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Phosphate uptake and release by *Acinetobacter johnsonii* in continuous culture and coupling of phosphate release to heavy metal accumulation

CD Boswell[1], RE Dick[2], H Eccles[3] and LE Macaskie

School of Biosciences, The University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

A strain of polyphosphate-synthesizing, phosphate-releasing *Acinetobacter johnsonii* was isolated from a wastewater treatment plant operating enhanced biological phosphate removal (EBPR) and was used to remove La^{3+} from solution *via* precipitation of cell-bound $LaPO_4$. The effect of repeated aerobic–anaerobic cycles on the carbon and phosphate metabolism of the organism was studied in attempts to promote increased phosphate flux using a three-stage, continuous bioreactor comprising aerobic, anaerobic and settling vessels. The bioreactor was operated in two modes: In flow-through mode, cells were grown aerobically with acetate as the sole carbon source, promoting excess phosphate uptake (up to 5.0 mmol/l=3.0 mmol/g protein). Cells were diluted into the anaerobic vessel where phosphate was released (up to 1.0 mmol/l=0.3 mmol/g protein), and thence to waste. The system was initially operated to steady state in flow-through mode, then switched to recycle mode. Here the anaerobic vessel output passed to a settling vessel from which settled cells were returned to the aerobic vessel. Carbon source (acetate) was supplied only to the anaerobic vessel; increased anaerobic acetate uptake was observed during recycle, which was sustained when the system was returned to flow-through mode and was related to increased cellular lipid inclusions by flow cytometry and electron microscopy. These phenomena may represent adaptation of cells to aerobic–anaerobic cycling with aerobic carbon/energy limitation. Addition of La^{3+} to the anaerobic vessel during recycle mode promoted removal of 95% of the La^{3+} from a 0.1 to 0.3 mM (14–42 ppm) solution at the expense of biogenic phosphate. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 333–340.

Keywords: *Acinetobacter* sp.; enhanced biological phosphate removal; polyphosphate; heavy metals; bioremediation

Introduction

Acinetobacter spp. have been implicated, along with other microorganisms, in enhanced biological phosphate removal (EBPR) [3,4,11], being commonly isolated from wastewater treatment plants operating EBPR and shown to take up and release phosphate (Pi) under aerobic and anaerobic conditions, respectively [35,36]. Recent work on the molecular ecology of wastewater treatment suggests that, numerically, *Acinetobacter* may not be dominant in all EBPR processes [37,38] and other bacteria present in wastewater treatment plants can, similarly, accumulate polyphosphate (polyP) [1]. Because *Acinetobacter* spp. can comprise a significant proportion of EBPR microflora [9,20,30], are known to carry out the functions required for EBPR [36] and were the predominant polyP-accumulating organisms isolated from activated sludge (R.E. Dick, unpublished) *Acinetobacter* spp. were chosen as suitable test organisms for the investigation of polyphosphate cycling and its possible coupling to metal-wastewater treatment. *Acinetobacter* spp. can accumulate polyP aerobically [16]. Subsequent exposure to anaerobic conditions promotes polyP degradation with concomitant release of phosphate into the medium, which has been coupled to bioprecipitation of heavy metal phosphates [10,18], providing a novel approach to the treatment of 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.

In general, EBPR processes use a configuration in which the influent mixed liquor is exposed to an anaerobic stage before the standard aerobic treatment in which (enhanced) phosphate uptake then takes place, generating a sludge that is enriched with phosphate. In the "Phostrip process" [17], phosphate stored in sludge microorganisms is "stripped" (released) anaerobically, producing a low-volume, concentrated solution of inorganic phosphate that can be precipitated chemically, generating less sludge and using less precipitant than conventional processes. The biomass is recycled, Pi-enriched sludge may be divided, with some sent to waste and the remainder recycled ("Bardenpho" and "UCT" processes [2]). More complex configurations have since been developed to allow combined removal of COD, nitrogen and phosphorus [28].

The objective of this "proof of principle" study was to evaluate the ability of *Acinetobacter* to take up and release Pi under aerobic and anaerobic conditions, respectively, in a biphasic system and to couple Pi release to heavy metal removal in a continuous process. A previous approach [10] utilized immobilized biomass with metals accumulated *via* temporally separated, alternating anaerobic and aerobic cycles. The present study aimed to develop a series of spatially separated modules with continuous metal removal

Correspondence: Dr LE Macaskie, School of Biosciences, The University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

[1]Present address: School of Chemical Engineering, The University of Birmingham, Birmingham B15 2TT, UK.

