Foam Separation of Pseudomonas fluorescens and Bacillus subtilis var. niger

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ABSTRACT

An experimental investigation established the effect of the presence of inorganic salts on the foam separation of Pseudomonas fluorescens and of Bacillus subtilis var. niger (B. globigii) from aqueous suspension by use of a cationic surfactant. For P. fluorescens, 5.0 µeq/ml of NaCl, KCl, Na₂SO₄, K₂SO₄, CaCl₂, CaSO₄, MgCl₂ or MgSO₄ produced increases in the cell concentration in the residual suspension (not carried into the foam) from 2.9 × 10⁷ up to 1.6 × 10⁸ to 2.8 × 10⁸ cells per milliliter (initial suspensions contain from 3.3 × 10⁶ to 4.8 × 10⁸ cells per milliliter). The exceptional influence of magnesium was overcome by bringing the cells into contact first with the surfactant and then the salt. For B. subtilis, the presence of 5.0 µeq/ml of any of the eight salts increased the residual cell concentration by one order of magnitude from 1.2 × 10⁶ to about 4.0 × 10⁷ cells per milliliter. This occurred regardless of the sequence of contact as long as the surfactant contact period was sufficient. The presence of salts increased collapsed foam volumes with P. fluorescens and decreased collapsed foam volumes with B. subtilis.

Foam separation may provide a valuable technique to the water treatment engineer for the concentration of bacteria from dilute suspensions to enable more accurate cell counts on the concentrated foam and for the removal of bacteria from dilute suspensions to permit the reduction in disinfectant dosages. Since a foam separation process involves the interaction of surfactants at or in bacterial surfaces, studies should provide a further understanding of the nature of interfacial phenomena associated with microorganisms.

A number of investigations have been carried out on the foam separation of bacteria, including five recent studies on Escherichia coli (2, 5, 6, 9, 11). The spores of Bacillus subtilis (7) and of B. anthracis and of other bacteria (1) have been floated, and a comparative investigation (R. B. Grieses and S. L. Wang, Biotech. Bioeng., in press) has included B. cereus, B. subtilis var. niger, Pseudomonas fluorescens, Proteus vulgaris, and Serratia marcescens. Since most bacterial suspensions behave as negatively charged hydrophilic colloids (10, 13), the best flotation agent should be a cationic surfactant. By use of ethylhexadecyltrimethylammonium bromide (EHDA-Br), E. coli has been foam-separated successfully (2, 9), and the effects of foaming time, of initial cell concentration, of initial surfactant concentration, of gas rate, and of foam port height have been established. However, it has also been shown that the presence of inorganic salts provides a reduction in efficiency, unless the surfactant is added in stages. With the surfactant added in stages, residual cell concentrations are higher in the presence of salts, but lower volumes of collapsed foam provide more concentrated foams. It would be of considerable value to determine whether salts produce similar effects on the foam separation of other bacterial species and to try to elucidate the mode of interaction.

The objective of this investigation was to determine quantitatively the effects of the presence of inorganic salts in the initial suspensions on the foam separation efficiency of P. fluorescens (gram-negative) and of B. subtilis (gram-positive). Both species have been floated most efficiently from neutral distilled water suspensions of the pure cultures. It was also our objective to determine the modifications caused by varying salt and surfactant contact times and by varying the sequence of contact.

MATERIALS AND METHODS

The organisms, P. fluorescens (ATCC-13525) and B. subtilis var. niger (B. globigii), were supplied by H. W. Bretz of the Biology Department at the Illinois Institute of Technology. They were grown on HYT medium containing 1.2% Heart Infusion (Difco),
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0.5% yeast extract (Difco), 0.5% Trypticase (BBL), and 0.1% glucose. Twelve-hour-old _P. fluorescens_ cells grown at 28 °C were harvested and washed in distilled water with centrifugation before being suspended in 500 ml of distilled water at an optical density of 0.1 at 620 m
d. Sixteen-hour-old _B. subtilis_ cells (broth-aerated while culturing) grown at 37 °C were harvested and washed in 0.0067 M phosphate buffer before being treated similarly.

