Influence of Phosphate Starvation on Cultures of
Pseudomonas aeruginosa

CYNTHIA I. HOU, AUDREY F. GRONLUND, AND J. J. R. CAMPBELL

Laboratory of Dairying, University of British Columbia, Vancouver, British Columbia, Canada

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Abstract

HOU, CYNTHIA I. (University of British Columbia, Vancouver, B.C., Canada), AUDREY F. GRONLUND, AND J. J. R. CAMPBELL. Influence of phosphate starvation on cultures of Pseudomonas aeruginosa. J. Bacteriol. 92:851–855. 1966.—The changes occurring in Pseudomonas aeruginosa during phosphate starvation in a phosphate-deficient medium were assessed by measuring alterations in optical density, viable-cell count, chemical composition, and ribosome patterns. After a 24-hr period of starvation, optical density, protein, and deoxyribonucleic acid per milliliter of culture increased, whereas ribonucleic acid decreased. Extensive ribosomal degradation was apparent from sucrose density gradient centrifugation patterns. The induction of an alkaline phosphomonoesterase during phosphate starvation was observed. A linear response of phosphate-starved cells to low levels of phosphate supplied exogenously was evident from optical-density measurements, and a threshold requirement for phosphate analogous to the "energy of maintenance" was not detected.

McGrew and Mallette (11) reported the utilization of small amounts of glucose by Escherichia coli for the maintenance of viability without concurrent growth. A reproducible intercept was evident in a plot of turbidity increment against the amount of glucose supplied in the medium. This finding provided evidence for an "energy of maintenance" not previously detected in microorganisms, and raised the question of whether a similar phenomenon might occur for other nutrients. The growth and survival of E. coli in phosphate-limiting medium was accordingly studied by Mallette, Cowan, and Campbell (12), who were unable to detect a threshold requirement for phosphate analogous to the "energy of maintenance" requirement found for glucose.

In the work presented here, the approach of Mallette et al. (12) was applied to a similar study of Pseudomonas aeruginosa under conditions of phosphate limitation. This study was extended to include an investigation of changes in the chemical composition and ribosomal patterns of the cells after phosphate starvation and after addition of phosphate. The starved cells were also assayed for the presence of a phosphate-repressible phosphomonoesterase, analogous to that reported in other microorganisms and studied in detail in E. coli (3, 6, 15).

Materials and Methods

Growth and phosphate starvation of the organism. P. aeruginosa (ATCC 9027) was grown in Roux flasks in a glucose-ammonium-salts medium of the following composition: 0.02 M NH₄Cl, 0.001 M KCl, 0.05 M tris(hydroxymethyl)aminomethane (Tris)-HCl buffer, 0.005 M KH₂PO₄, 0.015 M Na₂HPO₄, 0.011 M glucose, 0.0016 M MgSO₄·7H₂O. The medium was prepared from separately autoclaved solutions, as described by Mallette et al. (12), and was adjusted to pH 7.4 with HCl before it was autoclaved. The same medium, with the phosphate salts omitted, was used to wash the cells free from exogenous phosphate and as the suspending fluid in starvation experiments, and is referred to as phosphate (Pi)-deficient medium.

In the initial experiments involving the measurement of optical density (OD) of cultures, cells for starvation were harvested aseptically from the complete medium after 8 hr of incubation at 37 C. For the chemical, ribosomal, and enzymatic studies, cells were harvested aseptically after 18 hr at 30 C. In each case, the cells were washed four times with cold sterile Pi-deficient medium and then were suspended in the same medium.

OD of cultures. Cell concentrations were adjusted to give an initial OD at 400 nm of approximately 0.1, when 1.0 ml of suspension was added to 4.8 ml of Pi-deficient medium supplemented, as required, with phosphate solution. The suspensions were shaken at 37 C, and a separate sample was used for each measurement. In some experiments, cells were starved of phosphate by preliminary shaking of the
washed suspensions at 37 C for 21 to 22 hr. The corresponding nonstarved suspensions were stored at 4 to 6 C for the same period of time.

OD measurements were made at 400 mλ with a spectrophotometer (model DU; Beckman Instruments, Inc., Fullerton, Calif.). [An OD of 1.0 is equivalent to approximately 0.4 mg (dry weight) of cells per ml.] Viable-cell counts were determined on cell suspensions diluted in 10^{-4} M phosphate buffer (pH 7.2) and plated as described by McGrew and Mallette (11).

