The effect of heavy metals and other environmental conditions on the anaerobic phosphate metabolism of *Acinetobacter johnsonii*

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A strain of *Acinetobacter* with potential for bioremediation of heavy metal-contaminated waters was isolated from a wastewater-treatment plant operating an enhanced biological phosphate removal process. NMR and extractive methods showed that polyphosphate accumulated aerobically was degraded under anaerobic conditions both in the presence and absence of cadmium or uranium (0.2–0.5 mM). NMR showed that free phosphate was formed at the expense of polyphosphate, and an extractive technique indicated that this reaction could be stimulated by the presence of UO_2^{2+} under these conditions. Energy-dispersive X-ray microanalysis demonstrated that only cadmium could enter the cells, and co-localized with intra-cellular granules containing phosphate and other divalent metals. The effects of other environmental parameters on the anaerobic phosphate metabolism were also investigated. Between pH 5.5 and 8.0, phosphate release increased with increasing pH. Between 4 °C and 37 °C, phosphate release increased with increasing temperature. The presence of nitrate at concentrations of 10 mM and above inhibited anoxic phosphate release, but supplying tungstate in the growth medium prior to anoxic incubation reduced the production of active nitrate reductase and alleviated this effect.

Keywords: *Acinetobacter* sp., polyphosphate, heavy metal, toxicity

INTRODUCTION

Acinetobacter spp. have been implicated in enhanced biological phosphate removal (EBPR) in wastewater-treatment plants operating these processes. The basis for this is their isolation from such plants (Fuhs & Chen, 1975; Barnard, 1976; Beacham, 1992) and the demonstration of the ability of certain strains to take up, store, then release inorganic phosphate (Pi) under the appropriate conditions (van Groenestijn *et al.*, 1985, 1989). Recent studies (Wagner *et al.*, 1994a, b; Bond *et*

al., 1995; Christensson *et al.*, 1998) have demonstrated that *Acinetobacter* spp. are not necessarily the principal organisms effecting EBPR and that members of the *Rhodocyclus* group may have a greater role in the process than previously thought. However, *Acinetobacter* spp. formed a significant proportion of polyphosphate (polyP)-accumulating organisms isolated from activated sludge in our own studies (C. D. Boswell & R. E. Dick, unpublished). The isolate used in our studies represents *Acinetobacter* spp. from activated sludge capable of phosphate cycling and which may effect EBPR in at least some wastewater-treatment processes.

Aerobically, some *Acinetobacter* spp. can accumulate phosphate as polyP (Kornberg, 1995). Subsequent exposure to anaerobic conditions promotes polyP degradation with concomitant release of phosphate into the medium. This release has been coupled to bio-precipitation of heavy metals as cell-bound metal phosphates (Boswell *et al.*, 1998) and could provide a novel

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Abbreviations: EBPR, enhanced biological phosphate removal; EDAX, energy-dispersive X-ray micro-analysis; MSM, minimal salts medium; Pi, inorganic phosphate; polyP, polyphosphate; TEM, transmission electron microscopy.

approach to the co-treatment of phosphate- and heavy metal-contaminated wastewaters where the concentration of metal and phosphate is too low to exceed the solubility product of the metal phosphate(s) in the bulk solution. Here, localized phosphate release in close proximity to nucleation sites on the cells promotes the bio-crystallization of metals as MHPO_4 (M, metal cation; Dick *et al.*, 1995).

In addition to the toxicity of heavy metals, other co-contaminants and various environmental factors may affect cellular metabolism and polyP degradation/Pi release. This study aims to identify some parameters which may be critical both to EBPR processes and to *Acinetobacter*-based bioremediation systems.

Evaluation of polyP metabolism is difficult in the presence of heavy metals: precipitation of Pi as metal phosphate prevents direct measurement of phosphate release. Extraction and quantification of residual polyPs was used, therefore, as an evaluation criterion in conjunction with ^{31}P -NMR to investigate the effect of Cd^{2+} and UO_2^{2+} on anaerobic phosphate metabolism. The cations were used at concentrations in the range which had previously been found to exhibit toxic effects on this organism (Boswell *et al.*, 1998).

METHODS

Organism, growth conditions and preparation of cells. A strain of *Acinetobacter johnsonii*, designated 'M45' was isolated on minimal salts agar (see below) from a wastewater-treatment plant (Severn Trent pilot wastewater-treatment plant, Milcote, Warwickshire, UK) operating an EBPR process. The isolate was selected for its polyP-accumulating properties on the basis of a positive reaction to the Neisser stain (Gurr, 1963) and was identified using the Analytical Profile Index for non-enteric bacteria (API NE) kit (bio-Mérieux), negative Gram reaction and non-motile, coccoid morphology (Towner *et al.*, 1991). The strain was maintained on agar plates of minimal salts medium (below) solidified with 1.5% (w/v) granulated agar (Becton Dickinson). Plates were stored at 4 °C and subcultured every two weeks.