[2]Present address: QUESTOR Centre, Department of Chemical Engineering, The Queen's University of Belfast, David Keir Building, Stranmillis Road, Belfast BT9 5AG, Northern Ireland.

[3]Permanent address: BNFL, Salwick, Preston PR4 OXJ, UK.
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anaerobically and conservation of biomass *via* recycle. A secondary objective was to select for organisms within the population with enhanced magnitude of phosphate flux. Such cells would accumulate more metal per cycle, settle more rapidly in the settling stage and hence enter the recycle loop for repeated selection. The ability of *Acinetobacter* to take up acetate and store carbon (as polyhydroxyalkanoates, PHAs) under anaerobic conditions to provide energy for subsequent phosphate deposition as polyP aerobically was also considered because this determines the amount of Pi potentially available for anaerobic efflux and coupling to heavy metal removal on the next cycle.

Methods

Organism, growth and preparation of cells

A strain of *A. johnsonii* ("M45") was isolated from the Severn–Trent pilot wastewater treatment plant, Milcote, Warwickshire, UK operating EBPR. The isolate was selected for its polyP-accumulating properties on the basis of a positive reaction to the Neisser stain [13] and was identified using the Analytical Profile Index (API NE) kit (bioMérieux, Marcy L'Étoile, France), negative Gram reaction and nonmotile, cocco-bacillary morphology. The strain was maintained on agar slopes made from minimal salts medium (below) solidified with 1.5% (w/v) granulated agar (BBL, Becton Dickinson, Oxford, UK). Slopes were stored at 4°C and subcultured every 2 weeks.

Media and batch cell growth

Minimal salts medium (MSM:aerobic medium) buffered at pH 7.1 with 50 mM Tris (hydroxymethyl) aminomethane/HCl contained the following components (per liter): sodium acetate (trihydrate); 20 g, $(NH_4)_2SO_4$; 1.75 g, $MgSO_4 \cdot 7H_2O$; 0.16 g, $CaCl_2 \cdot 2H_2O$; 0.08 g, KCl; 0.6 g, K_2HPO_4; 0.65 g, EDTA (disodium salt); 0.25 mg, trace elements solution; 1 ml (containing, per liter: $ZnSO_4 \cdot 7H_2O$, 10 mg; $MnCl_2 \cdot 4H_2O$, 3 mg; H_3BO_3, 30 mg; $CoCl_2 \cdot 6H_2O$, 20 mg; $CuCl_2 \cdot 6H_2O$, 1 μg; $NiCl_2 \cdot 6H_2O$, 2 mg; $Na_2MoO_4 \cdot 2H_2O$, 3 mg). Phosphate stock solution was sterilized separately and added after it had cooled. Anaerobic medium was prepared as above except that phosphate was omitted, and the concentration of sodium acetate (trihydrate) was reduced to 1.3 g/l. During recycle operation, sodium acetate was omitted from the aerobic medium. Antifoam (polypropylene glycol) was added to all media at approximately 100 mg/l. Unless stated otherwise, cells were grown batchwise in 25 ml of MSM medium in 250-ml Erlenmeyer flasks shaken (170 rpm) at 30°C. Batches were inoculated from a starter culture grown as above, which in turn was inoculated from a maintenance agar slope.

Bioreactor operation

The continuous culture system (30°C), operated aseptically, comprised an aerobic vessel, an anaerobic vessel, and a settling vessel (Figure 1). The aerobic vessel (New Brunswick C30 "Bioflo" reactor, working volume of 300 ml) was stirred magnetically (600 rpm) and aerated using on-line compressed air at a flow rate of 1 l/min (3.33 vessel volumes per minute). The mean cell residence time was 3.0 h. (aerobic medium feed was at D=0.33). The dissolved oxygen concentration was maintained at 70–80% of saturation (polarographic DO_2 probe constructed in the School of Chemical Engineering, University of