Chemical, ribosomal, and enzymatic studies. Washed-cell suspensions were prepared at 10 times growth concentration; 3 ml of suspensions [ca. 8 mg (dry weight) of cells] was used to inoculate P_i-deficient medium, which established an initial OD at 660 mλ of approximately 0.2. The P_i-deficient cultures were subsequently incubated at 30 C for 24 hr. In some experiments, phosphate, at the concentration used in the complete growth medium (0.01 m), was supplied to the starved cells, and the cultures were incubated an additional 6 hr at 30 C.

OD measurements were recorded at 660 mλ with a spectrophotometer (model B; Beckman Instruments, Inc.). Viable-cell counts were determined on cell suspensions diluted in phosphate buffer by the pour-plate method.

Chemical fractionation of whole cells and analytical methods. Concentrated cell suspensions [1 to 4 mg (dry weight) per ml] were prepared from measured volumes of the cultures and were fractionated by the following modification of the procedure of Hutchinson, Downte, and Muoro (9). A 1-ml amount of 1.4 N cold perchloric acid (PCA) was added to 1 ml of cell suspension; the material was mixed, held in ice for 20 min, and centrifuged at 7,500 x g at 6 C. The supernatant fluid (cold PCA-soluble fraction) was removed; the pellet was suspended in 2 ml of 0.7 N PCA, heated at 90 C for 15 min, cooled, and centri- fuged. The supernatant fluid (hot PCA-soluble fraction) was removed, and the pellet (protein residue) was dissolved in 0.1 N NaOH.

Protein was determined by the method of Lowry et al. (10); deoxyribonucleic acid (DNA), by the Schneider (14) technique; ribonucleic acid (RNA), by a modification of the method of Schneider (14) which involved heating for 45 min in a boiling-water bath for maximal color development; and P_i, by the method of Chen, Toribara, and Warner (2). RNA values were corrected for interference from DNA.

Preparation of cell-free extracts and ribosome patterns. Concentrated cell suspensions [ca. 10 to 12 mg of protein, 17 to 20 mg (dry weight) per ml] of cells were prepared by washing cells twice in 0.85% NaCl (pH 7.4) and by resuspending them in either 0.05 M Tris-HCl buffer (pH 7.4), containing 10^{-4} M MgCl_2 for ribosomal studies, or 0.1 M Tris-HCl buffer (pH 7.4) for enzymatic studies. Deoxyribo- nuclease (0.05 ml (1 mg/ml)) was added to the sus- pensions, and cell-free extracts were prepared with a French pressure cell. Whole cells were removed from the extracts by centrifugation at 5,000 x g for 10 min at 6 C.

Ribosome patterns were obtained by the procedure of Gronlund and Campbell (5).

Determination of alkaline phosphatase activity. The assay used was based on the procedure described by Torriani (15). Reaction mixtures contained the following: 0.5 ml of Tris-HCl buffer (1.0 m, pH 8.0), 0.1 ml of p-nitrophenyl phosphate (2 mg/ml), 0.2 ml of cell-free extract for nonstarved cells or 10 to 25 ml of starved cells, and water to 1.0 ml. Com- ponents exclusive of substrate were brought to 35 C in a 1-ml cuvette and placed in the warm chamber of the Beckman model DU spectrophotometer. The reaction was started by the addition of warmed substrate, and the increase in OD was measured at 400 mλ.

RESULTS AND DISCUSSION

OD of P_i-starved cultures. In the preliminary experiments, the course of starvation in P_i-deficient medium was followed by observing changes in OD and in numbers of viable cells of suspensions shaken for 30 hr (Fig. 1). The viable-cell count rose sharply at first, but with a decreasing rate over the entire period, and reached a plateau at approximately 24 hr. An eightfold increase in viable-cell numbers was observed. Measured over the same period, OD at 400 mλ rose in a linear fashion, also tapered off somewhat between 24 and 30 hr, and reached, at this point, a value more than double the initial level. In a longer experiment, the time required to attain a maximal viable-cell count was confirmed to be approximately 24 hr. The fact that glucose was not limiting in these experiments was indicated by the failure of glucose when added (at 24 hr) to the

![FIG. 1. Increase in viable cells and optical density during phosphate starvation of Pseudomonas aerugi- nosa in shaken cultures.](http://jb.asm.org/)
level of an additional 0.011 m, to increase OD or viable-cell counts.