Aerobic batch cultures (170 r.p.m.) and phosphate-uptake tests used a defined minimal salts medium (MSM) in 20 mM MOPS/NaOH (pH 7.0; 50 ml) containing the following components per litre: sodium acetate (tri-hydrate), 20 g; $(\text{NH}_4)_2\text{SO}_4$, 1.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.16 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.08 g; KCl, 0.6 g; K_2HPO_4 , 0.65 g; EDTA (disodium salt), 0.25 mg; trace elements solution, 1.0 ml (containing per litre: $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 3 mg; H_3BO_3 , 30 mg; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 20 mg; $\text{CuCl}_2 \cdot 6\text{H}_2\text{O}$, 1.0 µg; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 2 mg; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 3 mg).

Exposure of growing cells to heavy metals. Cells were grown to exponential phase (OD_{600} 0.3) as above. At this point a concentrated (10 mM), filter-sterilized stock solution of cadmium nitrate or uranyl nitrate was added as required to a final concentration of 0.1 mM. Cultures and metal-unsupplemented controls were incubated for a further 2.5 h then sampled (1.5 ml) for electron microscopy and energy-dispersive X-ray micro-analysis (EDAX).

Anaerobic phosphate-release experiments. Cells were pre-grown aerobically (50 ml, 16 h, OD_{600} 1.5, 30 °C, 180 r.p.m.)

and stored on ice. Cells were harvested by centrifugation, washed with ice-cold 0.85% (w/v) NaCl and resuspended in the NaCl to 10% of the final working volume of the experiments. Aliquots of the concentrated cells (1.0 ml each) were placed in 10 ml glass anaerobic bottles and 9.0 ml 20 mM MOPS/NaOH, pH 7.0 (except where otherwise stated; see below) was added. Vials were sealed with butyl rubber caps and gassed with oxygen-free nitrogen (10 min) via a hypodermic needle. The needle was used to withdraw samples and the removed volume was simultaneously replaced with oxygen-free nitrogen via a second needle. Supernatants for analyses were produced by centrifugation (12000 r.p.m., 4 °C, MSE Micro Centaur) of culture samples. Cell pellets were stored at -20 °C prior to polyP analysis.

Detection of Pi and polyP using ^{31}P -NMR. Cells were exposed to cadmium and uranium under anaerobic conditions by the addition of filter-sterilized stock solutions of either uranyl nitrate or cadmium nitrate to 0.2 mM as appropriate immediately prior to anaerobic incubation as described above. For ^{31}P -NMR spectroscopy, cells were harvested at the times shown in Fig. 1. Cell pellets or concentrates (e.g. OD_{600} 2.09; 292 µg protein ml⁻¹) were kept on ice and analysed within 10 min of harvesting. NMR spectra were acquired on a Bruker AMX-400 spectrometer at a frequency of 161.98 MHz. The sweep width was 20 kHz and spectra were acquired with a 4.5 µs pulse, 1.0 s acquisition time and 31.3 µs recycle delay. The standard was 85% (v/v) H_3PO_4 and D_2O was used as the field frequency lock via a capillary insert. In the case of uranyl, residual metal in the supernatant was analysed using arsenazo III (Tolley, 1993; Dick *et al.*, 1995).

Extraction, purification and quantification of polyP fractions. The method of Clark *et al.* (1986) was used for polyP extraction and analysis, and was modified according to the method of Bayly *et al.* (1991), in which separation of polyPs from nucleic acids was not undertaken. To inhibit phosphatases, 100 mM NaF was added after each washing step to give a final concentration of 1.0 mM (Rao *et al.*, 1985) and samples were kept on ice between steps. The procedure, described below, produced three fractions: 1, considered to contain short-chain or surface (periplasmic) polyPs; 2, considered to contain long-chain soluble polyPs, and 3, considered to contain long-chain granular polyPs as demonstrated previously by polyacrylamide gel electrophoresis (Clark *et al.*, 1986). Cells were harvested by centrifugation (12000 r.p.m., 4 °C, MSE Micro Centaur) in pre-weighed 1.5 ml micro-centrifuge tubes, washed with 0.85% (w/v) sodium chloride and combined as appropriate to give pellets of 0.1 g wet weight of cells. Pellets were washed in 0.3 ml ice cold trichloroacetic acid (TCA: 2%, w/v, aq.). The retained supernatant was denoted 'Fraction 1'. The pellet was washed in 0.2 ml ice cold 0.7% (w/v, aq.) TCA:67% (v/v, aq.) acetone and the supernatant combined with Fraction 1. The cell pellet was then washed in 0.25 ml ice cold 67% (v/v, aq.) acetone then 0.25 ml 2 mM EDTA (sodium salt). The cell suspension in EDTA was neutralized to pH 7–8 by the addition of 0.2 M LiOH. Cells were harvested and the retained supernatant was denoted 'Fraction 2'. The residual pellet was washed in 0.25 ml 2 mM EDTA, neutralized to pH 7–8 with LiOH as before, and an equal volume (approx. 0.2 ml) of ice cold 1:1 (v/v) phenol/chloroform was added, mixed thoroughly, then centrifuged for 10 min (12000 r.p.m., 4 °C, MSE Micro Centaur). The aqueous layer was removed and denoted 'Fraction 3'.

