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Survey of Extreme Solvent Tolerance in Gram-Positive Cocci: Membrane Fatty Acid Changes in *Staphylococcus haemolyticus* Grown in Toluene

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Environments having high concentrations (10 to 50%, vol/vol) of organic solvents are considered extreme (1), and bacteria that are able to tolerate such environments have recently been recognized as a subgroup of the extremophiles (1). Lipophilic hydrocarbons are harmful to bacteria because they accumulate in membrane lipid bilayers, thus affecting the structural and functional properties of the membranes (25), and cytoplasmic membranes are the primary site of cellular damage by both organic solvents (25) and anionic detergents, such as sodium dodecyl sulfate (SDS) (17). Recently, our laboratory has pioneered the study of oil fly larval guts as a source of solvent-tolerant bacteria (12, 13). Larvae of the oil fly, *Helicopsyche petrolei*, are found exclusively submerged in oil, where they ingest large quantities of oil and asphalt. Thus, any bacteria isolated from oil fly larval guts have been naturally selected for solvent tolerance. Larvae isolated from the asphalt seeps of Rancho LaBrea in Los Angeles, Calif., contained ca. 2 × 10^9 heterotrophic bacteria per larva (13). Most of these bacteria were identified as *Providence retgeri* and *Acinetobacter* isolates (13), and most of the *Providence* strains were subsequently shown to be naturally resistant to many antibiotics and organic solvents (12).

Significantly, all of the bacteria identified from oil fly larvae at that time were gram-negative bacteria; no sporeformers or gram-positive bacteria were found (13). Presumably, the outer membrane protects the cytoplasmic membrane by reducing the periplasmic concentrations of harmful chemicals to acceptable levels. Our finding that almost all of the bacteria in oil fly larvae were gram negative agreed with the generalization of Aono and Inoue (1) that gram-negative bacteria show higher levels of organic solvent tolerance. It also agreed with previous conclusions from our laboratory that only gram-negative bacteria could tolerate high levels (5 to 25%) of SDS or other anionic detergents (15, 17, 22). We decided to investigate further the importance of the outer membranes in organic solvent tolerance by looking explicitly for anaerobic bacteria in oil fly larval gut contents. In the process we identified the highly solvent-tolerant gram-positive *Enterococcus* and *Staphylococcus* strains which are described in this paper.

Organic solvents have many detrimental effects on microbes, and some solvents are more harmful than others. To classify the intrinsic toxicity of a solvent, the logarithm of its partition coefficient in n-octanol and water was measured and termed log *P*~*ow~. Solvents with a low log *P*~*ow~ (1.5 to 4.0) are considered extremely toxic, while solvents with a higher log *P*~*ow~ are less toxic (7). The reasoning for this toxicity index lies in the fact that solvents with lower log *P*~*ow~ values tend to partition into the cytoplasmic membranes of organisms, compromising the structure and destroying vital functions. In recent years, a growing number of bacterial species able to overcome these toxic effects have been found (1).

Most solvent-tolerant bacterial species are gram negative. Gram-negative bacteria have the advantage of having an additional outer membrane, which allows quick modifications...
and adaptations in the lipopolysaccharides, efflux pumps, and/or fatty acid composition (23). Due to the inherent disadvantage of lacking an outer membrane, only a few gram-positive organisms have been reported to exhibit solvent tolerance, including species of *Bacillus*, *Rhodococcus*, and *Enterococcus* (8, 18). Mechanisms of solvent tolerance have not been proposed yet for gram-positive species, although it has been suggested that these organisms may use mechanisms similar to those used by gram-negative bacteria (23).

In their review, Sardessai and Bhosle (24) paid particular attention to the limited reports of solvent tolerance among gram-positive bacteria. These authors concluded that (i) there is a large void in the available data on solvent tolerance mechanisms in gram-positive bacteria; (ii) there should be studies to determine whether molecular mechanisms of solvent tolerance elucidated in gram-negative bacteria are also conserved in gram-positive bacteria; and (iii) new habitats need to be explored to isolate other species displaying such tolerance (24).