From each suspension 500 ml was hand-agitated with 495 ml of distilled water and with 5 ml of a salt solution of NaCl, KCl, Na_2SO_4, K_2SO_4, CaCl_2, CaSO_4, MgCl_2·6H_2O, or MgSO_4·7H_2O, or with 5 ml of distilled water (controls). The mixing period was 30 sec, the resultant salt concentrations were 5.0 µeq/ml, and the resultant cell concentrations were in the range of 1.6 × 10^8 to 4.8 × 10^8 cells per milliliter. We added 15 ml of a 2.0 mg/ml solution of EHDA-Br, a cationic surfactant, to the suspensions, providing a concentration of 30 µg/ml of surfactant. The mixing and contact period with the surfactant was 60 sec (±5 sec) before each foam separation experiment was initiated. The suspension was then placed in the column and the experiment was begun immediately. In later experiments, the salt and surfactant contact periods were lengthened and the sequence of addition was reversed. The pH of each initial suspension was approximately 7.0.

The foam separation unit has been described previously (2). Some brief details follow, including some modifications from the previous operating procedure (2) to make the unit operable for both species. The foaming column was 9.6 cm in diameter, 82 cm in height, and was made of Pyrex. Filtered nitrogen gas was metered with a calibrated rotameter and diffused through twin sintered-glass aerators of 30-µm porosity. The 1,015 ml of initial suspension containing bacteria, salts, and surfactant was placed in the column, and the nitrogen flow was begun and was maintained at 13.5 liters per min. Foam was removed continuously from a port located 35.5 cm above the base of the column, that is, 20.5 cm above the initial suspension level. The length of the foam runs was 15 min with temperature held at 25 ± 1 °C. At the end of each experiment, the residual suspension was taken for total cell count and surfactant analysis. The EHDA-Br was determined by a two-phase titration technique (3, 12) which was accurate to within ±1.0 µg/ml. The volume of the residual suspension was measured carefully. After each experiment, the column was rinsed several times with tap water and with distilled water.

The membrane-filter technique for total cell count (4) was employed as described previously (9). The only modifications were the use of a saline-picric acid solution as a fixing agent and the use of diluted acid fuchsin as a staining solution before direct microscopic observation.

**RESULTS AND DISCUSSION**

For each foam separation experiment, the following material balances can be written:

\[
V_1 = V_r + V_f
\]  

\[z_i V_1 = z_i V_r + z_i V_f \quad (2)\]

\[x_i V_1 = x_i V_r + x_i V_f \quad (3)\]

\[
V_i, V_r, \text{ and } V_f \text{ are the volumes in milliliters of the initial suspension, the residual suspension (after the foaming experiment, not carried into the foam), and the collapsed foam suspension, respectively. The cell concentration, } z, \text{ is the number of cells per milliliter, and the surfactant (EHDA-Br) concentration, } x, \text{ is the number of micrograms per milliliter. The subscripts refer to the appropriate suspensions. In the presentation of results below, a second subscript, } s, \text{ is added to } V_r, V_t, z_r, z_t, x_r, \text{ and } x_t \text{ to designate experiments for which salts are present in the initial suspensions. Equations 1, 2, and 3 may be used to calculate the concentrations of cells and of EHDA-Br in the collapsed foam suspension resulting from any experiment for which } V_r, z_r, \text{ and } x_r \text{ have been measured. The quantities } z_i V_1 \text{ and } x_i V_1 \text{ represent the total number of cells and the total weight of surfactant removed from the initial suspension by foam separation.}

**Effect of salts on _P. fluorescens_.** The first series of experiments was conducted to establish the effect of salts on the foam separation of _P. fluorescens_. Results are presented in Table 1 as average values for the number of experiments indicated. The first two rows in Table 1 show the influence of cells without salts upon the foam separation of surfactant solutions; the collapsed foam volume is reduced markedly by the presence of cells, whereas the residual surfactant concentration is virtually unchanged, thereby producing a foam considerably richer in surfactant. As noted previously (2, 9; R. B. Griewe and S. L. Wang, in press), a certain fraction of the surfactant in the initial suspension is adsorbed or bound by the cells.

The last eight rows in Table 1 show the effect of salts on the foam separation of _P. fluorescens_ as indicated by several parameters. Values of \(z_n/z_r, z_n V_n/z_r V_r, \text{ and } (z_1 - z_n)/(z_1 - z_t)\) were calculated by comparing each salt experiment with a corresponding control containing no salt but run identically. (The average of the controls is presented in the second row.) The parameters were then averaged, and the values found are given in the table. All salts decreased the efficiency of the foam separation process from the standpoint of residual cell concentrations; divalent calcium and magnesium salts clearly produced more marked changes than monovalent sodium and potassium salts. The presence of sulfate compared to chloride appeared to have little influence. Magnesium sulfate, as already noted for _E. coli_ (2), also produced the most severe interference for
P. fluorescens, a fact indicated by the values for \( z_{r_1} / z_r \) and \( z_{r_1} V_{r_1} / z_r V_r \). The interference is not indicated by the parameter \( (z_1 - z_{r_1}) / (z_1 - z_r) \), which is less sensitive and expresses the effect of salt by means of comparative concentration differences.