The obvious ability of the cells to survive and, in fact, proliferate under conditions of phosphate starvation was clearly demonstrated. Similar results were obtained by Mallette et al. (12) and by Horiuchi (8), who found that a marked increase in viable counts of *E. coli* occurred within a few hours of phosphate exhaustion of the medium. The absence of inorganic phosphate from the P_{1}-deficient medium was confirmed by the extrapolation through the origin of a plot of OD (attained during 24 hr of starvation) against the size of inoculum used, a criterion suggested by Mallette et al. (12) (Fig. 2). This was further substantiated by the inability to measure P_{1} present in the medium by the method of Chen et al. (2), which is capable of detecting levels of phosphate as low as 0.025 μmole/ml. Phosphate which permitted the observed increase in numbers of cells must, therefore, have been endogenous in origin.

The response of starved and nonstarved cells to levels of phosphate up to 0.5 μmole/ml was determined by OD measurements of suspensions shaken for 12 hr (a period of incubation found in preliminary experiments to produce maximal response to added phosphate). To establish that the response of the starved cells was not limited by nutrients other than phosphate nor inhibited by metabolic by-products which had accumulated during phosphate starvation, samples of the starved cells were also harvested by centrifugation and suspended to the same volume in fresh P_{1}-deficient medium before use in the response experiments (Fig. 3).

The response obtained was linear at low levels of phosphate with both starved and nonstarved cells. At higher levels of phosphate, however, the response of the starved cells was significantly less than that of the nonstarved cells. Starved cells suspended in fresh phosphate-deficient medium responded to P_{1} addition in the same manner as the starved cells not suspended in fresh medium; this confirmed that the OD responses observed were controlled solely by added P_{1}.

The failure of starved cells to respond to P_{1} addition as fully as nonstarved cells was observed also by Mallette et al. (12) in studies on *E. coli* and was indicated as not resulting from a loss of viability during the period of incubation with exogenous phosphate. These authors suggested that the inability of the starved cells to divide in the glucose-salts medium may have resulted from the loss of one or more components which were necessary for cell division and which were resupplied in the complex plating medium used.

Although, at the higher levels of phosphate, *P. aeruginosa* tended to form clumps and pellets, which gave rise to difficulties in obtaining highly

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![Graph 1](image1.png)

**Fig. 2.** Influence of size of inoculum on optical density of phosphate-starved shaken cultures of *Pseudomonas aeruginosa* after 24 hr of incubation.

![Graph 2](image2.png)

**Fig. 3.** Influence of added phosphate on the response of shaken cultures in phosphate-deficient medium.
accurate OD measurements, the results of the foregoing experiments paralleled those of Mallette et al. (12) with E. coli. In the P₇₁-response experiments, the plots for both starved and nonstarved cells extrapolated through the zero level of phosphate, which indicated that the smallest amount of phosphate supplied exogenously was capable of producing a measurable response, and thus ruled out the existence of a threshold requirement for exogenous phosphate detectable by this method of investigation.

Changes in the chemical composition of phosphate-starved cells. Chemical analyses performed on cells at zero time and after 24 hr of phosphate starvation demonstrated that both protein and DNA increased, and that RNA decreased, on the basis of micrograms per milliliter of culture during the starvation period (Table 1). The RNA values given represent the total RNA in both the hot and cold PCA-soluble fractions. The ratio of RNA to DNA decreased from the zero time value of 3.14 to 1.87 during the starvation period. The overall results are consistent with those of Horiuchi (8), who observed a similar synthesis of protein and DNA and degradation of RNA during phosphate starvation of E. coli, and who also implied that a large portion of the phosphate released from RNA was utilized for the synthesis of DNA.

The addition of phosphate to the cells after 24 hr of starvation resulted in increases in OD, viable-cell count, and dry weight, and in the concentrations of protein, DNA, and RNA per milliliter of culture when measured 6 hr later (Table 2). The percentage increase in RNA over the 24-hr level was particularly high, which suggests a preferential resynthesis of the compound degraded during phosphate starvation. The ratio of RNA to DNA increased from the 24-hr value of 1.87 to 3.57.

