Phosphatase Synthesis in a *Citrobacter* sp. Growing in Continuous Culture

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When a *Citrobacter* sp. was grown aerobically at $D = 0.5 \mu_{\max}$ in glycerol-limited chemostat culture, maximum phosphatase specific activity was obtained at 30 °C and pH 7.0. This was a four-fold increase on the maximum activity obtained in batch culture and detailed tests implicated an atypical acid-type phosphatase. The activity was very resistant to cadmium, confirming an earlier postulate that a cadmium-resistant phosphatase was indispensable for the high uptakes of cadmium (as CdHPO_4) obtained with this strain. Cobalt and nickel also had little effect on activity, but zinc was inhibitory at pH 7.0 and copper at pH 5.0 and 7.0. The enzyme was not activated by Mg^{2+} and the cells could be stored in saline for at least 17 d without any loss of phosphatase activity.

INTRODUCTION

A strain of *Citrobacter* isolated from soil heavily polluted with lead (Macaskie & Dean, 1982) grew in cadmium-supplemented minimal medium and accumulated the metal during growth (Macaskie & Dean, 1984a). Washed cells pre-grown in cadmium-free medium showed enhanced metal accumulation upon subsequent exposure in the 'resting' state. This ability, retained when the cells were immobilized, has led to the development of a process for the removal of cadmium (Macaskie & Dean, 1984b; Macaskie *et al.*, 1987a) and other metals (Macaskie & Dean, 1984c, 1985a, b, 1987) from aqueous solutions, with considerable potential for the removal of heavy metals from liquid industrial wastes (Macaskie *et al.*, 1987a; Macaskie & Dean, 1987).

Cadmium uptake is critically dependent on the presence of an organic phosphate (Macaskie & Dean, 1984a, b; Michel *et al.*, 1986) in the metal solution, and since uptake increased linearly with time an enzymic mechanism was implicated. It seemed likely that a surface-located phosphatase, produced during pre-growth, continued to cleave organic phosphate in the resting and whole-cell immobilized states to precipitate the metal with liberated inorganic phosphate at the cell surface. This has been demonstrated with another *Citrobacter* sp. able to accumulate lead as PbHPO_4 (Aickin *et al.*, 1979), but this latter strain accumulated very little cadmium (Macaskie & Dean, 1982, 1984b). Since cadmium is inhibitory to many enzymes (Vallee & Ulmer, 1972) it is likely that the phosphatase of the lead-accumulating strain was inhibited by Cd^{2+} ; it was suggested that the ability of the metal-non-specific *Citrobacter* sp. to accumulate cadmium was due to a reduced susceptibility of its phosphatase to cadmium toxicity (Macaskie & Dean, 1984a). This would also account for the ability of the organism to grow in Cd^{2+} -supplemented media (Macaskie & Dean, 1982, 1984a) by removal of the metal from the cellular micro-environment by precipitation as CdHPO_4 (Macaskie *et al.*, 1987b).

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Previous studies have been performed using cells pre-grown in batch culture, but the large biomass required for an economic industrial process necessitates the use of continuous culture (Tempest, 1984). It has been established that immobilized cells taken from continuous cultures are as effective in cadmium removal as batch-pregrown cells (Michel *et al.*, 1986), and in this investigation we compare the phosphatase titres obtained with both types of culture. The effect of cadmium and other heavy metals on the enzyme activity is also reported and related to the known performance of whole cells in metal accumulation. The involvement of a highly atypical phosphatase is suggested and this conclusion is further substantiated by an investigation into the effects of various compounds reported to activate or inhibit classical phosphatases.

METHODS

Growth of the organism. The *Citrobacter* sp. was grown in carbon-limiting continuous culture in medium having the following composition (g l^{-1}): Tris, 12.0; $(\text{NH}_4)_2\text{SO}_4$, 0.96; glycerol 2-phosphate (disodium salt hydrate, 5.5 H_2O) 0.67; KCl, 0.62; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.063; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.00032, glycerol, 0.6; with the pH adjusted, as necessary, with 2 M-HCl (usually to pH 7 or as otherwise stated in the text). Polypropylene glycol antifoam was incorporated at 1 ml per 10 l medium. The cultures were maintained at 30 °C, except where otherwise specified, in a New Brunswick 'Bioflo' apparatus (agitation 400 r.p.m.; air flow 0.5 l min^{-1}) at dilution rates (D) from 0.048 to 0.40 h^{-1} initially and at $D = 0.5 \mu_{\text{max}}$ (0.25 h^{-1}) in later experiments. The μ_{max} (maximum specific growth rate) was (mean $\pm$ SEM, three experiments) 0.41 ± 0.00 , 0.51 ± 0.01 and 0.53 ± 0.02 at 25, 30 and 35 °C respectively. These growth rates were determined in batch culture using the medium described above except that the glycerol concentration was increased to 1.5 g l^{-1} . Steady states were reached in the chemostats after 24 generations of growth after which the chemostat outflow was collected at 0 °C.