PolyP was purified from each fraction by the addition of an equal volume of ice cold phenol/chloroform/isoamyl alcohol

(25:24:1, saturated with 0.1 M ammonium acetate, pH 6.5), centrifugation as above and retention of the aqueous phase. This was followed by three successive extractions using ice-cold chloroform. The aqueous fraction was retained for polyP determination. Each of the polyP-containing fractions was hydrolysed separately by boiling (15 min) in an equal volume of 2 M HCl. Samples were diluted as appropriate with distilled water and the *ortho*-phosphate content determined using the colorimetric (molybdenum blue) method modified from the method of Pierpoint (1957) (see below).

Electron microscopy and EDAX analysis. Samples (1 ml) were centrifuged (12000 r.p.m., Heraeus Sepatech Biofuge A, 10 min, ambient temperature), fixed by resuspension in 2.5 % v/v aqueous glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2, 1 h), recentrifuged and resuspended in 0.85 % (w/v) NaCl. Fixed samples were stored at 4 °C until use, centrifuged and resuspended in 1 % w/v osmium tetroxide in 0.1 M phosphate buffer (pH 7.2, 1 h), dehydrated progressively in an ethanol/water series (v/v: 70, 90, 100 and 100 %) and dried with 100 % ethanol (15 min each step). Two 15 min washes in propylene preceded embedding in epoxy resin under vacuum (20 min). The resin was then left to polymerize (24 h, 60 °C). For EDAX analysis, sections (100–150 nm) were cut using a Reichart-Jung Ultracut E microtome and placed onto a carbon-coated copper grid. Sections were viewed using a JEOL 120CX2 transmission electron microscope (accelerating voltage 100 kV) fitted with a Link ISI EDAX system (JEOL). The cells were probed in intracellular regions corresponding to electron-opaque areas, non-electron-opaque areas, cell-wall regions and also in background areas in the intercellular resin. The limit of resolution of the EDAX microprobe was approximately 0.1 µm. For routine transmission electron microscopy (TEM), 70 nm sections cut from the same block were viewed using a JEOL Ex transmission electron microscope (accelerating voltage 80 kV).

The effect of pH, temperature and nitrate on anaerobic phosphate release. The following buffers were used (20 mM): MES/NaOH (pH 5.5, 6.0), PIPES/NaOH (pH 6.5), MOPS/NaOH (pH 7.0, 7.5), HEPES/NaOH (pH 8.0). Experiments to test the effect of temperature and nitrate (10 and 100 mM sodium nitrate) were in a background of 20 mM MOPS/NaOH, pH 7.0; procedures were as above.

Preparation of nitrate reductase-deficient cells. Cells deficient in nitrate reduction were prepared by growth with tungstate, an analogue of molybdate which is required for the function of nitrate reductase (Stewart, 1988). Cells were incubated as described previously for aerobic phosphate uptake in MSM without molybdate but with filter-sterilized sodium tungstate (5 mM). Controls were grown in tungstate-unsupplemented MSM as described above. Tungstate-treated and control cells were harvested and incubated anoxically at 30 °C as described above, with and without 10 mM sodium nitrate. Samples were taken periodically for phosphate and nitrite analysis of supernatants. Residual nitrate reductase activity was quantified by analysis of nitrite formation.

Phosphate and nitrite analysis. Pi in culture supernatants was measured using the method of Pierpoint (1957), modified for use with 3 ml micro-cuvettes (Sarstedt). Test or standard solution (0.3 ml) distilled, deionized water (1.0 ml) 7.5 % (w/v aq.) sodium molybdate mixed in a 2:1 (v/v) ratio with 2.5 M sulphuric acid (0.6 ml) 60 % (w/v) SnCl₂ in concentrated (11.8 M) HCl, diluted 0.25 % (v/v) in 1 M HCl immediately prior to use (0.4 ml) were mixed thoroughly and the A₇₂₀ determined.