Observation of the next to the last column in Table 1 reveals that the presence of salts in the initial suspensions produced increases in collapsed foam volumes with no correlation between the magnitudes of the volumes and the valence of the cations or anions present. Thus, salts not only raised the residual cell concentration, but they also produced larger and more dilute foams. This behavior is in direct contrast to that of E. coli (2), with which salts produced decreases in collapsed foam volumes and more concentrated foams. Salts produced definite decreases in the surfactant (EHDA-Br) concentrations in the residual suspensions, as indicated by the last column in Table 1. This behavior is analogous to E. coli (2).

*Effect of salts on B. subtilis.* Results of the second series of experiments are given in Table 2. From the first two rows, it can be seen that cells of *B. subtilis* had a quite different effect on the foam separation of EHDA-Br than did cells of *P. fluorescens*.

### Table 1. Effect of salts on foam separation of Pseudomonas fluorescens

<table>
<thead>
<tr>
<th>Salt</th>
<th>No. of experiments</th>
<th>( z_r )</th>
<th>( z_{r_1} / z_r )</th>
<th>( z_{r_1} V_{r_1} / z_r V_r )</th>
<th>( (z_1 - z_{r_1}) / (z_1 - z_r) )</th>
<th>( V_f )</th>
<th>( x_{r_1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4</td>
<td></td>
<td>-</td>
<td>-</td>
<td>417</td>
<td>10.6</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>5</td>
<td>2.9 \times 10^5</td>
<td>-</td>
<td>-</td>
<td>18</td>
<td>11.0</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>2</td>
<td>2.2 \times 10^4</td>
<td>8.6</td>
<td>8.0</td>
<td>0.95</td>
<td>93</td>
<td>2.8</td>
</tr>
<tr>
<td>KCl</td>
<td>2</td>
<td>2.7 \times 10^4</td>
<td>5.7</td>
<td>5.3</td>
<td>0.95</td>
<td>85</td>
<td>1.2</td>
</tr>
<tr>
<td>Na_2SO_4</td>
<td>3</td>
<td>1.6 \times 10^4</td>
<td>7.1</td>
<td>7.3</td>
<td>0.96</td>
<td>130</td>
<td>0.8</td>
</tr>
<tr>
<td>K_2SO_4</td>
<td>2</td>
<td>2.8 \times 10^4</td>
<td>6.0</td>
<td>5.2</td>
<td>0.94</td>
<td>128</td>
<td>1.0</td>
</tr>
<tr>
<td>CaCl_2</td>
<td>2</td>
<td>2.3 \times 10^4</td>
<td>48.9</td>
<td>43.6</td>
<td>0.44</td>
<td>114</td>
<td>1.7</td>
</tr>
<tr>
<td>MgCl_2</td>
<td>2</td>
<td>1.8 \times 10^4</td>
<td>48.6</td>
<td>43.8</td>
<td>0.63</td>
<td>132</td>
<td>3.3</td>
</tr>
<tr>
<td>CaSO_4</td>
<td>2</td>
<td>2.8 \times 10^4</td>
<td>59.6</td>
<td>50.9</td>
<td>0.32</td>
<td>152</td>
<td>0.7</td>
</tr>
<tr>
<td>MgSO_4</td>
<td>2</td>
<td>2.2 \times 10^4</td>
<td>115.8</td>
<td>100.1</td>
<td>0.33</td>
<td>157</td>
<td>0.8</td>
</tr>
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</table>

*Initial cell concentration = 3.3 \times 10^7 to 4.8 \times 10^7 cells per milliliter; initial salt concentration = 5.0 \mu g/ml; initial surfactant concentration = 30 \mu g/ml. Results are average values for number of experiments indicated.

b No cells present in initial suspensions.