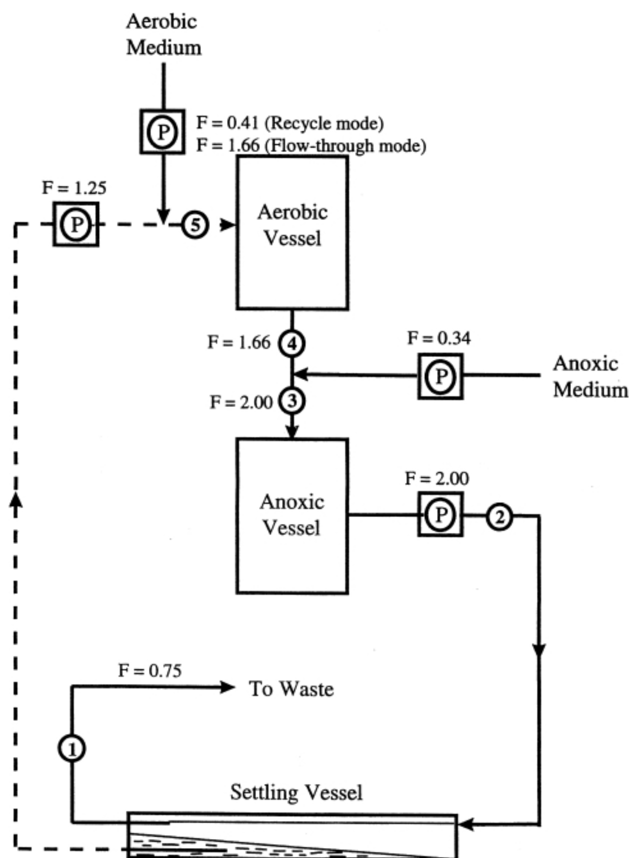

Figure 1 Schematic representation of the three-stage, continuous cycling bioreactor during recycle operation. Circled numbers indicate sampling points: 1, settler overflow; 2, anaerobic effluent; 3, anaerobic influent; 4, aerobic effluent, 5, aerobic influent. P: pump, F: volumetric flow rate (ml min^{-1}). During flow-through mode, the anaerobic vessel overflow was sent to waste and the flow rate of the aerobic influent was 1.66 ml min^{-1}.

Birmingham). The anaerobic vessel (working volume 300 ml) was stirred magnetically (120 rpm) and anaerobic conditions were promoted by sparging with oxygen-free nitrogen. The mean cell residence time was 2.5 h (anaerobic medium feed was at D=0.4). The settling vessel comprising a glass tube (30 mm×400 mm) received outflow from the anaerobic vessel. At the distal end, a weir maintained the liquid volume at 250 ml. Settled cells withdrawn through a second outlet at the base were returned to the aerobic vessel as required. The settler was placed after the anaerobic stage to select for the fastest-settling cells (i.e., those with more loaded metal and thus, greater phosphate release). The approximate mean cell residence time (unstirred) was 2 h. Initially the system was operated in flow-through mode; cells passed from the aerobic vessel to the anaerobic vessel, to the settling vessel and then to waste. Subsequently, the system was operated in recycle mode; settled cells were recycled as above with the overflow from the settler passing to waste. In recycle mode, the proportion of recycled medium and fresh medium was 1.25:0.41 (combined flow rate was 1.66 ml/min, Figure 1). In flow-through mode, the flow comprised aerobic medium only at a flow rate of 1.66 ml/min. Samples (5 ml) were taken at the five sampling points shown in Figure 1, at the times indicated (arrowed) on Figure 2. Samples were divided, 1 ml was centrifuged immediately and the supernatant was stored