For determination of nitrite in culture supernatants, a Griess (sulfanilamide/naphthylenediamine dihydrochloride) reaction (Tomsett & Garrett, 1980; modified for micro-cuvettes) was used: 25 µl sample or standard solution was added to 1.85 ml 1 % (w/v) sulphanilamide (Sigma) in 1.0 M HCl, 0.5 ml 0.01 % (w/v) naphthylenediamine dihydrochloride (Sigma) was added and the contents of the cuvette mixed well. After incubation (30 min, 15–20 °C), the A₅₄₀ was measured and compared to freshly prepared standards (0.1–5.0 mM sodium nitrite) in the appropriate medium.

RESULTS

Mobilization of polyP in the presence of Cd²⁺ and UO₂²⁺

PolyPs can be distinguished from Pi *in vivo* using ³¹P-NMR due to the difference in the molecular environment of the ³¹P atom in the two types of molecule (Suresh *et al.*, 1986; Keasling & Hupf, 1996). As described previously (Suresh *et al.*, 1986), the polyP and Pi resonances were detected between –23 and –22 p.p.m., and between 2 and 3 p.p.m., respectively (Fig. 1a). Although at a relatively low signal-to-noise ratio, the disappearance of polyP from intact cells and the appearance of Pi was observed (Fig. 1b). A similar result was obtained in the presence of UO₂²⁺ (Fig. 1c) and Cd²⁺ (Fig. 1d). In control cells (Fig. 1b), polyP was still detectable after 1–2 h but following incubation with metals (Fig. 1c, d) polyP was not detectable after 1 h, suggesting possible metal-promotion of polyP mobilization in the presence of metals. Variation in the linewidth and intensity of the polyP signal in the presence of UO₂²⁺ was observed; the paramagnetic uranium nucleus is known to quench the NMR signal (Tjissen & van Steveninck, 1984), making quantitative analysis difficult.

Pi release was therefore also estimated in the absence and presence of UO₂²⁺. In the absence of UO₂²⁺, after 1 and 2 h incubation, the amount of Pi release was 0.104 (±0.013) and 0.253 (±0.013) mmol l⁻¹ respectively. After 1 and 2 h incubation in the presence of 0.2 mM UO₂²⁺, Pi release was 0.070 (±0.01) and 0.121 (±0.006) mmol l⁻¹ respectively. Although the total Pi released was less in the presence of UO₂²⁺, assuming a molar ratio of U:P of 1:1 in the precipitated H₂UO₂PO₄ (Dick *et al.*, 1995), the accountable free Pi recovered in the supernatant after 1 h (0.070 mM), together with additional Pi accountable with the corresponding loss of UO₂²⁺ (0.18 mM) made a total Pi release of 0.25 mM, i.e. the rate of polyP mobilization was approximately doubled in the presence of 0.2 mM UO₂²⁺. By 2 h all of the uranyl ion was removed from solution.

Extractive method for the determination of anaerobic polyP degradation in the presence of heavy metals

³¹P-NMR spectroscopy is a useful technique for the study of phosphate species *in vivo*, but it is relatively insensitive and does not provide information on all polyP species present (Suresh *et al.*, 1986). The extractive

Fig. 1. ^{31}P -NMR analysis. (a) Cell-free extract of polyP from *A. johnsonii* M45 prepared using the method of Clark *et al.* (1986) (top) and polyP in intact cells of *A. johnsonii* M45 (bottom). Intact cells were incubated anaerobically at 30 °C in (b) buffer (20 mM MOPS/NaOH, pH 7.0) only; (c) buffer with 0.2 mM UO_2^{2+} ; (d) buffer with 0.2 mM Cd^{2+} . The times indicate the incubation period prior to sampling.

method for measurement of cellular polyP (Fig. 2) showed that phosphate was present in each of the three fractions and that degradation of polyPs occurred during 2 h anaerobic incubation. Disappearance of phosphate from Fraction 1 (short-chain, periplasmic polyP) both in the presence and absence of UO_2^{2+} or Cd^{2+} was apparent. In both cases, less phosphate remained in Fraction 1 following incubation at the higher metal concentration (0.5 mM) compared with the lower (0.2 mM). Degradation of Fraction 1 was approximately 75 % greater at 0.5 mM UO_2^{2+} than at 0.2 mM. In the case of Cd^{2+} this increase was approximately 35 %. A decrease in the phosphate content of Fraction 2 (long-chain, soluble polyP) over 2 h was observed for control cells and was enhanced in cells exposed to 0.2 mM uranium, but there was no significant decrease in the phosphate content of Fraction 2 for cells exposed to cadmium at either concentration. Degradation of long-chain, granular polyP (Fraction 3) was only greater than the control in cells exposed to 0.5 mM uranium (significant at $P = 0.95$).