In the present paper we describe our use of oil fly larvae as a novel source of solvent-tolerant gram-positive bacteria. We then examined the fatty acid and phospholipid compositions of one isolate (*Staphylococcus haemolyticus*) grown in the presence and absence of saturating levels of toluene. We isolated an *S. haemolyticus* strain, as identified by MIDI and 16S RNA sequence analyses, which is able to tolerate levels of cyclohexane, toluene, benzene, and *p*-xylene on plate overlays that are greater than the levels tolerated by any other previously isolated gram-positive bacterium. We also examined these bacteria when they were grown in monophasic (no phase separation) liquid cultures. Below we describe changes in fatty acid composition as a possible mechanism of solvent tolerance for *S. haemolyticus*. We postulate that when grown in toluene-saturated media, *S. haemolyticus* is similar to its gram-negative counterparts in that it alters its fatty acid membrane composition but is dissimilar in that the membrane becomes more fluid rather than less fluid. A survey of 13 strains of *Staphylococcus aureus* and *Staphylococcus epidermidis*, which had not previously been exposed to solvent selection, revealed solvent tolerance similar to that of *S. haemolyticus*.

**MATERIALS AND METHODS**

**Isolation of solvent-tolerant anaerobes from *H. petrolei* larvae.** All steps were performed under strictly anaerobic conditions in a Coy (Grass Lake, Mich.) anaerobic chamber containing a mixture of 5% hydrogen and 95% nitrogen. *Four H. petrolei* larvae were surface sterilized as previously described (13). The larvae were placed in a sterilized hand-held Potter-Elvehjem homogenizer containing 2 ml of *Clostridium* isolation broth (CIB) (2). The larvae were homogenized for 5 min, after which 0.1 ml was used to inoculate four tubes containing 9.9 ml of CIB. Growth and spor formation were monitored daily by phase-contrast microscopy. Each of the growing cultures was subcultured in CIB containing 5% (vol/vol) acetone or butanol and incubated anaerobically at 28°C for 48 h. For the cultures exhibiting growth, we used a series of dilution tubes and plating on CIB agar to isolate single colonies. Two isolated colonies were subcultured in CIB, and their purity and morphology were assessed by Gram stain and phase-contrast microscopy. Each of the growing cultures was subcultured in CIB containing 2 ml of another 24 h. The solvents tested included cyclohexane, toluene, and benzene. Growth was scored as follows: ++, confluent growth; +, some growth; –, no growth; ?, variable results. All data were based on at least three independent replicates.

**Optimization of media for solvent growth.** For the cultures exhibiting growth, we used a series of dilution tubes and plating on CIB agar to isolate the organisms. Each of the growing cultures was subcultured in CIB containing 2 ml of another 24 h. The solvents tested included cyclohexane, toluene, and benzene. Growth was scored as follows: ++, confluent growth; +, some growth; –, no growth; ?, variable results. All data were based on at least three independent replicates.

**E. faecalis** did not tolerate 100% *p*-xylene, toluene, or benzene; the maximum levels tolerated were 50, 35, and 30%, respectively.

**Measurement of solvent tolerance.** Plate overlay solvent tolerance was determined by spot inoculating 20 μl (10⁶ cells) of *S. haemolyticus* from an overnight culture grown in LB broth at 37°C on glass petri plates containing LB agar. The spots were then allowed to dry for 20 to 30 min at 37°C. The plates were transferred to a properly ventilated room temperature area, and solvent was directly poured on top of the agar plate surface to a depth of 5 mm. After 8 h the solvent was pipetted off, and the plates were inverted and incubated at 37°C for another 24 h. The solvents tested included cyclohexane, toluene, and benzene. Reagent grade toluene was obtained from Matheson, Coleman and Bell Inc., Norwood, Ohio. All other chemicals were obtained from Sigma-Aldrich, St. Louis, Mo.

**Monophasic solvent medium.** A 100-μl aliquot of an overnight culture was transferred to 20 ml of 37°C prewarmed LB medium which contained the same solvent mixture, toluene, or benzene. Monophasic (saturated) medium contained 0.95 mM cyclohexane, 6.2 mM toluene, or 21 mM benzene (i.e., 208, 134, and 37.6 μl per 20 ml, respectively). To ensure that there was no loss of solvent due to volatilization, flasks were capped with aluminum foil-covered rubber stoppers. Cells were grown at 37°C in 250-ml sidearm naphthalafflasks (Bellco Glass, Vineland, N.J.) with rotary shaking at 200 rpm. Optical density readings were taken every 2 h for 30 h using a Klett-Summerson colorimeter (New York, NY) with a red filter. This arrangement permitted us to measure cell density without opening the flasks.

**Measurement of detergent resistance.** Cultures were grown overnight in LB medium and inoculated into naphthalafflasks containing LB medium and detergent. The detergents used were SDS and cetrimethylammonium bromide (CTAB). Each detergent was initially diluted in water, filter sterilized, and then added to the medium to the appropriate final concentration. Growth was measured every hour for 8 h with a Klett-Summerson colorimeter.