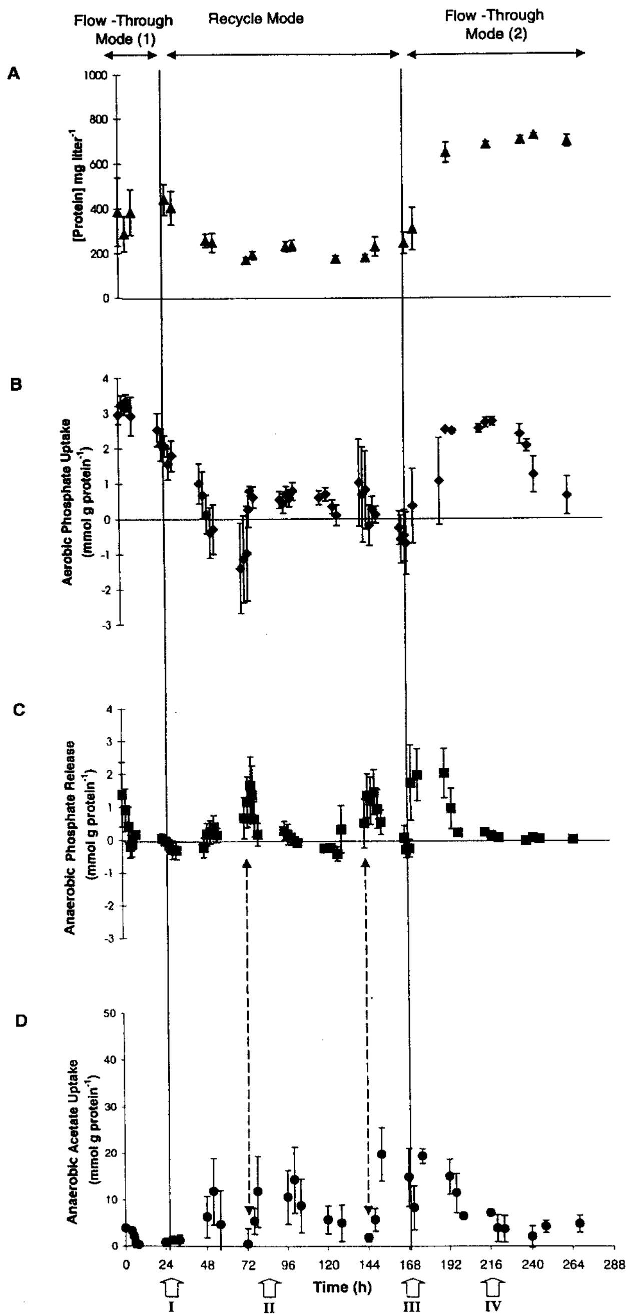

Figure 2 (A) Protein concentration; (B) aerobic phosphate uptake; (C) anaerobic phosphate release; and (D) anaerobic acetate uptake by cells of *A. johnsonii* M45 in a three-stage, aerobic–anaerobic cycling bioreactor. Flow-through refers to the exposure of the biomass to one period of aerobic incubation (3.0 h) followed by a period of anaerobic incubation (2.5 h) and thence to waste. Recycle refers to repeated aerobic (3.0 h)–anaerobic (2.5 h) periods with a settling period following each anaerobic period (see text). Symbols are mean of three independent experiments using separate bioreactors, error bars, ± 1 standard error. Open arrows (samples I–IV) indicate times at which samples were removed for flow cytometry and electron microscopy. Vertical broken arrows are periodic minima of acetate uptake (D) corresponding to onset of periodic increases in phosphate release (C).

at -20°C before analysis. The remainder of the samples were used for protein analysis, and where appropriate, electron microscopy.

Lanthanum accumulation

Lanthanum (La^{3+}) was used as a model heavy metal [33], justified in this study because it tends to be less toxic than many other heavy metals and because preliminary tests using *Acinetobacter* spp. and activated sludge showed comparable phosphate release and heavy metal (Pb, La) accumulation in each (C.D. Boswell, R.E. Dick and L.E. Macaskie, unpublished). The system was operated as described above, initially in flow-through mode, then under recycle. The medium was as above except that the buffer used was 40 mM MOPS (2-(*N*-morpholino) propane sulphonic acid)–NaOH, pH 7.1. After 36 h operation in recycle mode 1 M $LaCl_3$ (filter sterilized) was fed into the anaerobic medium to give a final concentration (in the anaerobic vessel) of 0.1 mM La^{3+}. After one and two further 24-h periods, additional 1 M $LaCl_3$ was added to give 0.2 mM and 0.3 mM La^{3+}, respectively. Residual La^{3+} in supernatants was analyzed colorimetrically using Arsenazo III by a method modified from that of Tolley [32]. Sample (0.2 ml) or standard, 2.0 ml deionized water, 0.3 ml of 0.75M HCl, and 0.1 ml 0.15% (w/v) Arsenazo III (Fluka Poole, Dorset, UK) were added to a 3-ml disposable plastic cuvette (Sarstedt), mixed and the A_{652} measured immediately. Metal-free glassware was prepared by soaking it overnight in 2% Decon 90 (Decon Laboratories, Fisher Scientific, Loughborough, Leicestershire, UK) then rinsing it three times in distilled, deionized water.