Harvesting and storage of the cells. The cells in the culture outflow were separated by centrifugation (MSE Mistral, 5000 g, 15 min, 0 °C), washed once in isotonic saline (8.5 g NaCl l^{-1}) and stored at 4 °C prior to use. The pellets were resuspended in isotonic saline to 1.2–2.4 mg bacterial dry weight ml^{-1} for assay, usually within 3–5 d of harvest.

Assay of phosphatase activity. Phosphatase activity was estimated by the liberation of *p*-nitrophenol from *p*-nitrophenyl orthophosphate (disodium salt, BDH) as described by Bolton & Dean (1972). Samples (1 ml) of the concentrated bacterial suspension (see above) were diluted to 10 ml in several buffers: 0.2 M-sodium acetate/NaOH, pH 5.0; 0.2 M-MES/NaOH, pH 5.0; 0.2 M-Tris/HCl, pH 7.0 or 8.5; 0.2 M-MOPS/NaOH, pH 7.0; or 0.2 M-Tricine/NaOH, pH 8.5. Magnesium chloride (0.1 ml of 0.15 mM- $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) was added and the mixture was pre-equilibrated at 30 °C for 15 min prior to the initiation of the reaction by the addition of 45.6 mM-*p*-nitrophenyl disodium orthophosphate (2 ml). Timed samples (2 ml) were quenched in 4 ml 0.2 M-NaOH and the liberated *p*-nitrophenol was estimated by measuring A_{405} . Phosphatase specific activity is defined as nmol *p*-nitrophenol liberated min^{-1} (mg bacterial protein) $^{-1}$, with bacterial protein determined by the Lowry method.

To test the effect of a heavy metal, activator or inhibitor, the cells were pre-equilibrated for 1.5 h in the reaction mixture containing the agent under test prior to the addition of *p*-nitrophenyl phosphate. Metal solutions were freshly prepared prior to their use and controls were performed to ensure that no metal precipitation in cell-free media occurred. Where the phosphatase reaction yielded a metal phosphate precipitate the solid material settled out within 1–2 h, and the clear supernatant could then be decanted prior to the estimation of *p*-nitrophenol.

RESULTS AND DISCUSSION

Buffer system for phosphatase assay

The *Citrobacter* sp. was grown in carbon-limited continuous culture at dilution rates in the range 0.048–0.4 h^{-1} (corresponding to 0.09–0.78 μ_{max} at 30 °C), and its phosphatase activity was determined at pH 5.0, pH 7.0 and pH 8.5. Several buffers were used each appropriate over a limited pH range. At pH 5.0 acetate buffer was used but since this was potentially metabolizable the non-metabolizable buffer MES (Good *et al.*, 1966) was also used. The results obtained were very similar and both buffers were used subsequently. MOPS and Tris buffers gave identical results at pH 7.0. Tris was also used at pH 8.5 since other commonly used alkaline-range buffers may inhibit (glycine and ethanolamine) or enhance (ethylaminoethanol and diethanolamine) phosphatase activity (Hausamen *et al.*, 1967; McComb & Bowers, 1972; Mossner *et al.*, 1980). In some later experiments Tricine buffer was substituted for Tris. This did not affect the phosphatase activity.

Phosphatase activity of the cells

Irrespective of the assay pH the phosphatase specific activity increased linearly with the dilution rate at which the cells were grown. The protein content per mg bacterial dry weight was invariant with the dilution rate. For convenience a dilution rate of $0.25\text{--}0.26\text{ h}^{-1}$ ($0.5\ \mu_{\max}$) was chosen for all subsequent experiments. The phosphatase specific activity of cells thus grown (assayed at pH 7.0) was 1349 ± 71.75 units (mean $\pm$ SEM from five chemostats), giving a standard error between chemostats of within 6% of the mean. The phosphatase specific activity of glycerol-batch-grown cells similarly assayed was 326.96 ± 3.43 units (mean $\pm$ SEM; three cultures); chemostat culture thus conferred a fourfold elevation of phosphatase activity as compared to batch culture for cells maintained continuously at $0.5\ \mu_{\max}$; at $0.78\ \mu_{\max}$ this ratio was increased to 5.4-fold.