**TABLE 1. Solvent tolerance of 14 *Staphylococcus* strains**

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Cyclohexane</th>
<th><em>p</em>-Xylene</th>
<th>Toluene</th>
<th>Benzene</th>
</tr>
</thead>
<tbody>
<tr>
<td>M SSA 476</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>?</td>
</tr>
<tr>
<td>RN 6930</td>
<td>++</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>SH 1000</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>MRSA 252</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>MW 2</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>RF 122</td>
<td>++</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>25923</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>213</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SE 29</td>
<td>++</td>
<td>++</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>SE 220</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>ATCC 14990</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>ATCC 12298</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>—</td>
</tr>
<tr>
<td>S. haemolyticus</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

* S. haemolyticus and E. faecalis were isolated as part of our oil fly screening, and *S. aureus* strains 213 and 25923 were obtained from Eugene Martin. All other strains were obtained from Greg Somerville (23).

Log Pₐ is a measure of the intrinsic solvent toxicity depending on the partition ratios of the solvent into E. coli membranes. Growth was scored as follows: ++, confluent growth; +, some growth; –, no growth; ?, variable results. All data were based on at least three independent replicates.

* E. faecalis did not tolerate 100% *p*-xylene, toluene, or benzene; the maximum levels tolerated were 50, 35, and 30%, respectively.
Growth conditions for lipid extraction. *S. haemolyticus* was grown in LB broth (200 ml in l-liter flasks) at 37°C either with or without toluene. For the cells grown with solvent 50 μl of toluene was added to the LB broth every 3 h to maintain continuous exposure to the solvent. All treatments were replicated four times. Cells were harvested for lipid analysis when they reached an optical density at 600 nm of 0.60.

**Lipid extraction.** Lipids were extracted using a modification of the Bligh-Dyer method (3). Two hundred milliliters of cells was grown as described above. The cells were centrifuged, and the lipids were extracted twice using 2 ml of methanol:chloroform (1:1). Extracts were combined, phase separated, and centrifuged, and the methanol layer was discarded. The chloroform layer was filtered (Whatman no. 40, 5.5 cm) and evaporated under nitrogen. Lipids were dissolved in 0.500 ml chloroform stabilized with amylenes for both phospholipid analysis and fatty acid methyl ester derivatization.

**Phospholipid analysis.** The phospholipid compositions were analyzed and compared by using the methods of Nahhas et al. (16). Briefly, lipid extracts from cells grown with and without toluene were spotted onto a Silica Gel 60 F254 (Whatman no. 40, 5.5 cm) and evaporated under nitrogen. The same expanding-ring appearance was noted in a study (11) of the known energy dependence of SDS resistance in enteric bacteria (22).

**RESULTS**

**Solvent tolerance.** Strains of *S. haemolyticus* and *E. faecalis* were isolated with solvent selection, and, as expected, they exhibited exceptional solvent tolerance (Table 1). In plate overlay assays (20 μl, ca. 10^6 cells), *S. haemolyticus* tolerated 100% cyclohexane, p-xylene, toluene, or benzene for 8 h (Table 1). After removal of the solvent and subsequent incubation at 37°C, confluent cell growth was seen after 24 h. Subsequently, eight strains of *S. aureus* and five strains of *S. epidermidis* were also examined to determine their solvent tolerance (Table 1). None of these strains had been previously exposed to solvent via solvent selection. All 13 of these *Staphylococcus* strains tolerated cyclohexane (log P_toluene < 3.3) overlays, and all of the strains except *S. aureus* strains RN6390 and RF122 tolerated p-xylene (log P_toluene < 3.1). Toluene (log P_toluene = 2.6) had effects similar to those of p-xylene, while benzene (log P_toluene = 2.0) was the most detrimental of the solvents tested (Table 1).

**Growth under solvent overlays.** Of the solvent-tolerant bacteria described in Table 1, only *S. aureus* SH1000 showed visible growth under all of the solvents tested. The other strains merely tolerated the solvents. Visual evidence for growth under solvent was provided (Fig. 1A) by the expanding-ring appearance of *S. aureus* SH1000 under 100% toluene overlay. The same expanding-ring appearance was noted in a study (11) of the gram-negative organism *P. rettgeri* growing under 10% xylene/90% cyclohexane (Fig. 1B). One explanation for the ring-like growth seen under a solvent is that solvent tolerance is an energy-dependent phenomenon and the ring consists of metabolically active cells generating sufficient energy to maintain solvent tolerance. This model is adapted from the well-known energy dependence of SDS resistance in enteric bacteria (22).

