Localization of enzymically enhanced heavy metal accumulation by *Citrobacter* sp. and metal accumulation *in vitro* by liposomes containing entrapped enzyme

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A heavy-metal-accumulating *Citrobacter* sp. has been used for the treatment of metal-laden industrial wastes. Metal uptake is mediated via a cell-bound phosphatase that liberates inorganic phosphate which precipitates with heavy metals as cell-bound metal phosphate. A phosphatase-deficient mutant accumulated little UO$_2^{2+}$, while a phosphatase-overproducing mutant accumulated correspondingly more metal, with a uranium loading equivalent to the bacterial dry weight achieved after 6 h exposure of resting cells to uranyl ion in the presence of phosphatase substrate (glycerol 2-phosphate). The phosphatase, visualized by immunogold labelling in the parent and overproducing strains, but not seen in the deficient mutant, was held within the periplasmic space with, in some cells, a higher concentration at the polar regions. Enzyme was also associated with the outer membrane and found extracellularly. Accumulated uranyl phosphate was visible as cell-surface- and polar-localized deposits, identified by energy-dispersive X-ray analysis (EDAX), proton-induced X-ray emission analysis (PIXE) and X-ray diffraction analysis (XRD) as polycrystalline UO$_2$PO$_4$·4H$_2$O. Nucleation sites for initiation of biocrystallization were identified at the cytoplasmic and outer membranes, prompting consideration of an *in vitro* biocatalytic system for metal waste remediation. Phosphatidylcholine-based liposomes with entrapped phosphatase released phosphate comparably to whole cells, as shown by $^{31}$P NMR spectroscopy in the presence of NMR-silent $^{125}$I$_2$Cl$^+$. Application of liposome-immobilized enzyme to the decontamination of uranyl solutions was, however, limited by rapid fouling of the biocatalyst by deposited uranyl phosphate. It is suggested that the architecture of the bacterial cell surface provides a means of access of uranyl ion to the inner and outer membranes and enzymically liberated phosphate in a way that minimizes fouling in whole cells.

**Keywords:** *Citrobacter* sp. phosphatase, liposomes, uranium uptake

**INTRODUCTION**

The use of micro-organisms for the accumulation of heavy metals from solution is becoming increasingly attractive for the bioremediation of wastes and sites contaminated with metals. Metals can be removed from wastes upstream by biosorption (Volesky, 1990) or can be precipitated via ligands produced as a result of microbial activity. One example (Barnes et al., 1991, 1992) utilizes H$_2$S produced from the metabolic activity of sulphate-reducing bacteria (SRB) to precipitate metals as cell-bound metal sulphides. H$_2$S production is mandatory; in the absence of this the SRB are metal-sensitive (Postgate, 1979). An alternative bioprecipitation approach has utilized the plasmid-borne metal resistance
mechanisms of organisms such as *Alcaligenes eutrophus* (Mergeay et al., 1985; Diels et al., 1995). Here, heavy metals are effluxed from the cell countercurrent to proton influx inward; the ensuing localized alkalization of the periplasmic space promotes deposition of heavy metals as hydroxides and carbonates in the cellular microenvironment. Both of these examples rely on continuing metabolic activity, which may be sensitive to adverse physical conditions such as the pH of the target waste stream and the toxicity of other co-contaminants. In this respect the use of non-growing (growth-decoupled) biomass may be necessary for prolonged activity and to overcome the constraints imposed by the waste stream.

Although the reductive bioprecipitation and removal of metal anions via growth-decoupled hydrogenase activity of SRB and of *Escherichia coli* has been documented (Lloyd & Macaskie, 1996; Lloyd et al., 1997; Macaskie et al., 1996), the use of non-growing cells for removal of metals by biocrySTALLization via a single enzymic step has received relatively little attention. In addition to a reduced dependence on physiologically permissive conditions (the sensitivity is limited to the enzyme catalysing ligand production), the use of growth-decoupled cells produces a sludge which is compact and attractive in terms of the low organic content and high enrichment for the metal for recycling.

The most extensively documented example of growth-decoupled metal removal is by a strain of *Citrobacter* sp. originally isolated from metal-contaminated soil (Macaskie, 1990; Macaskie et al., 1992, 1995a; Yong & Macaskie, 1995; Tolley et al., 1995). This organism has been applied to the removal of uranium from mine water (Roig et al., 1995; Macaskie et al., 1996, 1997) and to the accumulation of radiotoxic elements such as americium and plutonium (Macaskie et al., 1994a, 1996). Metal uptake requires a cellular phosphatase, homologous to the *phoN* product of *Salmonella* and some other enterobacteria (Macaskie et al., 1994b), which liberates inorganic phosphate from a supplied organic phosphate substrate to precipitate HPO$_4^{2-}$ with metals (M) extensively as cell-bound MHPO$_4$ (Macaskie et al., 1992, 1996; Tolley et al., 1995; Yong & Macaskie, 1995). However, little is known of the cellular localization of this phosphatase in *vivo* and the mechanism(s) by which enzymic activity contributes to the metal bioaccumulation process. Factors other than phosphate release alone are implicated in effective metal removal (Macaskie et al., 1995a); indeed, some other enterobacteria containing *phoN* retained little heavy metal from solution (Macaskie et al., 1994b).

