Influence of Cultural Conditions on Phosphate Accumulation of *Arthrobacter globiformis* PAB-6

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Received August 8, 1979

The influence of cultural conditions was examined on phosphate accumulation of the high phosphate accumulating bacterium, *Arthrobacter globiformis* PAB-6. This bacterium utilized nitrate and nitrite, as well as ammonium salts, as nitrogen source, and polyphosphates, organic phosphates, as well as inorganic phosphate, as phosphorus source. Ferric and calcium ions were found to be essential for high phosphate accumulation. Both growth and phosphate uptake were stimulated by addition of certain amino acids. Phenylalanine had an especially highly stimulatory effect. Optimal temperature and pH for efficient phosphate uptake were 25°C and between 7 and 8, respectively.

In the preceding paper,¹⁾ we described the isolation of several high phosphate accumulating bacteria from nature and one of them was identified as *Arthrobacter globiformis* PAB-6 which accumulated as much as 20% phosphate per dry cell mass. In this paper, the influence of various cultural conditions was studied on growth and phosphate accumulation by the isolate, *A. globiformis* PAB-6 as a basis for application of this bacterium in activated sludge plants.

MATERIALS AND METHODS

Strain. *A. globiformis* PAB-6 described in the preceding paper¹⁾ was used throughout the study.

Growth medium. For cultivation of *A. globiformis* PAB-6, a chemically defined and simplified medium (basal medium) was used in all experiments to study the influence of various factors on bacterial phosphate uptake and growth. It contained (per liter) glucose 2 g, NH₄Cl 1 g, Na₂SO₄ 0.1 g, MgCl₂ 0.01 g, KCl 0.1 g, FeCl₃ 0.001 g, CaCl₂ 0.01 g, PO₄³⁻ 0.1~0.15 g and tris(hydroxymethyl)aminomethane 10 g; pH was adjusted to 7.6 with HCl. Antifoam agent (Toshiba silicone YMA 6509, Tokyo) was added, when necessary.

Analytical methods. Glucose concentration was assayed with Glucose-B Test (Wako Pure Chemical Industries Ltd., Osaka). Other methods were described in the preceding paper.¹⁾

Experimental apparatus. Most cultures were performed in shake flasks (working volume=50~100 ml). A mini-jar fermentor (Iwashiyama Co., Tokyo) (working volume=1 liter) was used when pH had to be precisely controlled. The cultivation temperature was 30°C, unless otherwise mentioned.

Chemicals. Peptone was "polypepton" from Dai-ichi Eiyō Kagaku Co. Meat extract and yeast extract were the products of Wako Chemical Co. and Difco Co., respectively.

RESULTS

Carbon sources

When glucose was used as a carbon source, the ratio of the amount of phosphate consumed by the cell to the amount of glucose consumed was almost constant (120 mg PO₄ consumed/2 g glucose consumed) irrespective of the initial phosphate and glucose concentrations. When arabinose, xylose, saccharose, maltose, glycerol, acetate and citrate were added as a carbon source at 0.2% each, 100 mg PO₄ was consumed by the time when growth was levelled off. The concentration of these carbon sources approximately corresponds to 0.2% glucose on the basis of carbon content. Lactose, sorbitol and mannitol were not metabolized. Though galactose (0.2%) was utilized, phosphate consumption decreased to 30~50 mg.

Nitrogen sources

Ammonium nitrate, ammonium sulfate, ammonium phosphate monobasic and urea were also utilized as nitrogen source and these nitrogen compounds showed no specific influence on growth and phosphate uptake at concentrations of 0.1~0.2%. However, when the concentration of ammonium salts increased to 1.0%, both PO_4 uptake and the final cell mass concentration decreased. But even at this high concentration of ammonium salt, the amount of PO_4 consumed per unit cell mass was almost constant, as illustrated in Fig. 1 for NH_4Cl . Similar patterns were obtained for other ammonium salts. It was found that nitrate and nitrite were also utilized as nitrogen sources by this bacterium, though growth rate and PO_4 consumption rate were somewhat reduced in comparison to when NH_4Cl was used as the nitrogen source (Fig. 2).

Metal ions and other chemicals

Fe^{3+} and Ca^{2+} ions were found to be effective and essential in the process of PO_4 uptake as shown in Fig. 3. Ions, such as Mn^{2+} , Zn^{2+} , Mo^{6+} , BO_3^{3-} had no specific effect on growth and PO_4 uptake of *A. globiformis* PAB-6 in concentrations between 1~10 ppm. How-

FIG. 1. Effect of NH_4Cl Concentration on Growth and Phosphate Uptake by *A. globiformis* PAB-6. Growth and PO_4 uptake were determined in various NH_4Cl concentrations of basal medium. A, final cell mass (OD₆₆₀) when glucose was exhausted; B, amount of inorganic phosphate consumed; C, maximum specific growth rate in logarithmic phase; D, phosphate consumption per unit cell mass.

ever, Cu^{2+} ion inhibited growth at a concentration of 4 ppm, while no such effect was observed at 0.4 ppm (Fig. 3).