The increased hydrolysis of Fractions 1 and 3 in cells exposed to 0.5 mM UO_2^{2+} compared with the control was reflected in the greater overall degradation of polyP, whereas the inhibition of total polyP degradation by cadmium was observed (Fig. 2a). The total polyP analysis is shown in Table 1

Table 1. Contribution of each of three fractions to the total polyP content of *A. johnsonii* M45

Fractions were extracted and analysed according to the method of Clark *et al.* (1986) (see text). Percentages are shown as phosphate content $\pm$ SEM and are from three experiments.

PolyP fraction	Percentage of total polyP
1 (Short chain, surface)	9.89 ± 2.19
2 (Long chain, soluble)	52.53 ± 3.07
3 (Long chain, granular)	37.58 ± 4.02

Fig. 2. Extraction and quantification of polyP in three fractions from *A. johnsonii* M45 immediately prior to (white) and following (black) 2 h anaerobic incubation (see Methods). (a) Total polyP. (b) Fraction 1, short-chain or surface (periplasmic) polyPs; (c) Fraction 2, long-chain soluble polyPs; (d) Fraction 3, long-chain granular polyPs. Error bars indicate SEM from three experiments. Data are expressed as percentage of the control value in each case.

Electron microscopy and EDAX of heavy metal-exposed cells

The growth of exponential-phase cells was inhibited by exposure to either 0.1 mM Cd^{2+} or to 0.1 mM UO_2^{2+} (Fig. 3a). Electron microscopy of cadmium-supplemented cells indicated the presence of intracellular, electron-opaque granules (Fig. 3c, arrows), with cadmium detected in the electron-opaque granules (EDAX data not shown) but not in the cell wall or the cytoplasm. In addition to cadmium, phosphorus, magnesium and calcium were detected in the granules (the copper signal was from the grid). Uranium was not detected in cell walls or within UO_2^{2+} -exposed cells. Although electron-opaque granules were less apparent in uranium-challenged cells (Fig. 3), the cells, overall, contained intracellular phosphate. This confirmed that intracellular phosphorus is detectable within the limit of sensitivity of the EDAX technique, hence confirming the lack of intracellular uranium; other metals were clearly detectable at these concentrations (above).

The effect of pH, temperature and nitrate on anaerobic phosphate release

A positive relationship between anaerobic Pi release and both increasing pH (Fig. 4a) and increasing temperature (Fig. 4b) was observed. The presence of nitrate (Fig. 4c)

had an inhibitory effect on anaerobic phosphate release measured at concentrations of 10 mM and above.

The effect of nitrate on anoxic phosphate release by nitrate reductase-deficient cells

PolyP mobilization is triggered by a shift to anaerobiosis; provision of nitrate as an alternative electron acceptor to O_2 could inhibit this process. Using the nitrite assay described above, *A. johnsonii* M45 was found to convert nitrate to nitrite under anoxic conditions when an electron donor was provided. Further reduction of nitrite to gaseous N_2 was not detected. Attempts to produce useful nitrate reductase-deficient mutants were unsuccessful. Nitrate reductase-negative mutants were generated by selection techniques based on chlorate resistance (Tomsett & Garrett, 1980; Pascal *et al.*, 1982) but none of these could accumulate phosphate to high levels. Instead, formation of functional nitrate reductase was prevented by out-competition of molybdate with tungstate. For cells which were not grown with tungstate which were subsequently incubated anoxically with 1.0 mM KNO_3 , the supernatant concentration of nitrite at 0, 2 and 4 h was 0, 0.320 (± 0.025) and 0.658 (± 0.035) mmol l^{-1} , respectively. For cells grown with 5.0 mM sodium tungstate, the corresponding concentrations of nitrite (0, 2 and 4 h)

Fig. 3. (a) The effect of 0.1 mM Cd²⁺ (Δ) and 0.1 mM UO₂²⁺ ($\circ$) on the growth of *A. johnsonii* M45 (control, $\blacklozenge$, no added metal). Metals were added at the time indicated (OD₆₀₀ 0.3). Example fields from TEM are: (b) *A. johnsonii* incubated without heavy metal; (c) following 2.5 h incubation with 0.1 mM Cd²⁺; (d) following 2.5 h incubation with 0.1 mM UO₂²⁺. Arrows in (c) indicate electron-opaque granules. Bar, 500 nm.

were 0, 0.128 (± 0.035) and 0.282 (± 0.042) mmol l⁻¹, respectively. Thus, compared with control (tungstate unsupplemented) cells, an (approx.) 50% reduction in the production of nitrite was observed in tungstate-grown cells.