### Table 2. Effect of salts on foam separation of Bacillus subtilis

<table>
<thead>
<tr>
<th>Salt</th>
<th>No. of experiments</th>
<th>( z_r )</th>
<th>( z_{r_1} / z_r )</th>
<th>( z_{r_1} V_{r_1} / z_r V_r )</th>
<th>( (z_1 - z_{r_1}) / (z_1 - z_r) )</th>
<th>( V_f )</th>
<th>( x_{r_1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>10</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>428</td>
<td>8.3</td>
</tr>
<tr>
<td>None</td>
<td>11</td>
<td>1.2 \times 10^4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>388</td>
<td>5.7</td>
</tr>
<tr>
<td>NaCl</td>
<td>4</td>
<td>2.4 \times 10^4</td>
<td>23.8</td>
<td>28.9</td>
<td>0.99</td>
<td>200</td>
<td>1.0</td>
</tr>
<tr>
<td>KCl</td>
<td>3</td>
<td>1.4 \times 10^4</td>
<td>12.4</td>
<td>15.1</td>
<td>0.99</td>
<td>191</td>
<td>0.5</td>
</tr>
<tr>
<td>Na_2SO_4</td>
<td>2</td>
<td>4.7 \times 10^4</td>
<td>45.4</td>
<td>55.2</td>
<td>0.98</td>
<td>163</td>
<td>0.3</td>
</tr>
<tr>
<td>K_2SO_4</td>
<td>2</td>
<td>4.7 \times 10^4</td>
<td>23.5</td>
<td>29.2</td>
<td>0.99</td>
<td>152</td>
<td>0.3</td>
</tr>
<tr>
<td>CaCl_2</td>
<td>2</td>
<td>8.8 \times 10^4</td>
<td>44.0</td>
<td>52.5</td>
<td>0.97</td>
<td>185</td>
<td>1.5</td>
</tr>
<tr>
<td>MgCl_2</td>
<td>3</td>
<td>1.4 \times 10^4</td>
<td>142.9</td>
<td>178.3</td>
<td>0.94</td>
<td>208</td>
<td>0.8</td>
</tr>
<tr>
<td>CaSO_4</td>
<td>3</td>
<td>4.7 \times 10^4</td>
<td>42.7</td>
<td>50.5</td>
<td>0.98</td>
<td>173</td>
<td>0.9</td>
</tr>
<tr>
<td>MgSO_4</td>
<td>4</td>
<td>1.8 \times 10^4</td>
<td>235.0</td>
<td>330.5</td>
<td>0.91</td>
<td>197</td>
<td>0.7</td>
</tr>
</tbody>
</table>

*Initial cell concentration = 1.6 \times 10^7 to 3.1 \times 10^7 cells per milliliter; initial salt concentration = 5.0 \mu g/ml; initial surfactant concentration = 30 \mu g/ml. Results are average values for number of experiments indicated.

b No cells present in initial suspensions.
**P. fluorescens**; the collapsed foam volume is decreased but slightly, and a definite reduction in the residual surfactant concentration is observed. Comparing the second rows of Tables 1 and 2, residual cell concentrations of **B. subtilis** are better than an order of magnitude lower than those of **P. fluorescens**, generally due to the larger collapsed foam volumes, but also perhaps due to the nature of interaction between the EHDA cations and the cell surfaces (R. B. Grieves and S. L. Wang, in press).

From the last eight rows in Table 2, it can be seen that all salts interfered with the foam separation of **B. subtilis**. There is not a significant variation when comparing sodium and potassium with calcium; magnesium salts do, however, provide a more significant interference. Again comparing Tables 1 and 2, it is difficult to relate the effects of salt on the two species, particularly because of the different magnitudes of the residual cell concentrations that are involved. Perhaps the best parameter by which to compare the two species is the ratio of the residual cell concentrations (z1 - z2)/(z1 - z2), which shows that the effect of salts on the concentration reduction of **B. subtilis** is less marked than on that of **P. fluorescens**.

In direct contrast to **P. fluorescens**, salts in the initial suspensions decreased collapsed foam volumes for **B. subtilis** (and thus produced more concentrated foams), and the residual EHDA-Br concentrations were again markedly lower than those of the controls.

With a distilled water solution of a cationic surfactant (EHDA-Br), the presence of cells adsorbs or ties up a fraction of surfactant, thereby reducing the collapsed foam volume; the presence of salts generally provides increases in collapsed foam volumes (8). The presence of both salts and cells may produce increases or decreases because of the magnitude of the effect of salts on the extent and nature of interaction between the surfactant cations and cells, and because of the interaction between salts and cells. Again considering the four initial (surfactant-cell-salt) systems, the effect of cells on the residual surfactant concentration is overshadowed by the predominant influence of salts in producing lower concentrations.