A previous study indicated that the acid phosphatase of *Salmonella typhimurium* was not released easily by osmotic shock (Neu & Heppel, 1965; Nossal & Heppel, 1966; Kier et al., 1977). The *Citrobacter* enzyme was, similarly, poorly extracted (Jeong, 1992) in conflict with a proposed role for the enzyme in the accumulation of heavy metals within the periplasmic space. Immobilized phosphatase removed little metal from solution per se (B. C. Jeong, unpublished) and previous studies suggested a requirement for nucleation sites for initiation of crystal growth (Macaskie et al., 1996). This study aims to identify the site(s) of phosphatase localization, relative to the site(s) of metal uptake. Nucleation sites were suggested on the membrane phospholipid groups. Therefore, an *in vitro* system was constructed using artificial membrane vesicles (liposomes) with entrapped phosphatase to evaluate a possible application of the encapsulated enzyme for metal removal *in vitro*. This could forestall potential problems (e.g. biofouling) associated with the use of live bacteria in filtration systems and gain acceptability if genetic manipulation was employed to increase the level of phosphatase production further.

**METHODS**

**Bacterial strains and growth conditions.** *Citrobacter* sp. strain N14, originally isolated from metal-polluted soil, was as described previously (Macaskie et al., 1992, 1996; Tolley et al., 1995; Yong & Macaskie, 1995), by permission of Isis Innovation, Oxford. The phosphatase-overproducing (dc5c) and -deficient (lp4a) mutants were as described previously (Macaskie, 1995; Macaskie et al., 1998). The cultures (250 ml -3 l as appropriate) were grown at 30 °C in a Tris/glycerol 2-phosphate-based minimal medium with glycerol (2 g l$^{-1}$) as the carbon source and forced aeration (Tolley et al., 1995). For phosphatase preparation cultures were grown as above in two 15 l batches or in larger batches of 100 l in an adapted 130 l plastic drum. This was sterilized using propylene oxide under ambient conditions for 7 d with residual gaseous sterilant displaced with sterile air for several days. The culture was grown at 30 °C from an approximately 2% (v/v) overnight culture inoculum using an overhead stirrer and forced aeration, and harvested at ambient temperature (continuous flow centrifuge: Heraeus 17RS). Large quantities of enzyme for entrapment within liposomes or for studies using NMR were prepared from cultures (600 l) grown in an LS1 Biolafitte bioreactor in the Centre for Biochemical Engineering, University of Birmingham, in glycerol-based medium (Macaskie et al., 1995b).

**Purification of phosphatase and assay of phosphatase activity.** Cell disruption was achieved using a French pressure cell (SLMO AMINCO, SLM instruments Co.) or a cell disruptor (‘Bio Neb’ cell disruptor, Glas-Col Ltd) for the biomass harvested from the 600 l culture. The phosphatase was purified via (NH$_4$)$_2$SO$_4$ fractionation, anion and cation exchange column chromatography and fractionation on hydroxyapatite and phenyl-Sepharose columns (Jeong, 1992; Jeong et al., 1994), and was judged as pure by a single band on SDS-PAGE (Jeong, 1992). Phosphatase activity was assayed by the release of p-nitrophenol (PNP) from p-nitrophenyl phosphate (PNPP) (Tolley et al., 1995; Macaskie et al., 1995a,b). One unit of activity is defined as that liberating 1 nmol product min$^{-1}$ (mg bacterial protein)$^{-1}$, with protein assayed by the Lowry method and calculated from the OD$_{600}$ of the bacterial suspension via a conversion factor (Jeong, 1992). The molar extinction coefficient for PNP was 9025 cm$^{-1}$ M$^{-1}$, measured under the conditions of assay (1 cm path length; A$_{405}$).

