The effects of osmotic stress on survival and alkaline phosphatase activity of \textit{Aeromonas hydrophila}

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Abstract

\textit{Aeromonas hydrophila} was grown under low and high phosphate conditions and inoculated into microcosms containing filtered autoclaved lake water as a starvation medium. Sodium chloride was added to the microcosms to cause osmotic stress and the survival of the bacteria was measured by viable count and ability to respire. The survival of \textit{A. hydrophila} was dependent upon the concentration of sodium chloride added to the microcosm, with higher concentrations causing a more rapid decline in the viable count. The ability of cells to respire was also gradually lost, but in the microcosms containing 5\% and 7\% (w/v) sodium chloride, the viable count had fallen below the limits of detection when respiration measurements indicated that 30\% of the cells were capable of respiration. Alkaline phosphatase activity increased in the flasks amended with sodium chloride and the osmoprotectant betaine, in both cells with a derepressed alkaline phosphatase and with a repressed alkaline phosphatase. No comparable increase was seen in cells grown with either betaine or sodium chloride alone.

Keywords: \textit{Aeromonas hydrophila}; Osmotic stress; Alkaline phosphatase

1. Introduction

The enzyme activity of bacterial cells may have an important function in determining their response to different stress conditions. The ability of a cell to synthesise protective proteins or to repair damage through enzyme activity is obviously important to a stressed cell. Some catabolic enzymes show alterations in activity in bacteria which have been subjected to changing environmental conditions including changes such as osmotic stress, starvation stress, oxidation stress or heat stress. Alkaline phosphatase is usually produced in response to starvation stress, in particular in response to the absence of inorganic phosphate from the growth medium or the environment. The enzyme can also be produced in response to other forms of stress, such as heat shock and osmotic shock, and may be an important component of global stress response networks [1,2]. Consequently, the enzyme may be involved in the survival of bacteria under stress conditions either through its role in the scavenging of phosphate or through a non-specific function such as aiding the permeability...
of the cell through the action of the porin proteins co-synthesised with alkaline phosphatase.

Osmotic stress caused by increasing concentrations of sodium chloride is an effective inducer of general stress proteins [3]. Osmotic stress is an important factor in the survival of *Escherichia coli* and *Aeromonas hydrophila* in untreated sewage or in sewage effluents discharged into the sea. Changes in osmolarity have been shown to affect the activity of alkaline phosphatase of *E. coli* [2]. *E. coli* also responds to changes in osmolarity by taking up osmoprotectants such as proline and the amino acid analogue, betaine [4], changing internal potassium ion concentration [5], the amount of polysaccharide in the periplasmic space [6], or the relative concentrations of outer membrane proteins Omp F and OmpC [7]. Proline, glutamate and glycine betaine are rapidly taken up under conditions of osmotic stress and can protect cells from the deleterious effects of sea water [8-10]. Although betaine could not be used by *E. coli* as a carbon or nitrogen source, the maintenance of viability under osmotic stress was conditional upon the bacteria being able to accumulate this compound and other amino acids [9].

We have investigated the effects of osmotic stress on the survival and alkaline phosphatase activity of *A. hydrophila* grown in a high or low phosphate medium. The aim was not to mimic survival of this organism in the marine environment but to investigate the relationship between the response of the cell to osmotic stress and the synthesis of alkaline phosphatase.

### 2. Materials and methods

#### 2.1. Bacterial strain and cultivation

*A. hydrophila* (ATCC 7966) was used throughout this study. It was routinely grown on nutrient agar (Oxoid) plates. High phosphate-grown cells were cultured to stationary phase at 30°C in nutrient broth (Oxoid). Low phosphate-grown cells were cultured to stationary phase in a minimal medium containing 2 mg PO₄ \( \text{L}^{-1} \) [11]. 10 ml of culture were harvested by centrifugation and washed with sterile lake water three times. Cells were resuspended in sterile lake water and used to inoculate microcosms.