For each of 10 chemostat cultures held at $D = 0.25\text{--}0.26\text{ h}^{-1}$ the specific activity observed at an assay pH of 7.0 was afforded a value of 100; the specific activities at pH 5.0 and 8.5 relative to this were 100.17 ± 1.37 and 110.0 ± 2.25 respectively (mean $\pm$ SEM; 10 cultures). Thus while the phosphatase activities assayed at acid and neutral pH were identical, the activity at pH 8.5 was elevated by about 10%, in accordance with similar findings using batch-grown cells. These batch culture experiments also established that no well-defined barrier to substrate access exists since disrupting the cells did not increase the activity. For example, the phosphatase specific activity of a saline suspension of cells was 335 units when assayed at pH 8.5 after treatment for 6 min at 0°C in a Mullard-MSE ultrasonic disintegrator compared to 330 units before treatment. Very similar results were also obtained at assay pH values of 5.0 and 7.0, and increasing the time of treatment led to a decrease in activity at all assay pH values.

Phosphatase activity was generally assayed at 30°C , although enzyme activity was temperature-dependent. At all three pH values the activity increased linearly by 45–50% over a 10°C rise in assay temperature from 25 to 35°C . The enzyme was relatively heat stable, retaining 32% of its initial activity of 1349 units at pH 7.0 after storage at 46.5°C for 3 d.

The stability of the phosphatase in whole cells was further demonstrated. Mg^{2+} often confers enzyme stability (Van Belle, 1972) and is commonly included during phosphatase preparation. However, the cells could be stored as concentrated saline suspensions in the absence of added Mg^{2+} for up to 17 d (the longest time tested) without loss of activity. Similarly, although phosphatases are commonly activated by Mg^{2+} (Ahlers, 1974; Linden *et al.*, 1977; Sugiura *et al.*, 1981) the presence of added Mg^{2+} in the reaction mixture was unnecessary for full activity.

Effect of growth conditions on phosphatase activity

The $\mu_{\max}$ values for organisms grown at 30 and 35°C (see Methods) are not significantly different ($P = 0.95$) and hence a dilution rate of 0.26 h^{-1} (i.e. equivalent to $0.5\ \mu_{\max}$) was used at both of these temperatures. However, the growth rate was significantly lower at 25°C ($\mu_{\max} = 0.41\text{ h}^{-1}$) and for the experiments at this temperature the dilution rate was accordingly adjusted to 0.21 h^{-1} . Irrespective of the assay pH, the activity of cells grown at 25°C was reduced by about 20% as compared to cells grown at 30°C .

Although the growth rate was identical at 30 and 35°C , phosphatase activity was approximately sevenfold lower in cells grown at the higher temperature. Furthermore it varied considerably between chemostat cultures: under identical assay conditions the phosphatase specific activity of three cultures grown at 35°C was 238 ± 118 units (mean $\pm$ SEM; range 29–439 units), this variation occurring under all assay pH conditions. The culture with an activity of only 29 units did not wash out of the chemostat, and since phosphatase activity was therefore not growth-limiting it was calculated by comparison with the enzyme activity of cultures at 30°C that phosphatase must have been present to at least a 45-fold excess over growth requirements at the lower temperature. By deduction an excess of inorganic phosphate must have been present in the cultures grown at 25 and 30°C and the role of phosphate was investigated further. Replacing the glycerol 2-phosphate in the carbon-limited medium with inorganic phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 1.52 g l^{-1}) was without effect, at all three assay pH values. However, changing the nutrient status of the medium from carbon-limitation to phosphate-limitation (glycerol 2-phosphate disodium salt hydrate, 0.066 g l^{-1} ; glycerol, 3 g l^{-1}) gave low activities

which increased rapidly as the assay pH was increased above 5.0, to reach a maximum of 340 units at pH 8.0. This is only about 25% of the value obtained in carbon-limitation and again ultrasonication of the cells did not increase the activity. Phosphate-limitation was not used in any other experiments.

The growth pH also affected the phosphatase activity. For cells grown at pH 6.0, 7.0 and 8.0 the phosphatase specific activities (assayed at pH 7.0) were 1170 ± 63 , 1473 ± 34 and 589 ± 14 (mean $\pm$ SEM; six determinations using cells from two chemostats) units respectively; i.e. growth at pH 7.0 significantly elevated the activity ($P = 0.95$) over that obtained at the other two pH values while growth at pH 8.0 reduced it markedly. Similar results were obtained with each type of cell assayed at pH 6.0 and 8.0, and as with previous experiments the protein content per mg dry weight was invariant.