**Immobilized phosphatase column.** Purified phosphatase was immobilized onto cyanogen bromide (CNBr)-activated Sepharose CL-4B (all steps at 4 °C). CNBr-activated Sepharose CL-4B (2 ml; Sigma) was added to 0.5 ml phosphatase (8 mg ml$^{-1}$)
in 0.5 M sodium phosphate buffer, pH 7.5, and gently mixed overnight. The beads were washed twice with the phosphate buffer, then once with 0.05 M sodium phosphate buffer, pH 7.5, supplemented with 1 M NaCl. Ethanolamine buffer (0.1 M, pH 7.5, 10 times the volume of the beads) was added and the mixture gently mixed at 30 °C for 4 h. The beads were washed twice with PBS (1×: 8.0 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄, pH 7.2) and placed in a disposable column with a bed volume of 1.5 ml (Pierce) and washed with 0.01 M Tris/HCl buffer (15 ml, pH 7.5), 0.1 M glycine/HCl buffer (15 ml, pH 2.5), 0.01 M Tris/HCl buffer (15 ml, pH 8.8) and finally 0.1 M freshly prepared diethylamine/HCl buffer (15 ml, pH 11.5) followed by 0.01 M Tris/HCl buffer (15 ml, pH 7.5). After use the column was washed with 0.01 M Tris/HCl buffer (15 ml, pH 7.5) and stored at 4 °C in this buffer supplemented with 0.01% (w/v) merthiolate (Sigma).

**Preparation of phosphatase antibody and ELISA.** Purified phosphatase (1 ml, 4 mg) in PBS was mixed with 1 ml Freund’s complete adjuvant (Sigma) and emulsified by passing through two 10-gauge needles until the emulsion was thick and did not disperse when a drop was placed on the surface of PBS. Blood (10 ml) was collected from two female rabbits (New Zealand White; University of Oxford) which were injected subcutaneously with a 500 µg protein sample. A boost was given in Freund’s incomplete adjuvant (Sigma) after 4 weeks with subsequent boosts at 2-week intervals. Blood samples were allowed to clot for 1 h at 30 °C, then overnight at 4 °C, and the serum was centrifuged (6600 g, 10 min, 4 °C). The crude antibody solution was stirred at 4 °C with 0.5 vols saturated (NH₄)₂SO₄ [761 g (NH₄)₂SO₄ in 1 l litre distilled water] added slowly and left unstirred for 2 h at 4 °C, followed by centrifugation (19 300 g, 20 min, 4 °C). Saturated (NH₄)₂SO₄ (0.5 vols of the starting volume) was added to the supernatant. After 4 h at 4 °C the preparation was centrifuged (19 300 g, 20 min, 4 °C). The pellet was resuspended in PBS (volume equal to the starting volume), dialysed against four changes of PBS (50 vols of the antibody solution) overnight at 4 °C and centrifuged (13 700 g, 10 min, ambient temperature). Samples (1 ml) were passed through the phosphatase affinity column three times, under gravity. The column was washed with 0.01 M Tris/HCl buffer (20 ml, pH 7.5) and then 20 ml of the same buffer supplemented with 300 mM NaCl. The antibody bound by acid-sensitive interactions was eluted with 0.1 M glycine/HCl buffer (15 ml, pH 2.5) into a tube containing 1 M Tris/HCl buffer (15 ml, pH 8.0). The column was then washed with 0.01 M Tris/HCl buffer (15 ml, pH 8.8). The antibody bound by base-sensitive interactions was eluted with 0.1 M freshly prepared diethylamine/HCl buffer (15 ml, pH 11.5) into a tube containing 1 M Tris/HCl buffer (15 ml, pH 8.0). Fractions were assayed using ELISA (see below), pooled and dialysed against four changes of PBS (50 vols of the antibody fractions) overnight. For ELISA, 50 µl phosphatase (10 µg ml⁻¹ solution in PBS) was added to wells of a microtitre plate and left for 2 h at ambient temperature in a humid atmosphere, washed three times (PBS), dried by inversion and washed three times with blocking buffer [3% (w/v) non-fat dried milk (Marvel) in PBS]. Primary antibody solution (serum or column eluate; 50 µl) was added to each well, incubated and washed as described above. Horseradish peroxidase (HRP)-labelled goat anti-rabbit antibody (50 µl of a 1:1000 dilution; Dakopatts, supplied by Agar Scientific Ltd) was added, incubated and washed as above. Dilutions of primary and secondary antibody were done in blocking buffer. For detection, chromogenic substrate for HRP [0.1 mg 3′,3′,5′,5′-tetramethylbenzidine (TMBl; Sigma) was dissolved in 0.1 ml DMSO. Sodium acetate buffer (0.1 M, pH 6.0, 9.9 ml) was added, the mixture was filtered through a 0.22 µm filter (Millipore) and 30% H₂O₂ (Sigma) was added to a final concentration of 0.01% (v/v). Substrate solution (50 µl) was added to each well and left for 15 min at ambient temperature. The reaction (pale blue positive) was stopped by addition of 30 µl 1 M H₂SO₄ (bright yellow positive). The titre of the serum or column eluate was defined as the highest dilution to give a negative reaction against controls with no enzyme or primary antibody.