#### 2.2. Viable counts

Viable counts were determined using the surface spread plate method on nutrient agar (Oxoid) plates. Serial dilutions were performed in quarter strength Ringers solution. Inoculated plates were incubated for at least 24 h at 30°C and viable counts expressed as colony forming units (cfu) per ml.

#### 2.3. Starvation experiments

Lake water was collected from Tocil Lake (OS ref. 303756), University of Warwick, in sterile containers. 100 ml of lake water were filtered through 0.45 μm membrane filters (Sartorius Ltd), added to a 250 ml Erlenmeyer flask wrapped in aluminium foil to exclude light and autoclaved at 121°C for 15 min. These flasks are referred to as filtered-autoclaved lake water microcosms. Cooled microcosms were inoculated with 100 μl of resuspended bacterial cells. Inoculated microcosms were incubated at 30°C without shaking. Samples were removed aseptically as required. Where appropriate, microcosms were amended aseptically with a filter-sterilised solution of betaine to give a final concentration of 5 mM and an autoclaved solution of sodium chloride to give a maximum final concentration of 7% (w/v).

#### 2.4. Alkaline phosphatase assay

All assays were carried out in 0.1 M Tris buffer, pH 9.0 using *p*-nitrophenyl phosphate (pNPP) as a colorimetric substrate [11]. The reaction mixture contained 2.6 ml sample from the microcosm, 0.3 ml Tris buffer and 0.1 ml 5% (w/v) pNPP. Reactions were carried out at 30°C and stopped by the addition of 0.1 ml 10 M sodium hydroxide. The final absorbance at 420 nm was recorded, corrected for non-enzymatic hydrolysis using a control containing sterile distilled water rather than lake water, and the activity expressed as μmol pNP released ml⁻¹ h⁻¹.

#### 2.5. Measurement of the percentage of cells capable of respiration

Epifluorescence microscopy was used to determine the percentage of the total population capable of respiration [12]. 4 ml samples from the inoculated
microcosms was incubated with 1 ml 0.2\% (w/v) 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT) (Sigma) for 30 min at 30°C. Samples were filtered through 0.2 μm polycarbonate filters (Nucleopore) stained with Sudan Black B. 1 ml of acridine orange was then pipetted onto the filter and removed after 5 min. The dried filter was examined in a Nikon epifluorescence microscope and the numbers of cells and respiring cells recorded using a graticuled eyepiece. 25 areas chosen at random were examined for each filter.

3. Results

Different concentrations of sodium chloride were added to filtered-autoclaved lake water microcosms to investigate the effects of osmotic stress on the viable count and the ability of cells to respire under starvation conditions at 30°C. The total count (recorded during the determination of the numbers of respiring cells in each sample) remained constant in all microcosms throughout the 28 day starvation period at 2.1 (± 0.36) × 10^6 cells ml^-1. The viable count on nutrient agar plates, however, declined in all cases (Table 1). The decrease in viable count was slightly faster in the presence of 0.5\% (w/v) sodium chloride compared with the control which contained no sodium chloride. The decrease in viable count was fastest at 3\%, 5\% and 7\% sodium chloride, with the viable count declining below the limits of detection within 27, 4 and 2 days respectively. A small, but not significant increase in the viable count was recorded in the control microcosm and in those amended with 0.5\% and 1.5\% (w/v) sodium chloride over the first 2 days of incubation. The number of viable cells remaining was proportional to the amount of sodium chloride added (Table 1).

Table 1 also shows the percentage of the total count of *A. hydrophila* capable of respiration. The percentage of cells capable of respiration activity declined, even in controls, over the 28 day starvation period until only 33\% of the total count was capable of respiration. Although the addition of 5\% and 7\% (w/v) sodium chloride to the microcosms reduced the viable count below the limits of detection within 4 and 2 days, respectively, respiration activity was still detectable even after 28 days. Obviously the ability to produce colonies on agar plates has little to do with the ability of cells to respire.