**Influence of modification of contact of cells with surfactant and salts.** Additional experiments were conducted to try to establish further the extent and mechanism of the salt effect on the foam separation of bacteria with cationic surfactants. First, experiments similar to those in Table 2 were carried out with **B. subtilis**, with NaCl or MgSO4 (5.0 μeq/ml), except that the contact period of the cell-salt suspension with the surfactant before foaming was varied. Instead of being held 1 min (see Materials and Methods), the materials were held together 0.75 min to 15.5 min, with the residual concentrations obtained from the resulting foam separation experiments in Fig. 1. After a surfactant contact period of 3 min or more, MgSO4 does not provide a significantly greater interference than NaCl. This indicates that any variation in residual cell concentrations with the type of salt may be produced by interference by the salt to the diffusion of the surfactant cations to the cell surfaces. Similar experiments with CaCl2 also showed that divalent cations did provide some resistance to diffusion, which may be overcome by lengthening the surfactant contact period. Lengthening of the 0.5-min salt contact period, with the succeeding surfactant contact period held at 1 min, produced no variations.

Next, with **B. subtilis** and 5.0 μeq/ml of MgSO4, the sequence of contact with surfactant and salt was reversed; the surfactant was added first and the salt second. For surfactant contact times varying from 0.5 to 10 min and salt contact times varying from 1 to 2.5 min, the residual cell counts ranged from $1.8 \times 10^6$ to $5.7 \times 10^6$ cells per milliliter in a random manner. This further emphasizes the feasibility of a diffusion interference by divalent cations, which of course would not exist if the surfactant was contacted with the cells before the salt.

For **B. subtilis**, the presence of 5.0 μeq/ml of...
any of eight salts tended to produce approximately the same interference ($x_{ra} = 3.0 \times 10^4$ compared to $x = 1.2 \times 10^4$ cells per milliliter) to the foam separation process. This occurred regardless of the order of salt and surfactant contact, as long as the surfactant contact period was 3 min or greater.

Finally, with *P. fluorescens* and 5.0 μeq/ml of MgSO₄, contact times and the sequence of contact were also varied; results in terms of residual cell concentrations are presented in Table 3. When the salt was added first (as in Table 1), increasing the surfactant contact time had no effect. This is in direct contrast to *B. subtilis*, for which a diffusion interference was postulated. When the surfactant was added first, time again had no effect, but the change in sequence of contact produced residual cell concentrations an order of magnitude lower. MgSO₄ (probably also true for MgCl₂ and thus caused by Mg²⁺) appears to react with surface sites on the bacteria, preventing the adsorption of the surfactant cations or modifying the orientation of the surfactant cations. This is substantially avoided by adding the surfactant first, where results are obtained similar to those found with other salts. The contrast between this behavior and that of *B. subtilis* may point to the differences between surface properties of two bacterial species, differences perhaps produced by factors similar to those making one species gram-positive and one gram-negative, and it may also point to other contrasting foam separation behavior. This possibility should be investigated for other species.

For virtually all of these experiments, the collapsed foam volumes and residual surfactant concentrations were insensitive to the sequence and duration of salt and surfactant contact. This may be explained by the fact that, although residual cell concentrations may vary considerably (for example, from $2.0 \times 10^6$ to $2.2 \times 10^7$ cells per milliliter), the total number of cells floated ($x_{ta}V_{ta}$) and thus present in the foam may vary to a lesser extent (from $3.47 \times 10^6$ to $3.17 \times 10^7$ cells for the above example with an initial cell concentration of approximately $3.5 \times 10^6$ cells per milliliter). This lack of variation would tend to keep foam volumes and residual surfactant concentrations approximately constant.

This investigation was conducted to establish the effect of the presence of inorganic salts upon the foam separation of a gram-positive species, *B. subtilis* var. *niger* (*Bacillus globigii*), and a gram-negative species, *P. fluorescens*, from neutral aqueous suspension by use of a cationic surfactant. For *B. subtilis*, the presence of 5.0 μeq/ml of any of the eight chloride or sulfate salts of sodium, potassium, calcium, and magnesium increased the residual cell concentration by an order of magnitude. This occurred regardless of the sequence of contact of salt and surfactant, as long as the surfactant contact period was 3 min or longer. Compared to experiments with cells suspended in distilled water, salts produced lesser foam volumes and foams more concentrated in cells. For *P. fluorescens*, salts had a more pronounced effect; 5.0 μeq/ml of MgSO₄ increased the residual cell concentration by two orders of magnitude. The exceptional influence of magnesium may be overcome by contacting cells with surfactant before the salt. Compared to experiments with distilled water, salts produced greater foam volumes and foams less concentrated in cells. The effect of salts on *E. coli* was not the same as on either species.

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**LITERATURE CITED**