**Fixation and embedding of cells for electron microscopy**

Fixation and embedding for cell staining. All steps were at ambient temperature unless otherwise stated. Cells (30 ml) harvested by centrifugation (5000 g, 10 min) in the mid-exponential phase were resuspended in 0.1 M sodium cacodylate buffer (15 ml, pH 7.2) with 2% (w/v) sucrose, fixed for 40 min in an equal volume of 2% (w/v) glutaraldehyde and 2% (w/v) paraformaldehyde in the same buffer and washed three times (10 min each) in 1 ml of the same buffer without fixatives. Osmium tetroxide (1 ml, 1% w/v, aqu.) was then added. After 1 h the mixture was centrifuged (5000 g, 10 min), the pellet was washed in 1 ml distilled water, incubated overnight at 4 °C in 1 ml uranyl citrate (0.5% w/v, aqu.) and dehydrated with 10, 20, 30, 50, 70 and 90% (v/v, aqu.) ethanol for 10–20 min each followed by absolute ethanol twice. Embedding was in absolute ethanol/Spurr (TAAB Laboratory Equipment Ltd) at ratios of 3:1, 1:1 and 1:3 (v/v) for 1 h each, and three changes of undiluted Spurr (1, 12 and 12 h, respectively). Samples were transferred from Eppendorf tubes to silicone rubber moulds (Agar Scientific Ltd) to polymerize for 10 h at 70 °C.

**Low temperature embedding in LR White**. Benzoic methyl ether (Aldrich) was added to LR White (final concentration 0.5% w/v; Agar Scientific Ltd) to accelerate hardening. Cells were resuspended in 0.1 M PIPES buffer (15 ml, pH 7.2), fixed and washed as above, then dehydrated in 10 and 20% (v/v, aqu.) ethanol at ambient temperature (10 min each), and in 30% ethanol at 0 °C and 50% ethanol at −20 °C (1 h each). Further dehydration used 70%, 90% and absolute ethanol at −20 °C (1 h each). Embedding was in LR White/absolute ethanol at ratios of 1:1, 2:1 and 3:1 (v/v) at −20 °C for 1 h each, followed by four changes of undiluted LR White at −35 °C (overnight, 8 h, overnight and 8 h, respectively). Polymerization (12 h at −20 °C and 12 h at 4 °C) was done using a UV lamp (Agar Scientific Ltd).

**Thin sectioning and mounting of sections**. Cells embedded in Spurr or LR White were thin-sectioned (80–100 nm) with a glass knife using a Reichert–Jung Knifemaker and a microscope (Ultracut E, Reichert–Jung). Sections from the normal fixation and embedding in Spurr were mounted on Cu grids (Agar Scientific Ltd) and stained with lead citrate for observation of cell structure. Sections from the low temperature embedding in LR White were mounted on Formvar-coated Ni grids (Agar Scientific Ltd) for enzyme localization (see below).

**Immunogold labelling.** This was done at room temperature with reagents filtered through a 0.22 µm sterile filter (Millipore) prior to use. Sections of LR White-embedded cells were blocked with PBST (PBS containing 1% (w/v, aqu.) globulin-free bovine serum albumin (PBSB: BSA from Sigma) and 0.2% (v/v, aqu.) Tween 80, pH 6.9 [Sigma]) for 10 min, then with PBSBG [PBS containing 1% (w/v, aqu.) globulin-free BSA and 0.02 M final concentration glycine, pH 6.9 (Sigma)] for 5 min. Sections were washed four times (5 min each) by floating the grid upside down on a droplet of PBSB solution. The grids
were incubated on a primary antibody droplet (15 μg rabbit anti-phosphatase ml⁻¹ in PBSB) for 1 h in a damp box, washed twice (5 min each) in PBSB and once in PBSBG (5 min), then incubated with gold (10 nm)-labelled goat anti-rabbit antibody (1:20 dilution in PBSB; British Biocell) for 1 h. The grids were washed three times (3 min each) in PBSB and three times (1 min each) in distilled water. For low phosphatase activity cells in some experiments, the concentrations of primary antibody and secondary antibody were increased to 75 pg ml⁻¹ in PBSB (see Methods). Data are from a representative experiment.

**Determination of metal removal by resuspended cells.** Cells harvested in the exponential phase (20–40 ml) were washed twice in isotonic saline (8-5 g NaCl 1⁻¹) and resuspended (OD₆₀₀ 0.3–0.4) in 2 ml citrate buffer, pH 6.9, 5 mM glycerol 2-phosphate, with the addition of uranyl nitrate to 1 mM at 30 °C. Timed samples (1-25 ml) were centrifuged (13,700 g, 5 min, ambient temperature) and the supernatants assayed for residual uranyl ion (Tolley et al., 1995). Uranium removal was calculated as a percentage of bacterial dry weight using a dry weight calibration of 0.495 mg ml⁻¹ for 1 unit of OD₆₀₀ (1 cm path length; P. Yong & L. E. Macaskie, unpublished). The uranium-loaded cell pellets (each equivalent to 5 ml culture) were washed twice in isotonic saline and once in water, resuspended in 0.2–0.4 ml water and mixed, and a drop was placed on a Formvar-coated electron microscope grid, air-dried overnight and examined with the electron microscope without further staining. For monitoring the intracellular uranium accumulation, uranium-loaded cells (equivalent to 5 ml culture) were washed twice in isotonic saline and water, fixed and embedded in LR White as described above and thin sections (80–100 nm) were examined, without further staining.