If *A. hydrophila* is grown in a minimal medium containing a limiting amount of inorganic phosphate then a derepressible alkaline phosphatase is synthesised. This alkaline phosphatase is not produced in the presence of high phosphate concentrations. In this respect, the derepression of the enzyme is similar to that reported previously for many other bacterial species [11]. When a microcosm was inoculated with *A. hydrophila* grown in either a high or low phosphate-containing medium, there was a marked increase in alkaline phosphatase activity over a 28 day incubation period in the microcosms amended with sodium chloride and betaine (Table 2). Sodium chloride alone also led to an increase in alkaline phosphatase activity compared to the control but the increase was only significant (Student’s *t*-test *P* < 0.05) in the case of the low phosphate grown cells (Table 2). With both the low phosphate and high phosphate grown cells there was a marked decline in the viable count in the presence of sodium chloride and a less marked decline if betaine was added to the microcosm as an osmoprotectant (Table 2). In the absence of osmoprotectant, the viable count dropped below the limits of detection between 21 and 28 days after inoculation of microcosms. With high and low phosphate grown cells, there was a slight increase in viable count over the first 24 h in the control and in the microcosm amended with betaine and sodium chloride. There was no further increase in viable count after day 7, when the alkaline phosphatase activity increased from 9.0 to 90 μmol pNP released h^-1 ml^-1 in the low phosphate grown cells, and from undetectable to 60 μmol pNP h^-1 ml^-1 released in the high phosphate grown cells in microcosms amended with sodium chloride and betaine. Betaine alone had no effect on alkaline phosphatase activity (data not shown).

4. Discussion

It has been shown previously that *A. hydrophila* produces a derepressible alkaline phosphatase [13] similar in its properties and derepression to the alkaline phosphatase of other bacteria, suggesting that this enzyme is part of the stress response of *A.
Table 1: The viable count and percentage of *Aeromomas hydrophilia* cells showing respiration activity in microcosms amended with different concentrations of sodium chloride. Viable counts are expressed as cfu ml⁻¹. The value for the initial viable count and percent of cells respiring was only determined for the control microcosm. Counts are the means of 4 determinations.

<table>
<thead>
<tr>
<th>Addition to microcosm</th>
<th>Day 0</th>
<th>Day 2</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
</tr>
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<tbody>
<tr>
<td>Viable count respiring</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Count</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.8×10⁶</td>
<td>3.2×10⁶</td>
<td>2.0×10⁶</td>
<td>1.0×10⁶</td>
<td>7.1×10⁵</td>
<td>1.0×10⁵</td>
</tr>
<tr>
<td>1.5% NaCl</td>
<td>3.2×10⁶</td>
<td>2.8×10⁶</td>
<td>2.8×10⁵</td>
<td>2.8×10⁴</td>
<td>5.6×10³</td>
<td>2.8×10³</td>
</tr>
<tr>
<td>2.0% NaCl</td>
<td>2.2×10⁶</td>
<td>1.0×10⁵</td>
<td>&lt;10⁴</td>
<td>&lt;10³</td>
<td>&lt;10⁴</td>
<td>&lt;10³</td>
</tr>
<tr>
<td>2.5% NaCl</td>
<td>2.0×10⁶</td>
<td>1.0×10⁵</td>
<td>&lt;10⁴</td>
<td>&lt;10³</td>
<td>&lt;10⁴</td>
<td>&lt;10³</td>
</tr>
<tr>
<td>5.0% NaCl</td>
<td>2.2×10⁶</td>
<td>1.0×10⁵</td>
<td>&lt;10⁴</td>
<td>&lt;10³</td>
<td>&lt;10⁴</td>
<td>&lt;10³</td>
</tr>
</tbody>
</table>