Electron microscopy

Samples (1 ml) were centrifuged (9000 rpm; Heraeus "Sepatech Biofuge A"; 10 min, ambient temperature), fixed by resuspension in 2.5% v/v glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2, 1 h), recentrifuged, and resuspended in 0.85% (w/v) sodium chloride. Fixed samples were stored at 4°C until used, centrifuged and resuspended in 1% w/v osmium tetroxide in 0.1 M phosphate buffer (pH 7.2, 1 h), dehydrated sequentially in an ethanol/water series (v/v): 70%, 90%, 100%, 100%, dried in 100% ethanol and embedded in a 1:1 (v/v) mixture of propylene oxide/Araldite resin. The resin was degassed and allowed to polymerize at 60°C (16 h). Ultrathin sections (70 nm) were cut using a Reichart–Jung Ultracut E microtome and stained with uranyl acetate and Reynolds' lead citrate [25]. The samples were examined using a JEOL Ex transmission electron microscope with an accelerating voltage of 80 kV. For examination using energy dispersive X-ray micro-analysis, sections (100–150 nm) were cut as above and 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 discrete electron-opaque areas, cell wall regions (to obtain a "background" signal attributable to Os in the sample: none was detected), and also in background areas in the intercellular resin.

Analysis of biomass protein and residual phosphate and acetate in culture fluids

Protein concentrations in cell pellets were determined using the Sigma protein test kit TPRO562 according to the manufacturer's instructions. The phosphate concentration of supernatants was determined as the reduced molybdo-complex by the method of Pierpoint [23] with minor modifications as described by Tolley [32]. Residual acetate was determined in supernatants by high-performance liquid chromatography (Waters-Millipore HPLC

Table 1 The effect of mode of operation[a] on correlation coefficient (r^2)[b] for anaerobic acetate uptake and anaerobic phosphate release

Bioreactor mode	r^2	n
Flow-through (I)	0.536	7
Recycle	0.023	17
Flow-through (II)	0.825	11

[a]See Methods.
[b]Correlations were calculated on the basis of n data points as shown: each value is the mean from three independent chemostats at a given time.

system: 490E UV Detector (254 nm), Waters Intelligent Sample Processor (WISP), 510 pump, pump control module and Millennium 2010 software for data processing) using a Hamilton PRP-X300 ion-exclusion (poly-(styrene-divinylbenzene) sulfonate) column for separations and a mobile phase of 0.5 mM H_2SO_4 (Sigma, St. Louis, MO; HPLC grade, flow rate 2 ml/min). The retention time of acetate was 3.7 min.

Flow cytometry

Cells were stained with the lipid-specific dye Nile red (Sigma) according to published methods [12,21]. Cells from the samples were harvested by centrifugation as above and resuspended in 2 ml of Dulbecco's buffered saline (DBS, containing, per liter: $CaCl_2$, 0.1 g; KCl, 0.2 g; KH_2PO_4, 0.2 g; $MgCl_2 \cdot 6H_2O$, 0.1 g; NaCl, 8.0 g; Na_2HPO_4, 1.15g). Nile red stock solution (20 μl; 10 μg/ml in acetone) was added to 2 ml of cell suspension (approx. OD_{600}=0.1) 30 min before analysis. Stained cells were diluted 100-fold in DBS to which 10 μl/ml (final concentration) of the Nile red stock solution was added immediately before analysis. Buffer and dye solutions were passed through a 0.2-μm pore size filter immediately before use. The fluorescence (λ_{em} max=625 nm) resulting from the interaction of Nile red with cellular lipids [12] was measured at 630 nm in a Coulter EPICS ELITE flow cytometer with 488-nm excitation from an argon-ion LASER at 15 mW [5]. WinMDI version 2.5 [34] was used for data processing.

Results

Bioreactor operation

Protein analysis (Figure 2A) showed that upon switching from flow-through to recycle mode the biomass protein initially decreased then subsequently remained at a constant level of approximately 200 mg/l. On return to flow-through mode, the biomass increased by >1.5-fold by comparison with the first flow-through period. Phosphate uptake and/or release in the three vessels is defined as the difference in Pi concentration between the respective influent and effluent culture supernatants and is expressed with respect to biomass concentration (mmol Pi/g protein). During the initial phase of flow-through operation (Figure 2B), Pi was taken up in the aerobic vessel (approx. 3.0 mmol/g protein). Approximately 10% (0.3 mmol/g protein) of Pi taken up aerobically was released in the anaerobic vessel (Figure 2C). Anaerobic acetate uptake was approximately 2 mmol/g protein (Figure 2D).