**Preparation of liposomes with entrapped phosphatase, phosphate release and metal uptake.** Phosphatidyldcholine (25 mg), cholesterol (2 mg) and stearic acid (1 mg) were dissolved in 10 ml chloroform/methanol (9:1, v/v). The solvent was evaporated under vacuum at room temperature. Phosphate (2.5 ml, 4.8 mg) in 20 mM MOPS buffer, pH 7, and six glass beads (2.5 mm diam.) were added and the flask agitated to mix. The flask was flushed with N₂ and sonicated (36 Hz, 6 x 30 s) and the liposomes were centrifuged (80,000 g, 20 min). The supernatant (untrapped enzyme) was removed and the liposomes were resuspended in 2:5 ml MOPS buffer (20 mM, pH 7) and re-centrifuged. The supernatant was tested for residual free enzyme using PNP. In some experiments the phosphatase was used after only the anion and cation exchange purification steps. Only minor contaminants remained at this stage. For assay, liposome-entrapped enzyme [0.08 ml liposome preparation of phosphatase; specific activity 980 nmol PNP min⁻¹ (mg protein⁻¹)] was added to 0.72 ml MOPS buffer (20 mM, pH 7) and 0.2 ml glycerol 2-phosphate (final concentration 1 mM) at 30 °C. Samples were analysed for liberated phosphate (P) in supernatants by a modification of the method of Pierpoint (1957). Where appropriate, Cd²⁺ or UO²²⁻ were added as the nitrate salts (final concentration 1 mM). Residual uranyl ion was assayed (Tolley et al., 1995) following removal of the liposomes by centrifugation as above.

**Examination of phosphate release using NMR.** Solution ³¹P NMR was performed using a Bruker 400 MHz Spectrometer at 161 MHz with a pulse time of 0.91 μs and a pulse recycle delay of 1 s. H₃PO₄ (85%, v/v) was used as a standard and D₂O as a field frequency lock. Liposome preparations, as described above, with or without 1 mM Cd²⁺, were incubated in an NMR tube. Initial experiments using 1 mM glycerol 2-phosphate (see above) were below the sensitivity of NMR; subsequent experiments therefore utilized 5 mM glycerol 2-phosphate.

**Examination of metal-loaded cells and liposomes using solid-state methods.** Whole cells, sectioned cells (above) and liposomes were examined by electron microscopy (EM), atomic force microscopy (AFM), energy-dispersive X-ray analysis (EDAX), proton-induced X-ray emission (PIXE) analysis and X-ray diffraction analysis (XRD), as appropriate. For EM the liposomes were resuspended in water, dropped onto a copper electron microscope grid, air-dried and examined using the transmission electron microscope (JEOL). AFM was done using wet or briefly air-dried mounts in water on glass slides in the laboratories of the Company Research Laboratory, BNFL, Preston, UK. The accumulated solid material was characterized using EDAX and PIXE as described previously (Bonthrone et al., 1996; Tolley et al., 1995; Yong & Macaskie, 1995), and the identity of the deposit was confirmed by XRD (Bonthrone et al., 1996; Yong & Macaskie, 1995) and comparison with appropriate reference databases (Bonthrone et al., 1996).

**RESULTS AND DISCUSSION**

**Phosphatase activity and uranyl ion accumulation by Citrobacter sp. strain N14 and mutants with altered enzyme activity**

The phosphatase deficiency of strain lp4a (specific activity 12–18 units) was confirmed by immunogold labelling. No phosphatase was visible in the mutant under conditions where the parent strain, N14, clearly expressed the enzyme. The low specific activity
Table 1. Comparison of uranium removal efficiency and phosphatase activity of *Citrobacter* N14 and the phosphatase-overproducing strain dc5c

<table>
<thead>
<tr>
<th>Sample</th>
<th>Uranium removal efficiency (phosphatase specific activity)*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>N14</td>
</tr>
<tr>
<td>Exponential phase</td>
<td>12.3 ± 1.4 (421 ± 71)</td>
</tr>
<tr>
<td>Stationary phase</td>
<td>16.0 ± 1.8 (587 ± 80)</td>
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Uranium removal efficiency is shown as percentage of the bacterial dry wt h⁻¹. See Methods for a definition of the units of phosphatase specific activity.

corresponded to poor removal of uranyl ion, where the parent strain accumulated UO₂⁺ to >100% of the bacterial dry weight after 10 h (Fig. 1). Conversely, the phosphatase-overproducing mutant, dc5c, showed an increased rate of uranyl ion uptake in a typical experiment (Fig. 1). This increase, less than two-fold in the example shown (despite a two-fold difference in phosphatase activity), was probably because at high phosphatase activities (above 700 units) the rate of P_i liberation ceases to be rate-limiting and biocrystallization constraints may prevail (Macaskie et al., 1994a). The reproducibility between three experiments is shown in Table 1, where the ratio between phosphatase activities and rates of uranyl uptake for the two strains are identical (1-6-fold).

