tryptic soy broth supplemented with 2% (wt/vol) NaCl (i.e., for *S. pealeana* and *S. woodyi*). Cultures were grown aerobically on a rotary shaker (150 rpm) at temperatures from 5 to 37°C and were harvested at a mid-exponential growth phase (optical density at 470 nm [OD470] = ~0.8).

**SDS-PAGE and nuclear magnetic resonance (NMR) LPS analyses.** Proteinase K-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze LPS (16, 24), and the gels were silver stained (41). Gels of *S. putrefaciens*, *S. oneidensis*, *S. baltica*, and *Shewanella* sp. strains MR-4 and CL 256/73 displayed only low-Mt bands when grown at 26°C. Gels did not display high-Mt bands or “laddering” (indicative of smooth LPS [S-LPS]), suggesting that these contained rough LPS (R-LPS) with core oligosaccharide and no O-side chains (Fig. 1, lane 1, is representative). *S. amazonensis*, *S. frigidimarina*, *S. pealeana*, *S. woodyi*, and most *S. algae* organisms produced S-LPS (or possibly capsular polysaccharides) as evidenced by bands located in the upper regions of the gel (Fig. 1, lanes 2 to 6, is representative). These bands sometimes were smeared (Fig. 1, lane 3) suggesting that each band possessed similar but not identical polymer lengths. At this point in our study, it was impossible to distinguish these S-LPSs from capsular material. By definition capsular macromolecules contain long-branched or unbranched homo- or heteropolysaccharides linked to a lipid substituent, which anchors them to the outer membrane. Capsular polymers can have either lipid A or entirely different lipid anchors, such as dia-cylglycerolphosphate, but the major differentiating property is polymer length (46). Capsular polysaccharides are longer than those of LPS and, as such, should not enter the gels. Strangely,
### TABLE 1. *Shewanella* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Collection no.</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. putrefaciens</em> CN32</td>
<td>ATCC BAA-453</td>
<td>Commonly used Fe oxide-reducing strain</td>
</tr>
<tr>
<td><em>S. putrefaciens</em></td>
<td>NCTC 10695</td>
<td>Reference strain for Owen’s homology group I</td>
</tr>
<tr>
<td><em>S. oneidensis</em> MR-1</td>
<td>ATCC 700580</td>
<td>Type strain</td>
</tr>
<tr>
<td><em>S. oneidensis</em> DLM-7</td>
<td>ATCC 51192</td>
<td>Type strain</td>
</tr>
<tr>
<td><em>S. algae</em> 136-2</td>
<td>NCTC 10738</td>
<td>Reference strain for Owen’s homology group IV</td>
</tr>
<tr>
<td><em>S. algae</em> BrY</td>
<td>ATCC 51181</td>
<td>Commonly used Fe oxide-reducing strain</td>
</tr>
<tr>
<td><em>S. algae</em> BCM-8</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Shewanella</em> sp. strain MR-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Shewanella</em> sp. strain CL 256/73</td>
<td>NCTC 12093</td>
<td>Reference strain for Owen’s homology group III</td>
</tr>
<tr>
<td><em>S. baltica</em> 63</td>
<td>ATCC 10735</td>
<td>Type strain</td>
</tr>
<tr>
<td><em>S. amazonensis</em> SB2B</td>
<td>ATCC 700329</td>
<td>Type strain</td>
</tr>
<tr>
<td><em>S. frigidimarina</em></td>
<td>ATCC 700345</td>
<td>Type strain</td>
</tr>
<tr>
<td><em>S. pealeana</em> ANG-SQ1</td>
<td>ATCC 700550</td>
<td>Type strain</td>
</tr>
<tr>
<td><em>S. woodyi</em> MS32</td>
<td>ATCC 51908</td>
<td>Type strain</td>
</tr>
</tbody>
</table>

*S. algae* BrY^{FC} lacked bands in the top region of the gel (Fig. 1, lane 1), being different from all other tested strains of *S. algae*, including BrY^{DIP}.