The influences of arsenate or arsenite upon phosphate uptake rate were investigated at concentrations of 1 mM and 5 mM of arsenate,

FIG. 2. Utilizability of Nitrate or Nitrite as Nitrogen Source.

Cells were grown in a shaking flask with basal medium containing 0.1% nitrate or nitrite, instead of NH_4Cl .

FIG. 3. Influences of Metal Ions on Growth and Phosphate Uptake.

A, basal medium; B, neither ferric nor calcium ions were added to basal medium; C, 0.4 ppm of copper ions was added to basal medium; D, 4 ppm of copper ions was added.

and at 0.01 mM and 1 mM of arsenite. Arsenate and arsenite are known to be transported into cells via the same transport system as phosphate.²⁾ Arsenite showed almost no inhibitory effect upon growth at these two concentrations, though a lag phase was observed at 1 mM. Arsenate showed an inhibitory effect upon growth of *A. globiformis* PAB-6 at a concentration of 5 mM, while no effect was observed at 1 mM.

The concentration effects of other chemicals, such as NaCl, Na_2SO_4 , MgCl_2 and KCl were also investigated. We could not determine the optimal concentrations necessary for growth or phosphate uptake for these chemicals. Therefore, the medium composition shown in MATERIALS AND METHODS was determined as a chemically defined basal medium for cultivation of our isolate.

Amino acids

Among 19 natural amino acids examined, asparagine, phenylalanine, glutamine, arginine, methionine, tyrosine, alanine and lysine showed a growth-stimulatory effect at 50~100 ppm. Phenylalanine had an especially highly stimulatory effect upon growth and PO_4 uptake rates, as shown in Fig. 4. When 50 ppm of

phenylalanine was added, the specific growth rate almost doubled. Threonine and cysteine inhibited growth. However, the PO_4 uptake per unit cell mass was almost the same as when cells were cultivated in basal medium, as shown in Table I.

TABLE I. EFFECT OF AMINO ACIDS ON GROWTH AND PHOSPHATE UPTAKE

Amino acid	Concentration (ppm)	Approximate doubling time (hr)	PO_4 (ppm)/ OD_{660}^a
Asn	50	2.6	39
Phe	100	1.8	35
Gln	50	2.9	40
Arg	100	2.6	34
Met	100	2.7	40
Tyr	50	2.5	45
Ala	100	2.4	40
Lys	100	2.6	42
Thr	100	4.1	37
Cys	50	5.8	46
No amino acid added		3.5	36

^a Amount of phosphate consumed per unit cell mass (OD_{660}).

FIG. 4. Effect of Phenylalanine on Growth and Phosphate Uptake of *A. globiformis* PAB-6. A, growth; B, phosphate uptake. The amount of phenylalanine added to basal medium was as follows: ○, 0 ppm; ●, 25 ppm; ▲, 50 ppm; ■, 100 ppm; ◇, 200 ppm.

FIG. 5. Influence of Nutrients on Growth and Phosphate Uptake.

A, growth and phosphate uptake with 100 ppm PO₄ and 0.2% polypepton as nutrient; B, growth and phosphate uptake with polypepton added to basal medium. ●, OD₆₆₀; ▲, inorganic phosphate concentration in the medium; △, organic phosphate concentration.

Phosphorus sources

The utilizability was studied of polyphosphates, such as pyrophosphate, triphosphate, and organic phosphates, such as glucose-6-phosphate, β -glycerophosphate, methyl ester of phosphoric acid and ethyl ester of phosphoric acid, as sole phosphorus source. The initial concentrations of these phosphate compounds were fixed to be nearly equal to 100 ppm of inorganic phosphate. The growth and glucose consumption curves in medium containing the above mentioned phosphates as phosphorous source showed similar pattern to when the bacterium was grown in medium with inorganic phosphate as the sole phosphorous source. Therefore, we assumed that this strain of bacterium utilized these phosphates as the sole phosphate source. This was verified when total phosphorus concentration analyzed after conversion of organic phosphates to inorganic phosphates decreased during growth of our isolate.

Organic nutrients

The effect of organic nutrients (namely, polypepton, meat extract and yeast extract at an initial concentration of 2 g/liter) on growth and phosphate uptake was investigated. Figure 5 shows the result for polypepton. As the inorganic phosphate concentration in medium decreased, the organic phosphate concentration increased (left part of Fig. 5). However, when the basal medium was supplemented with polypepton, no release of inorganic phosphate from cells to medium was observed, as shown in the right part of Fig. 5. Similar phenomena were observed when meat extract or yeast extract was used as nutrient.