Phosphatase localization was examined further on sections of cells using immunogold labelling (Fig. 2). Most of the enzyme was visible in the periplasmic space (Fig. 2a) and also associated with the outer membrane with, in some cells, a higher localization in the polar regions (Fig. 2b, arrowed), as seen previously in *E. coli* overexpressing alkaline phosphatase (Nesmeyanova et al., 1991). A higher concentration of immunogold label in late exponential phase cells was seen for both strains in accordance with the increased enzyme production reported in the mid–late exponential phase (Butler et al., 1991). Although some enzyme was apparent in the extracellular region, particularly in the overproducing strain (Fig. 2b), no evidence was obtained for the formation of outer-membrane vesicles as a mechanism for enzyme export as reported by Nesmeyanova et al. (1991), even though the specific activity of strain dc5c (946 units, Fig. 2b) was comparable to that of the overproducing *E. coli* (Nesmeyanova et al., 1991).

Further studies of uranyl ion uptake by strains N14 and dc5c

In accordance with previous studies (Macaskie et al., 1992), uranyl-unchallenged cells were indistinct when observed by electron microscopy; the cells were not stained (Fig. 3a, b). From the known phosphatase activity of a sample, it was possible to adjust the level of uranyl ion loading on the cells (given that these are correlated; Table 1) by the correct choice of sampling time from the uranyl-challenge solutions. Following uranyl accumulation, the cells became stained with an electron-opaque deposit which obliterated cellular detail (Fig. 3c, d). Occasionally broken cells were visible (Fig. 3e) showing clearly the cell-surface localization of the uranyl phosphate, the identity of which was confirmed as HUO₄PO₄₂·4H₂O using EDAX, PIXE and XRD, as reported previously (Macaskie et al., 1992; Yong & Macaskie, 1995; Bonthrone et al., 1996). The localization of HUO₄PO₄₂·4H₂O was in accordance with the
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Fig. 3. Uranyl uptake by whole cells of strains N14 and dc5c. Cells were harvested in the mid-exponential phase (OD₆₀₀ = 0.4-0.5) and resuspended in 2 mM citrate buffer/5 mM glycerol 2-phosphate, either uranium-free (a, b) or supplemented with 1 mM UO₂⁺ (c, d). a and c, Strain N14 (phosphatase specific activity 188 units, uranyl loading was 155% of the bacterial dry weight in c); b and d, strain dc5c (phosphatase specific activity 480 units, uranyl loading was 203% of the bacterial dry weight in d). In some cases broken cells were visible: (a) shows strain N14 (phosphatase specific activity 160 units, uranyl loading was 160% of the bacterial dry weight). Other cells in the cultures had dense polar deposits of uranyl phosphate (f). Bars, 500 nm.

cell-surface localization of the enzyme seen in Fig. 2. Some cells (Fig. 3f) had heavy polar deposits of uranyl phosphate, in accordance with the polar localization of the enzyme in some cells by immunogold labelling (Fig. 2). After drying and under examination in vacuo the uranyl-loaded cells appeared hard and crystalline. Attempts to visualize the precipitate on fully hydrated cells using AFM were unsuccessful. With wet preparations, movement of the cells under the probe gave very poor resolution. Partially air-dried samples gave clearly defined cells but the uranyl phosphate was not visible as clearly defined crystals.