Ribosome patterns. Patterns of cell-free extracts of nonstarved, starved, and P₇₁ “refed” cells obtained on ultracentrifugation through sucrose density gradients containing 10⁻⁴ M MgCl₂ are shown in Fig. 4. A marked decrease in 70S ribosomes was evident in the 24-hr starved sample; this illustrates that degradation of ribosomal material occurred during phosphate starvation. These results are consistent with observations of others (13), who have reported ribosomal degradation in microorganisms on the cessation of growth due to various nutritional deficiencies. The addition of phosphate to the starved cultures (“refed” cells) at 24 hr promoted recovery and, in

<table>
<thead>
<tr>
<th>TABLE 1. Changes in cultures of Pseudomonas aeruginosa during phosphate starvation.</th>
<th>Determination*</th>
<th>0 hr (non-starved)</th>
<th>24 hr (P₇₁-starved)</th>
<th>24 hr/0 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD, 660 mµ</td>
<td>0.115</td>
<td>0.155</td>
<td>135</td>
<td></td>
</tr>
<tr>
<td>Viable cells × 10⁻⁸</td>
<td>4.50</td>
<td>4.18</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>Dry weight, µg</td>
<td>29.6</td>
<td>44.4</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>Protein, µg</td>
<td>17.5</td>
<td>22.8</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td>Deoxyribonucleic acid µg</td>
<td>2.04</td>
<td>2.46</td>
<td>121</td>
<td></td>
</tr>
<tr>
<td>Ribonucleic acid, µg</td>
<td>6.4</td>
<td>4.6</td>
<td>72</td>
<td></td>
</tr>
</tbody>
</table>

* Per milliliter of culture.

Changes in phosphate-starved cultures of Pseudomonas aeruginosa after addition of inorganic phosphate

<table>
<thead>
<tr>
<th>Determination*</th>
<th>24 hr (Pi-starved)</th>
<th>30 hr (&quot;refed&quot;, Pi)</th>
<th>30 hr/24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD, 660 mµ</td>
<td>0.155</td>
<td>0.340</td>
<td>219</td>
</tr>
<tr>
<td>Viable cells × 10⁻⁴</td>
<td>4.18</td>
<td>7.68</td>
<td>184</td>
</tr>
<tr>
<td>Dry weight, µg</td>
<td>44.4</td>
<td>182.00</td>
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<tr>
<td>Protein, µg</td>
<td>22.8</td>
<td>75.1</td>
<td>330</td>
</tr>
<tr>
<td>Deoxyribonucleic acid</td>
<td>2.46</td>
<td>6.88</td>
<td>280</td>
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<tr>
<td>Ribonucleic acid, µg</td>
<td>4.6</td>
<td>24.6</td>
<td>535</td>
</tr>
</tbody>
</table>

* Per milliliter of culture.

Fig. 4. Ribosome patterns of cell-free extracts of nonstarved, phosphate-starved, and phosphate "refed" cells. Symbols: O, nonstarved; Δ, starved; □, "refed."
fact, a substantial increase in ribosomal material. These data support the results of the chemical analyses, which showed a disproportionate increase in the RNA content of P. "refed" cultures.

The increase in ribosomal material over the 0-hr value may reflect the fact that the growing "refed" cells were physiologically younger than those used as the inoculum at 0 hr, since a high RNA content is known to be characteristic of log-phase cells (7).

Alkaline phosphatase activity. The presence of an alkaline phosphomonoesterase in cell-free extracts, and preliminary experiments demonstrated that the specific activity of the enzyme increased several hundred fold during a 24-hr period of P. starvation of cultures of the organism (Table 3). A study of some of the characteristics of the enzyme has subsequently been undertaken and will be described in a later report.

The data presented here support the previous evidence of Campbell, Gronlund, and Duncan (1) and of Gronlund and Campbell (4, 5) for the role of ribosomal material as a primary endogenous reserve in P. aeruginosa. These authors have provided evidence for a decrease in ribosomal RNA and protein, and for the release of ammonia and material absorbing at 260 mμ when P. aeruginosa respired endogenously. The presence of constitutive enzymes capable of oxidizing various nucleosides, purines, pyrimidines, and degradation products of these compounds has also been demonstrated (1), and polynucleotide phosphor- ylase has been shown to be the degradative enzyme associated with the ribosomes (4, 5). Ribosomal material would appear to be a logical substrate capable of satisfying both the maintenance energy requirements of the cell and the need for metabolites, such as phosphate, when it is considered that the cessation of growth removes the requirement for a large complement of protein-synthesizing ribosomes.

ACKNOWLEDGMENTS

We are grateful to M. F. Mallette, Pennsylvania State University, for his interest and helpful discussions.

This investigation was supported by a research grant from the National Research Council of Canada.

| Table 3. Alkaline phosphatase activity in cell-free extracts of Pseudomonas aeruginosa |
|--------------------------------------|------------------|
| Cell-free extract                   | Δ OD per min per mg of protein |
| Nonstarved cells                    | 0.003             |
| P. starved cells                    | 1.580             |

LITERATURE CITED