Effect of phosphatase inhibitors

Adding inorganic phosphate to the assay mixture, which commonly causes competitive inhibition of alkaline phosphatases but not acid phosphatases (Fernley, 1971; Bolton & Dean, 1972; Kawabe *et al.*, 1981) had a negligible effect on the phosphatase activity of carbon-limited organisms: more than 95% of the initial activity was retained in the presence of 0.5–10.5 mM- HPO_4^{2-} irrespective of the assay pH (16 determinations, cells from two chemostats).

EDTA is also a potent inhibitor of alkaline phosphatases with, in typical mammalian systems, a 50% loss in activity at only 0.6 mM concentration (Van Belle, 1972). However, the phosphatase activity of the *Citrobacter* sp. at pH 5.0, pH 7.0 and pH 8.5 was unaffected by EDTA (0.15–3.10 mM), in accordance with the finding that exogenous Mg^{2+} is not required for activity (see above).

Other organic compounds also diagnostically inhibit alkaline phosphatases (Butterworth, 1983). These include theophylline (Fawaz & Tejjirian, 1972), imidazole (Brunel & Cathala, 1972) and L-phenylalanine (Ghosh & Fishman, 1966; Byers *et al.*, 1972), the latter exerting its activity in *Escherichia coli* by preventing active dimer formation (Neuman & Lustig, 1980). The phosphatase activity of the *Citrobacter* sp. was insensitive to phenylalanine (0.3–6.0 mM) at all pH values. Imidazole (Fig. 1*a*) and theophylline (Fig. 1*b*) had little effect at pH 7 at the concentrations tested and gave only slight inhibition at pH 8.5, while, surprisingly, a concentration-dependent stimulation of activity (small in the case of imidazole) was observed at pH 5.

These results argue against the participation of an alkaline phosphatase in the enzyme activity observed at pH 8.5 in carbon-limited cells. The broad pH optimum (pH 5.0–8.5), which contrasts with the much sharper peaks in activity observed at acid pH values only with the acid phosphatases of *Klebsiella aerogenes* (*K. pneumoniae*) (Pickett *et al.*, 1976) and of *Bacillus subtilis* 'subsp. niger' and a *Pseudomonas* sp. (Pickett & Dean, 1977), together with the very different pH-activity profile reported above for phosphate-limited organisms (in which alkaline phosphatase synthesis would be derepressed) compared to carbon-limited organisms, supports the conclusion that an atypical acid-type phosphatase operates at all pH values. The action of fluoride provided further evidence.

Inhibition by fluoride is a common property of bacterial acid phosphatases, and the *Citrobacter* enzyme was inhibited by this anion (Fig. 1*a*). At pH 5.0 about 90% of the initial activity was lost at a concentration of only 2.5 mM- F^- , higher concentrations having no further effect (Fig. 1*a*), findings similar to those of Torriani (1960) and Bolton & Dean (1972) with *E. coli* and *K. aerogenes* (*K. pneumoniae*) respectively. The degree of inhibition decreased at pH 7.0 and pH 8.5 (Fig. 1*a*). In contrast, formaldehyde was most inhibitory at pH 7.0 although marked inhibition was also apparent at pH 5.0 and pH 8.5 (Fig. 1*b*). This behaviour contrasts sharply with that of the acid phosphatases of *K. aerogenes* (*K. pneumoniae*) (Bolton & Dean, 1972) and of *B. subtilis* 'subsp. niger' and a *Pseudomonas* sp. (Pickett & Dean, 1977), which were unaffected by formaldehyde (130 mM), further illustrating the atypical nature of the *Citrobacter* enzyme.

Effect of heavy metals on phosphatase activity

Metals are required at very low levels as enzyme activators or as components of metallo-enzymes (Lazdunski *et al.*, 1969; Linden *et al.*, 1977), but they are inhibitory at high concentrations (Vallee & Ulmer, 1972).