Temperature

Temperature dependence of growth and phosphate consumption is illustrated in Fig. 6. It is interesting to see that the maximum specific growth rate, μ_{max} remained almost constant at the temperature range 15~37°C. PO_4 uptake per unit cell mass was maximum at 20~25°C. A fairly long lag time was observed when bacterium was grown at 15°C

FIG. 6. Effect of Temperature.

The bacterium was grown in basal medium at various temperatures. ■, maximum specific growth rate, μ_{max} ; Δ, cell yield; ●, phosphate uptake per unit cell mass; ▲, ratio of phosphate consumed to glucose consumed.

presumably because of the temperature shock involved in transfer from 30°C at preculture to 15°C at main culture.

pH

The effect of pH on growth and PO_4 uptake is shown in Fig. 7. Obviously, PO_4 uptake per unit cell mass and cell mass produced per unit of glucose consumed showed an inverse pattern. As seen from Fig. 7, the optimal pH range for efficient PO_4 uptake is between 7 and 8. Maximum specific growth rate has a tendency to decline as pH value increases.

Aeration

When the bacterium was cultivated in the jar fermentor with a dissolved oxygen control unit, growth and PO_4 uptake were not influenced at a dissolved oxygen concentration of about 1 ppm.

DISCUSSION

Our isolate, *A. globiformis* PAB-6 showed high phosphate uptake under various cultural

FIG. 7. Effect of pH.

Basal medium pH was varied and controlled with a pH control unit. Symbols are the same as those shown in Fig. 6.

conditions, and importantly the amount of PO_4 uptake per unit cell mass was found to be independent of growth rate change, even after medium composition changes in carbon source, nitrogen source or after the addition of amino acids (see Table I). This may be because phosphate accumulation in this bacterium was closely associated with cell growth.

One of the difficulties when phosphate is removed by activated sludge is that the cells release phosphate back into the medium, depending on the dissolved oxygen concentration.³⁾ One important feature of our isolate when grown in chemically defined medium is that it neither releases inorganic phosphate nor organic phosphate even after cessation of growth, while other type cultures examined, such as *Pseudomonas ovalis* IAM 1002 excreted phosphate back into the medium.¹⁾ However, in some cases, a release of phosphate from our isolate was found (as shown in Fig. 5) when the basal medium was replaced by organic nutrients, like polypepton, meat extract and yeast extract. Interestingly, the addition of basal medium to organic nutrients could stop the release of phosphate. This indicates that the excretion of phosphate compounds is dependent upon the composition of

the medium. Characterization of the factor(s) involved in phosphate release requires further investigation.

It has long been known that activated sludge has a capacity to remove phosphate from wastewaters to some extent and this phenomena were interpreted from different aspects. All living matters in activated sludge utilize certain amount of phosphate, since many essential cellular components have phosphorus.^{3,4)} On the contrary, another interpretation is that the chemical precipitation induced by cations in activated sludges was dominant in the removal of phosphate.⁵⁾ In our experiments, phosphate removed from medium was found intracellularly, but cations such as Fe^{3+} and Ca^{2+} were biologically important because the isolated bacterium requires these ions for efficient phosphate uptake, as shown in Fig. 3.

As the removal of BOD, nitrogen and phosphate is closely related in activated sludges, the utilizability of nitrogen sources is important. Though various kinds of ammonium salts were metabolized by cells, the cause of the decline in growth with increased ammonium salt concentration remains unknown. The fact that our isolate can also consume nitrate and nitrite gives this strain an additional advantage in applications in activated sludge plants, since both nitrate and nitrite are commonly known as intermediates in the biological nitrification and denitrification processes.^{5,6)}

Experimental results showing that the bacterium can grow and store phosphorous compounds even at low temperature and that the specific growth rate remains almost constant suggest that this bacterium can become dominant in outdoor activated sludge plants, as far as temperature is concerned. The optimal pH value for efficient phosphate uptake was around 7. Since this value is commonly observed in activated sludge plants, this is another advantageous feature of our isolate. The clearly inverse pattern of cell yield and phosphate uptake per unit cell mass, as seen in Fig. 7, suggests that at pH 7~8, the bacterium consumes cellular energy most efficiently for accumulation of phosphate at the expense of

cell growth.

As activated sludge systems generally consist of mixed populations and mixed substrates, studies on the behavior of the bacterium cultivated in mixed nutrients will be needed from biochemical and engineering viewpoints. Presently, the feasibility of utilizing this bacterium on a pilot scale is under investigation in activated sludge plants.

Acknowledgments. This research was supported by a Grant-in-Aid for Special Project Research from the Ministry of Education, Japan. The authors are greatly indebted to Mr. H. Tamatani and Miss A. Nomura for their experimental assistance.

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