In separate experiments, for control cells, incubation in the presence of 10 mM nitrate caused inhibition (approx. 50%) of Pi release after 2 h anaerobic incubation and approximately 75% after 4 h (Fig. 5). Pi release by cells grown with tungstate was similar to that of the control cells in the absence of nitrate. Inhibition by nitrate of Pi release by these cells was not apparent after 2 h, although approximately 50% inhibition of Pi release was observed after 4 h.

DISCUSSION

According to the work of Suresh *et al.* (1986), only surface polyPs are accessible to ³¹P-NMR. Following disappearance of the polyP signal in intact cells (as a result of polyP degradation), the signal could be restored by preparing cell-free extracts. In the present study, the NMR analysis (Fig. 1) should be compared with Fraction 1 obtained by the extractive method (Fig. 2). Both methods indicate that surface polyP is degraded both in the presence and absence of heavy metals. This is consistent with a study using flow cytometry and a membrane-impermeable nucleic acid stain, which

showed that although the growth of *Acinetobacter* sp. exposed to submillimolar concentrations of Cd²⁺ or UO₂²⁺ for periods exceeding 2 h was inhibited, most cells remained intact (Boswell *et al.*, 1998). The results of the methods for detection of polyP differ, however, in that NMR indicates that the rate of surface-polyP degradation is greater in the presence of 0.2 mM UO₂²⁺ than in the control (Fig. 1b, c) in accordance with assay of phosphate released (see above). From data obtained by the extractive method, the extent of surface-polyP degradation in the control was apparently greater than in the presence of 0.2 mM UO₂²⁺, but 0.5 mM UO₂²⁺ promoted this degradation (Fraction 1, Fig. 2a, b). Accurate evaluation of the extent of polyP degradation by ³¹P-NMR in the presence of UO₂²⁺ is difficult to assess because UO₂²⁺ quenches the NMR signal and has been used specifically for this purpose (Tijssen & van Steveninck, 1984). The low sensitivity of ³¹P-NMR also makes accurate quantification difficult, but no residual polyP was detected after 1 h in uranium-treated cells (residual polyP was detected in control cells, Fig. 1b). The ³¹P-NMR data, taken together with the analysis of Fraction 1 from 0.5 mM UO₂²⁺-challenged cells (Fig. 2b) and direct assay of Pi suggest that UO₂²⁺ promotes the breakdown of surface polyP, as suggested previously in the case of Cd²⁺ (Suresh *et al.*, 1986). In the present study, the evidence for such Cd²⁺ stimulation of surface-polyP breakdown is not clear, as degradation is within

Fig. 4. The effects of (a) pH, (b) temperature and (c) nitrate on anaerobic phosphate release by *A. johnsonii* M45. Error bars indicate SE (from three experiments). Cells were pre-grown aerobically (see Methods).

the limits of error of the control in the presence of both 0.2 mM and 0.5 mM Cd^{2+} ($P = 0.95$).

A decrease in the chain length of longer polyP molecules (Fractions 2 and 3 in Fig. 2c, d), resulting in the apparent transition of polyP between different pools, has been proposed by Clark *et al.* (1986) who found that the polyP molecules recovered during stationary phase in *Propionibacterium shermanii* were significantly shorter than those isolated from exponential-phase cells. It is possible that under conditions of polyP degradation (for example in *Acinetobacter* spp. during anaerobiosis) the progressive shortening of polyP due to enzymic hydrolysis results in an apparent flux from the pool of long-chain polyP to the short-chain pool.

The apparent difference between degradation of short-chain polyP detected by the extractive method and by ^{31}P -NMR may be due to the intracellular formation of short-chain polyP (through shortening of long-chain polyP) which would be detectable by the extractive method but not by ^{31}P -NMR. Thus the apparent inhibition of degradation of short-chain polyP in the presence of 0.2 mM UO_2^{2+} (Fig. 2a, b) could result from increased flux into short-chain polyP from the long-chain soluble pool during the incubation period. This would be consistent with the observed inhibition by

Fig. 5. The effect of aerobic pre-growth in the presence of 5.0 mM tungstate on inhibition of phosphate release by nitrate during subsequent anoxic incubation. □, no tungstate present during growth, anoxic incubation without NO_3^- ; ■, no tungstate present during growth, anoxic incubation with 10 mM NO_3^- ; ○, 5 mM tungstate present during growth, anoxic incubation without nitrate; ●, 5 mM tungstate present during growth, anoxic incubation with 10 mM NO_3^- . 100% Pi release corresponds to control cells pre-incubated without tungstate and incubated anaerobically for 2 h. Where they are not visible, error bars (± 1 SEM) are within the dimensions of the symbols.

cadmium of the mobilization of long-chain, soluble polyP (Fraction 2, Fig. 2c, d) which may be due to its inhibitory effect on the polyP-degrading enzyme(s), particularly as cadmium (but not uranium) was found to co-localize with (poly)phosphate.