Viable counts are expressed as cfu ml⁻¹. The value for the initial viable count and percent of cells respiring was only determined for the control microcosm. Counts are the means of 4 determinations.
<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LP grown</td>
<td>HP grown</td>
<td>LP grown</td>
<td>HP grown</td>
<td>LP grown</td>
<td>HP grown</td>
</tr>
<tr>
<td>Control</td>
<td>APase activity</td>
<td>7.3</td>
<td>0</td>
<td>9.5</td>
<td>0</td>
<td>12.5</td>
</tr>
<tr>
<td>Control</td>
<td>Viable Count</td>
<td>$3.2 \times 10^5$</td>
<td>$7.1 \times 10^6$</td>
<td>$1.3 \times 10^6$</td>
<td>$7.9 \times 10^6$</td>
<td>$1.1 \times 10^6$</td>
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<tr>
<td>+3% NaCl</td>
<td>APase activity</td>
<td>10.0</td>
<td>0</td>
<td>13.9</td>
<td>0</td>
<td>19.7</td>
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<tr>
<td>+3% NaCl</td>
<td>Viable Count</td>
<td>$3.2 \times 10^5$</td>
<td>$7.1 \times 10^6$</td>
<td>$3.2 \times 10^5$</td>
<td>$1.6 \times 10^6$</td>
<td>$2.0 \times 10^5$</td>
</tr>
<tr>
<td>+3% NaCl + 5 mM betaine</td>
<td>APase activity</td>
<td>10.0</td>
<td>0</td>
<td>24.2</td>
<td>32.4</td>
<td>35.7</td>
</tr>
<tr>
<td>+3% NaCl + 5 mM betaine</td>
<td>Viable Count</td>
<td>$3.2 \times 10^5$</td>
<td>$7.1 \times 10^6$</td>
<td>$1.6 \times 10^6$</td>
<td>$5.5 \times 10^6$</td>
<td>$1.3 \times 10^6$</td>
</tr>
</tbody>
</table>

Alkaline phosphatase activity is expressed as $\mu$mol pNP ml$^{-1}$ h$^{-1}$. Viable count is expressed as cfu ml$^{-1}$. LP, low phosphate medium. HP, high phosphate medium.
hydrophila to the absence of inorganic phosphate in the environment. Gauthier et al. [1] demonstrated that E. coli cells with high alkaline phosphatase activity survived for significantly longer periods of time than cells with no or little activity, although they also suggested that it was the ability of bacteria with a derepressed alkaline phosphatase to take up extracellular phosphate more rapidly which was responsible for their survival. However, they were unable to show that E. coli subjected to osmotic stress could actually synthesize alkaline phosphatase as a necessary means of prolonging their survival in response to osmotic shock. Here similar conclusions may be drawn for A. hydrophila exposed to osmotic stress. The survival of cells grown in the presence of high phosphate, but initially lacking alkaline phosphatase activity, was better than that of cells grown in a low phosphate environment. This suggests that it is the ability to accumulate inorganic phosphate that is important. The cells grown in a high phosphate environment will have a higher internal pool of inorganic phosphate than those grown under phosphate-limited conditions. However, the latter cells will have the higher alkaline phosphatase activity and will be able to scavenge more efficiently for traces of organic phosphate than cells grown in a high phosphate environment.

Although the viable count over the starvation period declined in all cases, there was a greater decline in the count than there was in the percentage of cells capable of respiring. This is a common phenomenon with starving bacteria, where the ability to produce colonies is lost before a cell loses its ability to respire [9,10,13]. Even in the microcosms amended with high concentrations of sodium chloride, the ability to respire was lost gradually, whereas the ability to produce colonies was rapidly lost.

Betaine, as expected, also increased the ability of cells to withstand osmotic shock with higher viable counts in the microcosms exposed to betaine plus sodium chloride than in those exposed to sodium chloride alone. The novel information here is the dramatic increase in alkaline phosphatase activity of cells grown initially in a high phosphate environment, but then exposed to osmotic shock in the presence of betaine. In comparison to previously reported experiments of osmotic stress, this is the first time that the synthesis of alkaline phosphatase in an essentially starvation medium amended with sodium chloride has been reported. The increase in activity in osmotically stressed cells once again highlights the importance of alkaline phosphatase and other proteins of the pho regulon in the global response of micro-organisms to stress.

References