Exposure of cells to repeated aerobic–anaerobic cycles (recycle mode: Figure 2) had differing effects on their carbon and phosphate metabolism; aerobic phosphate uptake (Figure 2B) fell by more than 3-fold, to <1 mmol/g protein. In the subsequent flow-through period, the level of phosphate uptake was not significantly different (P>0.1) from that in the first flow-through period. Following the switch from flow-through (single-pass) to recycle operation, a maximum of 1.97 (±0.39) mmol Pi/g protein was released anaerobically (Figure 2C) but this level was not sustained; some periodicity (approx. 72 h: broken arrows) was observed but repeated aerobic–anaerobic cycles did not have a significant effect (P>0.1) on the overall level of anaerobic phosphate release. Upon return to flow-through conditions, the level of anaerobic phosphate release did not differ significantly initially (P>0.1) from that observed in the first flow-through period, and was negligible after 192 h.

Uptake of acetate under anaerobic conditions (Figure 2D) increased approximately 4-fold from an average of 1.85 (±0.44) mmol/g protein during the first flow-through period, to an average of 7.26 (±0.55) mmol/g protein during recycle operation. There was a significant difference (P=0.05–0.1) between the levels of anaerobic acetate uptake before and after exposure of cells to repeated aerobic–anaerobic cycles. In addition, there was an interval of approximately 48 h (six cycles) before an increase in acetate uptake was observed. Acetate uptake fell significantly to minima, which mirrored the periods of onset of maximum phosphate release (Figure 2C and D, broken arrows). There was no correlation between anaerobic acetate uptake and anaerobic phosphate release during recycle, but on return to flow-through conditions a correlation was apparent that was greater than that observed during the initial flow-through period (Table 1).

Lanthanum accumulation

Where La^{3+} was incorporated, Pi release (0.5 mmol/l, corresponding to approx. 1 mmol/g protein) was observed initially in the anaerobic and settling stages. Following addition of La^{3+}, the soluble Pi was greatly reduced. Comparison of the La^{3+} concentration of fluid entering the anaerobic vessel and exiting the settling vessel (Figure 3) showed its removal from solution, even at the highest concentration tested (0.3 mM, see earlier). The free Pi concentration (i.e., non biomass-bound phosphate) of the fluid entering the anaerobic vessel was negligible (the maximum observed concentration was 29 μM). Ninety-five percent of the lanthanum added was retained in the system and the capacity of the

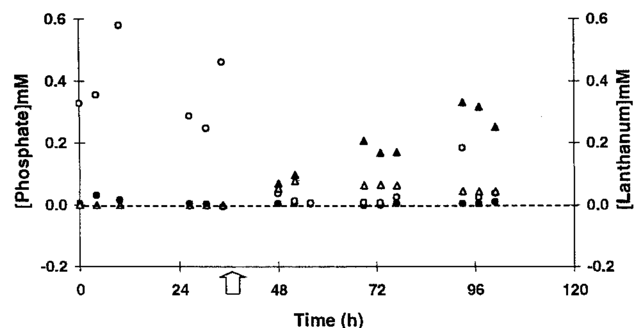

Figure 3 Concentrations of phosphate (●) and lanthanum (▲) in supernatants from fluid entering the anaerobic vessel and of phosphate (○) and lanthanum (△) in supernatants from fluid leaving settling vessel overflow. Open arrow indicates time at which lanthanum addition began.

biomass to accumulate La^{3+} was not exceeded in the course of the experiments.

Flow cytometry

Samples for flow cytometry were taken at the same time as those for electron microscopy. To detect differences between Nile red-induced fluorescence (resulting from changes in lipid content) of cells after aerobic and anaerobic incubation, samples from the aerobic and anaerobic vessel from each time point were mixed and treated as described above. The presence of a single population with respect to red fluorescence would show that there is no difference between the lipid content of the cells analyzed, whereas the presence of two or more populations would indicate that cells within the sample have differing lipid content. Figure 4 shows that with respect to red fluorescence the sample from the anaerobic influent (cells before the anaerobic phase) contained two