Fig. 1. Effect of phosphatase inhibitors on the phosphatase activity of *Citrobacter* sp. The cells were grown at $D = 0.5 \mu_{\max}$ and pre-exposed to the inhibitors for 1.5 h prior to assay for phosphatase activity. (a) Effect of fluoride (filled symbols) and imidazole (open symbols). (b) Effect of formaldehyde (filled symbols, lower scale) and theophylline (open symbols, upper scale). ●, ○, MES buffer (pH 5.0); ▲, △, MOPS buffer (pH 7.0); ■, □, Tricine buffer (pH 8.5). Results are mean values from two chemostats and the control (100%) value was 1349 units.

The sensitivity of the phosphatase of the *Citrobacter* sp. to heavy metals was investigated using MES (pH 5.0) and MOPS (pH 7.0), since these zwitterionic buffers are reported to complex heavy metals minimally (Good *et al.*, 1966), in contrast to acetate (see Nriagu, 1981) and Tris (Brignac & Mo, 1975). However, at pH 8.5 in non-complexing conditions, metal hydroxides would precipitate freely. A compromise was employed by using Tricine buffer which, although complexing with metals, especially copper, does so to a much smaller extent than Tris (Good *et al.*, 1966; Brignac & Mo, 1975).

At pH 5.0 and 8.5 cobalt and nickel had little effect on phosphatase activity, values within $\pm 8\%$ of the control (metal-unsupplemented) level being obtained at eight metal concentrations in the range 0.18–17.0 mM (cells from two chemostats). Both metals were slightly more inhibitory at pH 7.0 but the effect was again concentration-independent. Expressed as a percentage of the control activity (1349 units) the values obtained at pH 7.0 were $87.56\% \pm 2.38$ and $82.31\% \pm 1.89$ (means $\pm$ SEM; 16 determinations) for cobalt and nickel respectively. At pH 8.5 the copper-Tricine coordination complex which formed similarly caused only about 7% inhibition, independently of the copper concentration (Fig. 2a) up to 16 mM, the highest concentration tested. At pH 5.0 marked inhibition occurred (Fig. 2a), activity being reduced to about 30% at 0.8 mM-copper and to only 3% at concentrations above 10 mM. Copper was not very soluble in MOPS at pH 7 but nevertheless, even at the highest concentration possible (0.28 mM), activities of only 11% and 9% of the control values were obtained in two independent experiments.

Zinc is a potent non-competitive inhibitor of mammalian phosphatases (Linden *et al.*, 1977). However, the *Citrobacter* phosphatase was immune to zinc toxicity at pH 8.5, while at pH 5 about 15% of the activity was lost, the effects at both pH values being largely independent of the zinc concentration (Fig. 2a). In contrast, greater inhibition occurred at pH 7.0 (Fig. 2a), where only 62% of the control activity remained at 0.8 mM-zinc; activity decreased linearly with $\log_{10}$ (zinc concentration), to 6.5% of the control value at 3.8 mM-zinc.

As postulated earlier (see Introduction) the phosphatase was very resistant to cadmium. Very similar results were obtained at all three pH values up to about 10 mM-cadmium, where the residual activity was 80% of the metal-free control (Fig. 2b). At higher cadmium concentrations activity declined linearly with $\log_{10}$ (cadmium concentration). The effect of cadmium at

Fig. 2. Effect of heavy metals on the phosphatase activity of *Citrobacter* sp. Conditions were as in Fig. 1. (a) Effect of copper (filled symbols) and zinc (open symbols); (b) effect of cadmium. ●, ○, MES buffer (pH 5.0); ▲, △ MOPS buffer (pH 7.0); ■, □, Tricine buffer (pH 8.5). Results are mean values from two chemostats.

concentrations higher than 56 mM could not be tested at pH 8.5 due to its low solubility at alkaline pH, but up to this concentration the results obtained at pH 5 and 8.5 were very similar (Fig. 2b). The limited tolerance of the enzyme at acid pH corresponded to a cadmium concentration of 50 mM; above this concentration the activity fell linearly with log₁₀(cadmium concentration). However, it was only reduced to 50% of its initial level by 94.4 mM-cadmium (i.e. 10.6 g l⁻¹), while to reduce it to a negligible level required the presence of 290 mM-cadmium in the assay mixture (Fig. 2b). As might be expected for metals within the same group, the pattern of resistance to cadmium and zinc was similar (although the overall resistance to cadmium was greater): compare Figs 2(a) and 2(b).

The possibility that the high concentrations of cadmium employed (up to 290 mM) had an osmotic effect on the cells was discounted by the incorporation of an ionically equivalent amount of Na₂SO₄ into the assay buffers (cadmium-free). Under these conditions the phosphatase activity was unaffected and the inhibition reported above was concluded to be metal-mediated.

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