Freeze-Thawing of *Aquaspirillum magnetotacticum* Cells Selectively Releases Periplasmic Proteins

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Cells of the gram-negative bacterium *Aquaspirillum magnetotacticum*, when suspended in buffer and freeze-thawed, produced pinkish orange supernatant fluid. The fluid contained ~2.0% of total extractable outer membrane component 2-keto-3-deoxyoctonate or of the cytoplasmic membrane marker succinic dehydrogenase. Electrophoretic banding patterns and difference spectra of proteins and hemoproteins released by freeze-thawing cells were distinct from those of membrane-associated substances and similar to those of periplasmic substances obtained by applying conventional fractionation methods to this organism.

Freeze-thawing (FT) is undoubtedly important in defining indigenous soil bacteria populations in temperate regions. Morley et al. (11) observed a 40 to 60% decrease in bacterial viability in sandy loam soil as a direct result of FT. FT is known to have a profound effect on bacterial cells and is often used as a pretreatment for cell disruption (18, 19). Responses of gram-negative cells depend upon the cell genotype (3), the menstrum in which they are suspended, and the FT rates (4). Outer sheath material from an oral spirochete has been isolated by FT (10). Calcott and MacLeod (4) found that freeze-thawed lactose-limited *Escherichia coli* cells released considerable amounts of the periplasmic enzyme cyclic phosphodiesterase but not the cytoplasmic enzyme glucose-6-phosphate dehydrogenase. A small, constant quantity of 10 to 15% of total activity of β-galactosidase (normally cytoplasmic) released was attributed to a possible periplasmic form of this enzyme.

Periplasmic substances of *E. coli* have been separated from other cellular components by osmotic shock and spheroplast formation (12). Ames et al. (1) demonstrated selective release of periplasmic proteins from *E. coli* cells treated with chloroform.

FT of cell suspensions of *Aquaspirillum magnetotacticum* MS-1 in 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.4) or 10 to 50 mM potassium phosphate buffer caused the release of soluble cSS-type hemoproteins (15; W. O'Brien, M.S. thesis, University of New Hampshire, Durham, 1982). FT did not disrupt overall helical cell form. The objective of our study was to compare FT with other cell fractionation methods applied to this organism to determine the cellular origin of the substances released, including the soluble cSS-type hemoprotein. This method also allowed us to partially purify this hemoprotein. Periplasmic soluble c-type hemoproteins of unknown function have been detected in *Alcaligenes eutrophus* (17), *Aquaspirillum itersonii* (6), *Paracoccus denitrificans* (8), and *Haemophilus parasuis* (13).


Denitrifying *A. magnetotacticum* MS-1 cells (ATCC 31632) were batch cultured with a chemically defined medium (2) microaerobically at a dissolved oxygen tension of less than 1% saturation. Cells were harvested by filtration (15) when they reached a density of (4 x 10⁹)· ml⁻¹. Cells were washed once by centrifugation (8,000 x g, 30 min, 5°C) in at least 10 pellet volumes of 50 mM potassium phosphate buffer (pH 6.8) or 10 mM HEPES buffer (pH 7.4). Cells from a single 40-liter culture were suspended in 100 ml of potassium phosphate buffer or HEPES buffer, and equal portions were fractionated by the procedures described below.

The FT technique consisted of storing washed resuspended cells at -20°C overnight. The freezing rate was 0.7°C·min⁻¹. The sample was thawed at room temperature, and cells were pelleted by centrifugation (10,000 x g, 15 min, 5°C). The pinkish orange supernatant fluid was clarified by ultracentrifugation (100,000 x g, 1 h, 5°C) and concentrated by membrane dialysis (Spectra-Por membrane tubing; 6,000 to 8,000 molecular weight cutoff; Spectrum Medical Industries, Inc., Los Angeles, Calif.) on a bed of solid-flake polyethylene glycol (molecular weight, 20,000; J. T. Baker Chemical Co., Phillipsburg, N.J.) at 4°C.

Periplasmic proteins were obtained by two methods: osmotic shock (12) and chloroform extraction (1). The method of Schnaitman (19) was also used to separate outer membrane proteins, cytoplasmic membrane proteins, and soluble (cytoplasm and periplasm) proteins. Cells were disrupted in a French press (10,000 lb/in²) before treatment with 2% (vol/vol) Triton X-100 and 10 mM MgCl₂ in 10 mM HEPES buffer (pH 7.4). Each fraction was dialyzed overnight at 4°C against HEPES buffer before analysis.