$^{1}$H-, $^{13}$C-, and $^{31}$P-NMR was performed on selected strains and spectra analyzed according to the method described by Vinogradov et al. using a Varian Inova spectrometer (44). Most cores that have been studied possess high concentrations of carboxyl and phosphoryl sites, making them highly polar so as to interact with metal ions (10, 20) and inanimate surfaces (26). *S. putrefaciens* CN32 (and related strains [28, 47]) and *S. oneidensis* MR-1 have R-LPS (Table 2), which may aid their adhesion and close fit to iron oxide minerals. $^{1}$H-, $^{13}$C-, and $^{31}$P-NMR analyses on CN32 indicated that its oligosaccharide backbone consisted of β-Galf-(1-3)-β-Gal-(1-4)-β-Glc-α-DDHep2PETN-(1-5)-α-Kdo4P-(1-6)-β-GlcN4P-(1-6)-α-GlcNIP, which possesses phosphate and carboxylate groups (i.e., 3-deoxy-d-manno-2-octulosonic acid [Kdo]) suggesting that this region is polar and can be ionized. Phosphorylated Kdo is rare among extensively studied bacteria, such as enterobacteria, and difficult to detect by conventional colorimetric means (17). At this point, since so few *Shewanella* LPSs have been studied in detail (39, 40), it is impossible to say if phosphorylated Kdo could be a common trait of this genus or species.

From a dissimilatory metal-reducing bacterium point of view, it may be advantageous for a bacterium to have its length of LPS constrained so that the microbe can fit closely to the oxide, thereby more efficiently using the mineral as a terminal electron acceptor. Indeed, for a close fit to any inert surface, as long as the hydrophobicity and hydrophilicity properties are appropriate, short LPSs should be more beneficial. Dissimilatory reduction of metal oxides requires anaerobiosis, and our *Shewanella* was grown aerobically, but even under these conditions, close oxide-bacterium union is seen (14). Almost one-half of our strains possessed R-LPS (Table 2), suggesting that core oligosaccharide was the LPS terminus. Because of the high potential charge and relatively short terminus (core oligosaccharide) on CN32 LPS, it is attractive to suggest that these two characteristics allow a tight union between bacterium and iron oxide, allowing good contact of the putative outer membrane iron reductase so that electron flow is ensured (23, 29).

Many enterobacteria and pseudomonads are noted for the strong antigenicity that is expressed by their LPSs. Although endotoxemia is more a function of lipid A, O-side chains differentiate these bacteria into specific immunodominant groups. For example, *Pseudomonas aeruginosa* PAO1 belongs to serogroup O5 and expresses two separate LPSs under normal growth conditions, A-band LPS (“common antigen”) and B-
**Influence of growth temperature.** Growth temperature can markedly influence the polymeric organization of LPS (1, 18, 25, 27, 35, 42), and this was true with some *Shewanella* strains. Temperatures below 25°C resulted in S- to semiourg LPS in *S. oneidensis* MR-1, which corresponded to the appearance of one to three bands above the putative core (Fig. 2A). Maximum size heterogeneity of LPS was observed at 15 to 20°C. Most apparent was *S. frigidimarina*, where a characteristic ladderlike banding pattern appeared at 15°C (Fig. 2B). A further decrease of growth temperature caused a decrease in this LPS size heterogeneity; however, the LPS profiles of most strains were not affected by temperature. The low temperature effect on some strains was not surprising, since many *Shewanella* organisms are either psychrotolerant or psychrophilic (7, 43).

**Ultrastructural analyses.** For conventional embeddings for thin sections the glutaraldehyde-osmium tetroxide protocol of Beveridge et al. (5) was followed and cells were embedded in LR White. Ruthenium red was also used as suggested by Beveridge et al. (5). Freeze substitution was according to the method described by Graham et al. (15). Sections were imaged with a Philips EM300 under standard operating conditions. All conventionally processed strains possessed envelope profiles typical of gram-negative cells having an outer membrane, periplasmic space, peptidoglycan layer, and plasma membrane (Fig. 3A is representative). No specific surface structures, such as capsules, S-layers, exopolymeric substances, sheaths, spinæ, pili (fimbriae), or flagella (see references 4 and 6), could be discerned.

Since freeze substitution has been shown to preserve finely detailed surface structures such as capsules and LPS O-side chains (3, 15, 19), it was also used to examine the surfaces of these *Shewanella* organisms. All strains contained a periplasmic gel (3, 6). *S. oneidensis* strains revealed a fibrous fringe extending about 20 to 30 nm and 60 to 80 nm from the cell surfaces of MR-1 and DLM 7, respectively (Fig. 3B is representative). A more extensive fringe was seen on *S. algae* BrY⁵⁰ and BrY⁷⁰ reaching up to 60 to 90 nm. However, cell populations in the samples were very heterogeneous, since in each population, some cells possessed different fringe heights, some possessed only patches of fringe, and some had no fringe at all (Fig. 3C is representative), suggesting that there was unequal expression of fringe within a single culture. *Shewanella* sp. strain MR-4 possessed the most extensive fringe (70 to 130 nm) of all (Fig. 3D). Taken together, the variance of fringe thickness and patchiness and the heterogeneity of fringe expression corroborated the (sometimes) smeared appearance of bands within SDS-PAGE gels, since a variation of polymer lengths would be expected.