**Monophasic liquid culture.** *S. haemolyticus* also exhibited exceptional solvent tolerance during liquid monophasic growth. In LB medium containing saturating levels of solvent (21 mM benzene, 6.2 mM toluene, and 0.95 mM cyclohexane), *S. haemolyticus* exhibited no significant differences in growth rate or cell yield compared to cells grown without solvent (Fig. 2). Also, cell lysis was not observed during the stationary phase (Fig. 2). *S. haemolyticus* was chosen for further investigation of adaptations in the lipid portion of its cytoplasmic membrane during growth in solvent-saturated liquid media.

**Phospholipid analysis.** The phospholipids that occur most commonly in *Staphylococcus* species are diphosphatidylglycerol (cardiolipin), phosphatidylglycerol, lysophosphatidylglycerol, and another unidentified phospholipid (16). Characterization of the phospholipids in *S. haemolyticus* showed that the major classes of lipids were phosphatidylglycerol and another

![FIG. 1. Ring-like growth of bacteria under organic solvent overlays. (A) *S. aureus* SH1000 under 100% toluene. (B) *P. rettgeri* under 10% xylene/90% cyclohexane. Both cultures (15 μl) were spotted onto LB agar plus 2% glucose, allowed to dry for 10 to 15 min, overlaid with solvent to a depth of 2 mm, and incubated at 30°C for 6 to 8 h prior to photography.](image-url)
unknown glycolipid. These findings are consistent with phospholipids found in *S. haemolyticus* cells according to Nahaie et al. (16). Following exposure to toluene, small shifts in spots were noted. However, no changes were evident in the abundance or distribution of any phospholipid compared to non-toluene-exposed cells, as determined by the visual size and shape of the TLC spot. In particular, no spots due to lysylphosphatidylglycerol were observed for *S. haemolyticus* strains 213 and 25923 were tested for resistance to both anionic (SDS) and cationic (CTAB) detergents. None of the strains grew in the presence of ≥0.1% SDS or ≥0.04% CTAB. Thus, in gram-positive bacteria we observed no correspondence between organic solvent tolerance and SDS resistance.

**Detergent resistance.** *S. haemolyticus*, *E. faecalis*, and *S. aureus* strains 213 and 25923 were tested for resistance to both anionic (SDS) and cationic (CTAB) detergents. None of the strains grew in the presence of ≥0.1% SDS or ≥0.04% CTAB. Thus, in gram-positive bacteria we observed no correspondence between organic solvent tolerance and SDS resistance.

**DISCUSSION**

We exploited the unique ecological niche of oil fly larval guts to identify highly solvent-tolerant gram-positive bacteria. Clearly, an outer membrane is not needed for bacteria to tolerate organic solvents. The gram-positive bacteria studied were *E. faecalis*, *S. haemolyticus*, eight strains of *S. aureus*, and five strains of *S. epidermidis*. The first two species were isolated by positive solvent selection, whereas none of the *S. aureus* and *S. epidermidis* strains had ever previously been exposed to solvents. In 1991, *S. aureus* strain IFO was reported to be growth limited by solvents, with log *P*<sub>sw</sub> values less than 4.8, as measured by plate overlays (7). We found that all 14 of the *Staphylococcus* strains examined tolerated harsher solvents (Table 1) and that *S. haemolyticus* was able to grow in the presence of cyclohexane, benzene, and toluene, both in monophasic liquid cultures and in organic solvent plate overlays. Thus, *S. haemolyticus* can tolerate organic solvents with log *P*<sub>sw</sub> values as low as 2.0 (benzene). Given the ubiquitous nature of the bacteria identified, it seems likely that solvent tolerance is merely a previously unrecognized resistance characteristic and the designation extremophile is not warranted.

However, the prevalence of extreme solvent tolerance among the staphylococci is relevant to the efficacy of many chemical antiseptics in clinical use.

Several mechanisms of organic solvent tolerance have been found in gram-negative bacteria (23). Among these mecha-
tolerance mechanisms characteristic of the gram-negative bacteria, such as membrane efflux pumps (23, 24).

One target for future investigation should be the role of $\sigma^B$ in the solvent stress tolerance of *S. aureus* (6). The tolueone-sensitive organism strain RN6390 differs from the tolueone-resistant organism strain SH1000 only in that it contains an 11-bp deletion in rsbU, a positive regulator of $\sigma^B$ function in *S. aureus*, which causes a strong defect in $\sigma^B$ activity (6, 26). Similarly, the tolueone-sensitive organism *S. aureus* RF122 is also suspected of being a sigB mutant (Greg A. Somerville, personal communication).

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