Events during the initial stages of uranyl ion deposition

It was suggested previously that, for extensive metal accumulation, the phosphatase should be localized near to sites on the cell surface that can function as nucleation foci for initial complexation of the metal, and subsequent consolidation of crystal growth using Pi liberated by the enzyme in the vicinity of the heavy metals (Macaskie et al., 1994a). Membrane phospholipids could fulfil this nucleation role. This was tested by examination of sections of cells which were interrupted during accumulation of uranyl ion (Fig. 4). Identical results were obtained for both strains at corresponding uranyl loadings. Little electron opaque material was associated with sections of uranyl-unchallenged cells (Fig. 4a); the sections were, in this case, stained with uranyl citrate after the incubation period to provide contrast. Initially the accumulated metal was visible as electron dense deposits following the tracks of the double membrane surrounding the cells (Fig. 4b), in accordance with the cell-surface pattern of uranyl deposition seen previously with *Saccharomyces* (Strandberg et al., 1981) and *Pseudomonas* (Strandberg et al., 1981; Marques et al., 1991), where the uptake of uranyl was rapid and largely membrane-localized. To date, there is no evidence from the literature that uranyl ion is able to enter the cytoplasmic compartment of living cells (probably attributable to the large size and stereochemistry of UO₂²⁻ and its associated hydroxyl groups; Weigel, 1986), even though uranyl ion induces formation of the chelating agents probably responsible for iron acquisition in *Pseudomonas aeruginosa* (Premuzic et al., 1985). Strandberg et al. (1981) noted that uranyl uptake occurred rapidly in *P. aeruginosa*, but the mechanism of translocation of UO₂²⁻ across the outer membrane and to the cytoplasmic membrane is still unknown. Following the initial membrane-associated deposition more extensive uranyl uptake occurred (Fig. 4c) and at high uranyl loadings (150-200 % of the bacterial dry weight) the deposited metal was clearly visible in a matrix of wall material that became detached from the cells, leaving wall-free cell 'ghosts' (Fig. 4d). Thus, the cell surface can be regarded as an immobilizing matrix for the enzyme. This could provide an explanation for the high metal loads seen without cessation of activity, or saturation [e.g. 9 g uranium (g bacterial dry wt)⁻¹; Macaskie, 1990].
Localization of metal uptake by *Citrobacter*

Fig. 4. Time course and localization of uranyl accumulation by strains N14 and dc5c. The results were similar for both strains; representative results from multiple sections of each strain are shown. Cells harvested in the mid-exponential phase (OD₆₀₀ = 0.445, specific activities of N14 and dc5c were 188 and 480 units, respectively) were challenged with UO₂⁺ as described in the legend to Fig. 3, fixed with 1% glutaraldehyde and 1% paraformaldehyde, and embedded in LR White. Thin sections (80-100 nm) were examined. Uranyl-unchallenged cells (a) and cells loaded with UO₂⁺ to 28 (b), 84 (c) and 165% of the dry weight (d). Bars, 500 nm.

Since the cytoplasmic compartment does not appear to participate in uranyl phosphate deposition (Figs 3e and 4) and since the membranes appear to provide sites for metal deposition, metal uptake could occur by a phospholipid-based membrane surrounding entrapped enzyme. This was tested using column-immobilized and membrane-entrapped phosphatase in vitro.

**Phosphate release and metal uptake in vitro using immobilized phosphatase**

Initial tests in vitro utilized columns of CNBr-immobilized phosphatase (see Methods) to test for the uptake of UO₂⁺ from a 1 mM solution supplemented with 5 mM glycerol 2-phosphate. Phosphate release was observed from uranyl-unsupplemented solution but the enzyme was inactivated rapidly by UO₂⁺, as confirmed by testing for phosphate release from substrate in metal-free flow subsequent to the initial uranyl-challenge, and was in accordance with metal toxicity tests where UO₂⁺ gave 50% loss of phosphatase activity at a concentration of only 30 μM (Jeong & Macaskie, 1995). This short-lived activity was in contrast to columns containing whole cells, which retained phosphatase activity and uranyl removal over many weeks (Macaskie, 1990). It is likely that the enzyme held in the more protected periplasmic space matrix in whole cells is able to generate a protective localized layer of Pᵢ before penetration of UO₂⁺ across the outer membrane (note that uranyl uptake is not immediate, but is preceded by a short delay; Fig. 1).

Liposomes containing entrapped phosphatase were prepared according to published methods. The phosphatase (4.8 mg protein before immobilization) had a specific activity of 11263 nmol PNP min⁻¹ (mg protein)⁻¹; that recovered in the liposome preparation (325 mg liposome ml⁻¹ final concentration, containing 3 mg protein ml⁻¹) had a specific activity of 980 nmol PNP min⁻¹ (mg protein)⁻¹. Thus the yield recovered in the liposomes was only approx. 9% of that incorporated into the preparation, and was consistent between liposome batches, within 10-15%. Preliminary tests using liposome-entrapped phosphatase challenged with glycerol...
2-phosphate confirmed P₁ release; the specific activity was 8·6 nmol P₁ released min⁻¹ (mg liposome preparation)⁻¹ (measured gravimetrically), corresponding to 96 nmol min⁻¹ (mg enzyme)⁻¹ (0·09 mg enzyme immobilized (mg dry wt of liposome material)⁻¹). This is broadly similar to the specific activity of whole cells of strain N14 (e.g. legend to Fig. 2) and it is suggested that in terms of P₁ release per unit of mass of carrier the use of liposome-entrapped enzyme offers little or no advantage.