Some variations in fiber arrangement of the fringes were also observed. The fibers of MR-4 extended directly away from the cell envelope in quite an organized manner (Fig. 3D), while those of *S. oneidensis* and *S. algae* (Fig. 3B and C) were more randomly arranged, forming a netlike mesh; these could be a softer, less ordered matrix than that of MR-4. Taking into account the structure and substantial thickness of these three fibrous layers, it would be reasonable to designate them as comprising capsules based solely on polymer length. In contrast, the cell surfaces of *S. putrefaciens* (CN32 and NCTC 10695), *S. baltica*, and *S. amazonensis* were devoid of any fibrous material and were not capsulated (Fig. 3E).

Ruthenium red is a stain that is frequently used to contrast acidic surface polymeric substances, such as capsules (4, 5). Strangely, when this stain was used with all our strains, no fringes were seen, even on those strains shown to have capsules by freeze substitution (data not shown). It is possible that the capsules of *Shewanella* are too delicate to withstand conventional fixation-ruthenium red processing or that the capsules’ overall electronegative charge density is too low to bind the stain.

Because the electron microscopy and LPS analyses have produced many data points in our study, we have tabulated results in Table 2. Here it is revealed that ~50% of the strains have R-LPS and that about one-half of these possess a fringe (freeze substitution) that is presumably a capsule. The other strains possess S-LPS or low-M₅ polysaccharides (since they

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**Fig. 2.** (A) Silver-stained SDS-PAGE gel of LPS from *S. oneidensis* MR-1 cells cultured at 5°C (lane 1), 15°C (lane 2), 20°C (lane 3), 26°C (lane 4), and 30°C (lane 5); (B) gel of LPS from *S. frigidimarina* cells cultured at 5°C (lane 1), 15°C (lane 2), 22°C (lane 3), and 26°C (lane 4).
enter the SDS-PAGE gel) that can only infrequently be seen by freeze substitution.

**Possible implications.** Surface polysaccharides, whether they are capsular or LPS, strongly affect the physicochemistry and adhesion qualities of gram-negative bacteria (8, 10, 11, 12, 34, 38, 48). Often it has been assumed that strains possessing R-LPS are more hydrophobic than their S-LPS counterparts, but this is not necessarily true (19, 25). Rough strains often have more exposed ionizable groups, and O-side chains can be so rapidly in motion that long-lived interactions between ions or surfaces can be rare (3, 49). Capsules, which are typically considered to aid adhesion, can be either adhesive (via charge-charge interaction) or nonadhesive (34, 46). Chemistry and length of both capsular and O-polysaccharides and core oligosaccharides all come to bear as they help to produce the net physicochemistry of a bacterial surface. In some instances, strong polarity would be required for adhesion, whereas in others, strong hydrophobicity would be required. Phenotypic plasticity is important (36), and the most successful bacteria are those that can modulate their surface properties according to the environment and the chosen attachment surface. For this reason, we propose that certain *Shewanella* organisms, such as CN32, have developed short R-LPS of high charge character to aid close adherence to inanimate surfaces, such as iron oxides. Under anaerobic conditions this would allow the Fe(III)-Fe(II) couple to aid electron capture during respiration. MR-1, on the other hand, retains the R-LPS phenotype but also requires the aid of a capsule of low charge density to aid its adhesion. BrYDL has adapted itself to live under natural environmental conditions but can also be an opportunistic pathogen. Possibly, the O-side chains aid in pathogenicity, but its flexible malic acid hinge-like linkage could also allow close contact to hard materials. There is no doubt that the surfaces of the other *Shewanella* organisms in our study will utilize their structural combinations in similar self-serving ways.

We acknowledge the excellent technical assistance of Bob Harris, Dianne Moyle, and Sean Langley of our laboratory, and we thank Chris Whitfield of our Department for his helpful comments on capsular material.
This work was funded by NABIR-DOE grants to T.J.B. and Y.G. The electron microscopy was done in the NSERC C. Guelph Regional STEM Facility (GSRF), which is partially funded by an NSERC-Major Facilities Access grant to T.J.B.

REFERENCES