The relative activity of succinic dehydrogenase (SDH), an integral enzyme of the cytoplasmic membrane (5), and the concentration of 2-keto-3-deoxyoctonate (KDO), a constituent of outer membrane lipopolysaccharide, were used as indices of the purity of cell fractions (9). Proteins and molecular mass standards (Bio-Rad Laboratories, Richmond, Calif.) were solubilized and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (16) and stained with silver (14). Spectra of air-oxidized soluble protein fractions were subtracted from spectra of dithionite-reduced soluble protein fractions, both of which were at room temperature, as previously described (15). The ability of cells to survive FT was evaluated by using a standard plate assay. Logarithmic dilutions of thawed cells were prepared as pour plates in semisolid medium in triplicate. Plates were incubated for 1 week at room temperature microaerobically. Colony counts were compared with those of control (non-FT) cells plated similarly.

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TABLE 1. Membrane markers in *A. magnetotacticum* cell fractions

<table>
<thead>
<tr>
<th>Fractionation method (reference), fraction</th>
<th>Total KDO (mg)</th>
<th>Total SDH* (10^2 U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton X-100-MgCl₂ (18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OMPb</td>
<td>1,330</td>
<td>30</td>
</tr>
<tr>
<td>Periplasm-cytoplasm</td>
<td>6</td>
<td>0.9</td>
</tr>
<tr>
<td>CMPc</td>
<td>149</td>
<td>200</td>
</tr>
<tr>
<td>Chloroform (1), periplasm</td>
<td>11</td>
<td>0.9</td>
</tr>
<tr>
<td>Osmotic shock (12), periplasm</td>
<td>16</td>
<td>2.0</td>
</tr>
<tr>
<td>FT (this study), periplasm</td>
<td>20</td>
<td>5.0</td>
</tr>
</tbody>
</table>

* Micromoles of cytochrome c reduced · mg of protein⁻¹ · min⁻¹.
* OMP, Outer membrane protein.
* CMP, Cytoplasmic membrane protein.

Supernatant fluids obtained by FT contained 1.3% of the total SDH activity detected and 2.0% of the total KDO recovered (Table 1). These results suggest that FT did not markedly disrupt either the outer or the inner cell membranes with subsequent release of these markers. Soluble fractions obtained by chemical treatment (chloroform or lysozyme-EDTA) or mechanical disruption (French press) of strain MS-1 cells had comparable proportions of total detectable SDH activity and KDO (Table 1). Most (87%) of the total SDH activity and 90% of the total KDO recovered were in the cytoplasmic and outer membrane fractions, respectively, of strain MS-1 cells (Table 1).

Electrophoretograms of each soluble fraction (Fig. 1, lanes 4 to 7) exhibited similar protein-banding patterns. In each of these fractions, more than 60 proteins were evident, including several major proteins with molecular masses ranging between 28,000 and 85,000 daltons. Four proteins with apparent molecular masses of 29,000, 41,000, 44,500 and 45,000 daltons were unique to the periplasm (Fig. 1, lanes 4 to 7). The cytoplasmic membrane (Fig. 1, lane 3) contained three major proteins (16,500, 56,000, and 85,000 daltons) which were also present in the periplasmic fraction.

The outer membrane (Fig. 1, lane 2) and periplasmic fractions (Fig. 1, lanes 4 to 7) contained few proteins in common.

Solute fractions obtained by FT, chloroform treatment, osmotic shock, or French press disruption of strain MS-1 cells contained substances with absorption spectra (Fig. 2) typical of η551-type hemes (maxima at 419, 522, and 551 nm). Spent growth medium and cell-wash fluids of *A. magnetotacticum* concentrated 100-fold did not contain detectable quantities of protein or c-type hemoproteins.

The effects of FT on strain MS-1 cells were evaluated by plate assay and electron microscopy. Only 1 to 7% of control (non-FT) cells were recovered as CFU after FT. Survivors were magnetotactic. Freeze-thawed cells which were negatively stained with uranyl acetate and observed by electron microscopy lacked flagella but appeared otherwise structurally intact as compared with control cells. They retained their helical form and did not form spheroplasts or show blebbing.

Our results indicate that FT provides a rapid, simple, reproducible method of selectively releasing periplasmic substances, including the soluble η551-type hemoproteins, from *A. magnetotacticum* without recourse to chemical treatments.

We have applied FT to *Aquaspirillum itersonii* and *Azospirillum lipoferum* cells and obtained spectral evidence for release of c-type hemoproteins from these organisms as well (data not shown). Recently, FT was applied to cells of nine genera of gram-negative bacteria. The method was found to be comparable to the chloroform method (1) for the release of periplasm (B. E. Eribo, S. D. Lall, and J. M. Jay, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, 1152, p. 197).

FT had several advantages over conventional techniques used to obtain periplasm from *A. magnetotacticum*. These include the following: (i) the absence of chemical treatment, such as with lysozyme, chloroform, toluene, or EDTA; (ii) the rapid and selective recovery of periplasmic substances, including enzymes; and (iii) the lack of apparent gross cell damage. This method, if generally applicable to other gram-
negative species, would prove useful in obtaining periplasm with minimal cell handling, e.g., with pathogens.

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LITERATURE CITED