A representative time course in the presence of glycerol 2-phosphate showed substantial P₁ release which was unaffected by Cd²⁺ (approx. 8·9 nmol P₁ released min⁻¹ ml⁻¹ in each case), but was reduced by more than 75% in the presence of UO²⁺. Attempts to monitor total P₁ release by liposomes in the presence of UO²⁺ using ³¹P NMR were unsuccessful, probably due to quenching by the paramagnetic uranyl ion. Phosphate release experiments therefore utilized ‘NMR-silent’ ¹¹¹Cd²⁺. Little signal was given by the liposome-bound phosphate groups (phosphatidylcholine) which were below the detection limit; indeed, 5 mM glycerol 2-phosphate or P₁ was required for an acceptable signal to noise ratio (Fig. 5). Similar data were obtained in the absence and presence of Cd²⁺. This confirmed that degradation of glycerol 2-phosphate by liposomes with release of P₁ occurred within 24 min, was comparable to the reaction seen with whole cells and was independent of the presence of 1 mM Cd²⁺. Accurate quantification is difficult due to the low sensitivity of ³¹P NMR, but the appearance of P₁ at the expense of substrate was shown clearly in all cases. CdHPO₄ did not precipitate during the experiments (because the total phosphate was the same with and without Cd by NMR and was recovered as P₁ in the supernatant in each case: see above); possibly the Cd²⁺ was initially held as a complex with the phosphate groups of the phosphatidylcholine of the membrane. The presence of complexing ligands reduces the concentration of free metal (Hughes & Poole, 1991).

In the phosphate release experiments (not shown) and in those using NMR, the liposomes precipitated in the presence of uranyl ion. Precipitated liposomes gave an X-ray diffraction pattern indistinguishable from those published previously for cell-bound HUO₄PO₄ (Yong & Macaskie, 1995; Bonthrone et al., 1996). There was no residual uranyl ion in solution by assay. Since the uranyl ion removed (1 mM) exceeded the maximum concentration of P₁ liberated (up to 0·2 mM) it was assumed that UO²⁺ was deposited mainly onto the membrane phospholipid groups, in accordance with metal biocrystallization onto enzyme-free liposomes reported by Mann et al. (1986). Washed, uranyl-loaded liposomes gave negligible P₁ release subsequently. In contrast, liposomes previously exposed to Cd²⁺ had a phosphatase specific activity of (for example) 892 nmol PNP min⁻¹ (mg dry wt)⁻¹, i.e. a loss of activity of less than 10%. UO²⁺ is more toxic to the phosphatase than Cd²⁺ (Jeong & Macaskie, 1995) but it was not possible to conclude from these experiments whether activity was lost due to enzyme inhibition or to blockage by the accumulated metal.

**Examination of the metal-loaded liposomes using AFM and EM**

Attempts to examine native liposomes using AFM were unsuccessful. In the absence of metal the sticky liposomes gave distortion and dragging under the probe tip. Metal-loaded liposomes had a more granular appearance, but no structures were visible (not shown). Examination of the liposomes by EM after drying, but unstained, showed ill-defined oval vesicles of approx. 200 nm in length (Fig. 6a, arrowed). After uranyl-
Localization of metal uptake by *Citrobacter*

Challenge the liposomes appeared as contracted, electron-opaque deposits with little structural definition (Fig. 6b). Closer examination (Fig. 6c) showed a granular pattern of uranium deposition (arrowed; possibly attributable to sorption onto the surface phospholipids) with localized areas of heavy deposition (double arrows). In native liposomes radial discontinuities are visible (Fig. 6a, double arrows), possibly providing access to the liposome interior.

Conclusions, and implications for biotechnological metal removal

This study establishes that metal accumulation as cell-bound metal phosphate is mediated via a phosphatase, located periplasmically, in association with the outer membrane, and possibly also extracellularly. Uptake of UO$_2^{2+}$ is initially at the sites of the double membranes bounding the cells and subsequently throughout the cell wall layers. Membrane phospholipids function as sites for uranyl phosphate crystallization *in vitro* but encapsulation of enzyme within liposomes confers no advantage over previous studies using these as metal sorbents *per se* (Mann *et al.*, 1986). This is attributable to poor $P_i$ release by the liposomes in the presence of UO$_2^{2+}$, probably attributable to blockage of sustained $P_i$ release by the accumulated metal. In this respect the whole cells act as superior carriers of the enzyme, since the cell surface layers provide a framework for extensive uranyl phosphate deposition without fouling. A previous study has established that radio-killed cells retain the ability to accumulate metal (the phosphatase is radio-resistant; Strachan *et al.*, 1991). Thus, dead cells can function as enzyme carriers, simultaneously providing phospholipid nucleation sites, together with the spatial infrastructure to hold the accumulated metal phosphate as large crystals, without blockage. The combination of these characteristics provides a novel system for the extensive removal and recovery of heavy metals from contaminated solutions unconstrained by the physiological requirements of the living cell.

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