Calculation of the total polyP content of cells prior to and following anaerobic incubation (Fig. 2a) showed that, overall, polyP degradation varied according to the treatment. In the case of 0.5 mM UO_2^{2+} there was a significant stimulatory effect on polyP degradation ($P = 0.95$), whereas the presence of Cd^{2+} (0.2 mM) was inhibitory.

EDAX analysis of sections of cadmium- or uranyl-challenged cells (not shown) indicated that only the former can enter the cell. This might be expected given that the entry of Cd^{2+} into bacterial cells via either the Mn^{2+} (Laddaga *et al.*, 1985) or Zn^{2+} (Laddaga & Silver, 1985) transport systems has been shown. EDAX also demonstrated that, in addition to cadmium, the electron-opaque granules contained phosphorus and divalent cations, consistent with polyP granules described in other studies (Jones & Chambers, 1975).

Aiking *et al.* (1984) found that whilst cells of *Klebsiella aerogenes* exposed to cadmium accumulated greater amounts of phosphate, they did not contain polyP granules which were present in cells not exposed to the heavy metal. A detoxification mechanism whereby cadmium precipitates with phosphate released from polyP was proposed to account for the greater sensitivity of phosphate-limited cells which did not accumulate polyP. This mechanism is consistent with the work of Keasling & Hupf (1996) in which a genetically manipulated strain of *Escherichia coli* lacking exopolyphosphatase activity (Ppx^-) and unable to degrade previously accumulated polyP had greater sensitivity to cadmium

than the corresponding Ppx^+ strain which could. The relative sensitivity to heavy metals of *A. johnsonii* M45 cells lacking polyP remains to be examined.

No evidence for the entry of uranium into living bacterial cells via metabolic processes has been established; it is possible that the relatively large UO_2^{2+} oxyanion does not sufficiently resemble a species for which there is a transport system. Suresh *et al.* (1986) propose a mechanism whereby the active uptake of Cd^{2+} by *Acinetobacter* cells stimulates the degradation of surface polyPs which provides ATP for the metal-uptake mechanism. The work of van Groenestijn *et al.* (1987) demonstrates that such a phenomenon is possible but evidence of stimulation of surface polyP degradation by Cd^{2+} was not obtained in the present study. In the case of UO_2^{2+} , for which there is no evidence of cellular uptake, the coupling of metal uptake to surface-polyP (short-chain polyP) degradation is unlikely. A second mechanism should be invoked to account for the observed stimulation of polyP degradation by UO_2^{2+} . The enzyme(s) responsible for polyP hydrolysis is probably regulated by the concentration of free phosphate, for example in the case of polyphosphatase (van Groenestijn *et al.*, 1989; Vasiliadis *et al.*, 1990; Bayly *et al.*, 1991). A decrease in the free phosphate concentration by its precipitation with divalent cations in the periplasm or at the cell surface (Dick *et al.*, 1995) would alleviate inhibition of the hydrolysis reaction, resulting in an increase in the observed rate and extent of polyP degradation by removal of Pi.

Temperature, pH and the presence of nitrate were all found to affect anaerobic phosphate release by *A. johnsonii* M45. The stimulatory effect of temperature on anaerobic Pi release (Fig. 4a), is probably attributable to increased activity of polyP-degrading enzyme(s). The increase in anaerobic Pi release with increasing pH (Fig. 4b) is in agreement with previous work. Smolders *et al.* (1994a, b) found that for phosphorus-accumulating organisms in wastewater treatment, the energy requirement for carbon source (acetate) uptake increased with pH. The proposed source of additional energy (in the form of ATP) was hydrolysis of polyP, which in turn would result in greater Pi release at higher pH. In support of this, van Groenestijn *et al.* (1987) have demonstrated that ATP can be generated from polyP hydrolysis, leading to the release of Pi from cells. In studies on the phosphate transport of *Acinetobacter* spp., van Veen *et al.* (1993) found that a proton-motive force across the cytoplasmic membrane inhibited efflux of Pi whilst efflux increased with increasing (external) pH.

Nitrate has been identified as a common component of municipal and industrial wastewaters, e.g. the NO_3^- concentration of such wastewaters may range from less than 1 mM (62 mg l⁻¹) to greater than 200 mM (12.4 g l⁻¹) (Chen *et al.*, 1996; Zhou & Fang, 1997) whereas the nitrate concentration of uranium-bearing nuclear-fuel-cycle waste may exceed 1 M (62 g l⁻¹) (Tolley, 1993). Although little work has been done on anoxic res-

piration using nitrate as a terminal electron acceptor in *Acinetobacter*, the inhibitory effect of NO_3^- on 'anaerobic' phosphate release in activated sludge has previously been noted (Egli & Zehnder, 1994; Kroiss & Negm, 1994). In mixed cultures, such inhibition is probably a result of competition from denitrifying polyP bacteria (Kern-Jespersen *et al.*, 1994). No direct mechanism for the inhibition of phosphate release by nitrate has been clearly demonstrated, although Klebanoff (1993) found that nitrite had a bactericidal effect on *E. coli*, possibly through the generation of free radicals. In addition, Sijbesma *et al.* (1996) have demonstrated an uncoupling effect (dissipation of the proton-motive force) of nitrite on *Pseudomonas fluorescens* at concentrations exceeding 20 mM. Such an effect is unlikely to account for the inhibitory action of nitrate observed in the present study (Fig. 4c) as Pi efflux in *Acinetobacter* sp. was stimulated by dissipation of the proton-motive force (van Veen *et al.*, 1993). Also, the concentration of nitrite in the present experiments did not exceed 0.5 mM. Kortsee *et al.* (1994) have shown that nitric oxide has an inhibitory effect on phosphate release in activated sludge. Under such conditions, nitric oxide could be generated by denitrifying organisms. This would not explain the inhibitory effect of nitrate in pure culture as *A. johnsonii* M45 cannot denitrify completely (see above).

If the nitrate reductase of *A. johnsonii* M45 does have a respiratory function, it is possible that in the presence of nitrate, the proton-motive force can be maintained in the absence of molecular oxygen without the need for additional energy generation via polyP degradation. van Veen *et al.* (1993) have shown that the presence of a proton-motive force is inhibitory to Pi efflux, which may allow nitrate reduction (to nitrite, see Results) to occur preferentially to polyP degradation.

The antagonistic effect of tungstate pre-treatment towards nitrate inhibition (Fig. 5) is likely to result from the formation of a dysfunctional nitrate reductase due to substitution of tungsten for molybdenum, which is required for the function of the enzyme (Stewart, 1988; Hemschemeier *et al.*, 1991; Frunzke *et al.*, 1993). This effect is consistent with the above: when energy generation through respiratory electron transport to nitrate is inhibited (Table 1), the inhibitory effect of nitrate on Pi release would be diminished.

In conclusion, analysis by two methods has shown that polyP is degraded anaerobically by *A. johnsonii* M45 in the presence and absence of submillimolar concentrations of Cd^{2+} and UO_2^{2+} . ³¹P-NMR indicated that Pi was formed at the expense of polyP under these conditions, and an extractive method to quantify polyP showed that the extent of polyP degradation was dependent on the metal used. Calculation of total polyP showed that in the presence of 0.5 mM UO_2^{2+} there was a stimulatory effect on overall polyP degradation. Conversely, cadmium at 0.2 and 0.5 mM had a negative effect on the process, possibly as a result of the ability of Cd^{2+} to enter cells and inhibit the enzyme(s) responsible

for polyP hydrolysis. In accordance with published data for Cd^{2+} , the presence of UO_2^{2+} (but not, in this case, Cd^{2+}) stimulated degradation of surface polyP but since UO_2^{2+} probably cannot enter the cell this is likely to be attributable to phosphate sequestration into cell-surface-bound metal phosphate (Dick *et al.*, 1995), promoting breakdown of further polyP.

Thus, this study demonstrates that polyP degradation can occur in the presence of low concentrations of heavy metals. However, at higher metal concentrations the process of heavy metal accumulation may be limited by the toxic effects of the metal on polyP hydrolysis, particularly in the case of those metals that can enter cells. Nitrate may also be problematic and could necessitate the use of a nitrate reductase deficient (Nar^-) strain of polyP-accumulating *Acinetobacter*. Partial inhibition of nitrate reductase by tungstate suggested this possibility but initial attempts to obtain a Nar^- strain which could be used in the metal-accumulation process were unsuccessful due to pleiotropic effects associated with the Nar^- phenotype. Possible reasons for this would require further investigation.

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