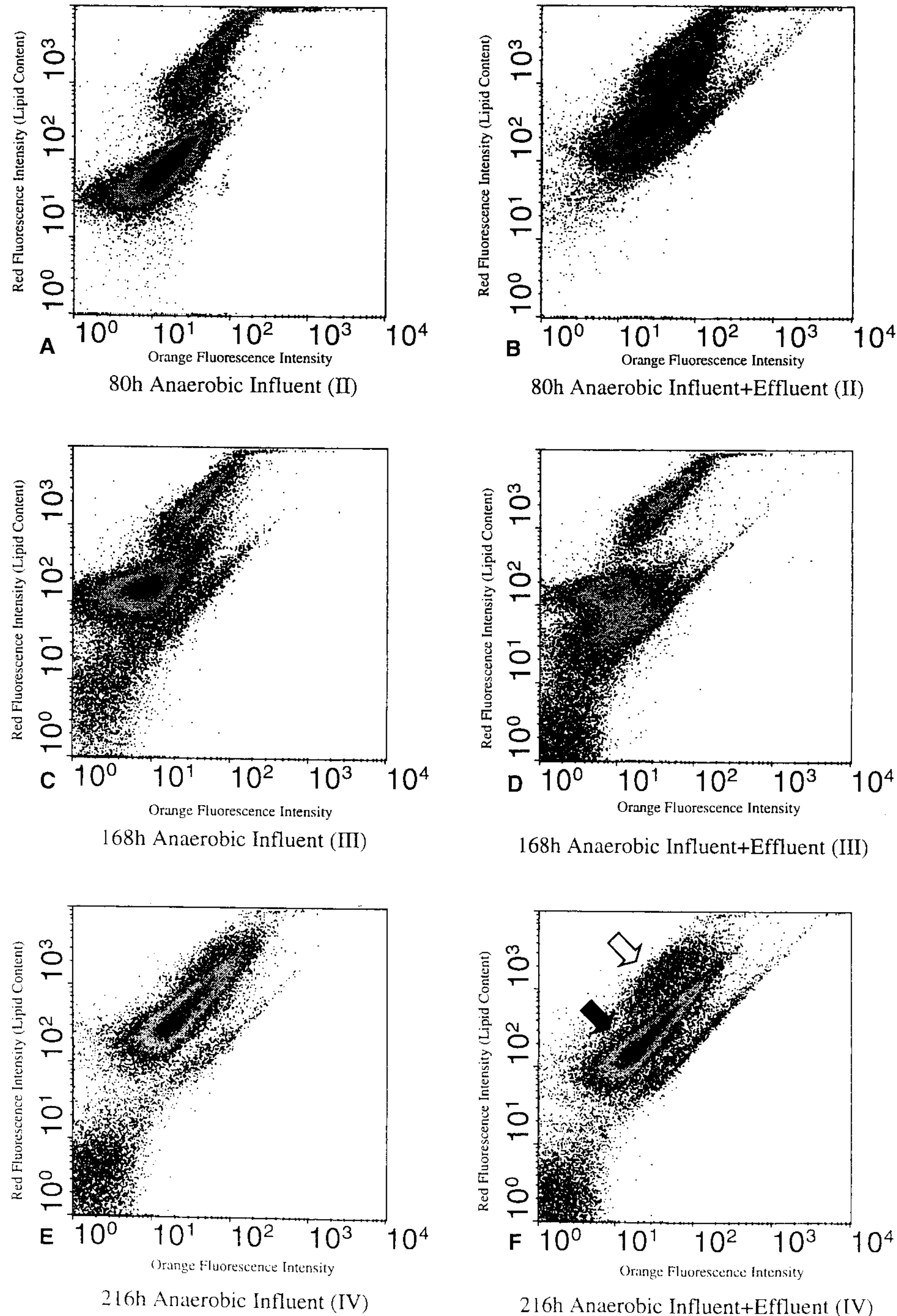

Figure 4 Detection of intracellular lipid-induced Nile red fluorescence in samples from two points in a three-stage, continuously cycling bioreactor by flow cytometry. Sampling points II–IV were as shown in Figure 2. II: (A) Anaerobic influent after 80 h (middle of recycle phase), (B) mixture of anaerobic influent and effluent after 80 h. III: (C) Anaerobic influent after 168 h (end of recycle phase), (D) mixture of anaerobic influent and effluent after 168 h. IV: (E) Anaerobic influent after 216 h (middle of second flow-through phase), (F) mixture of anaerobic influent and effluent after 216 h.

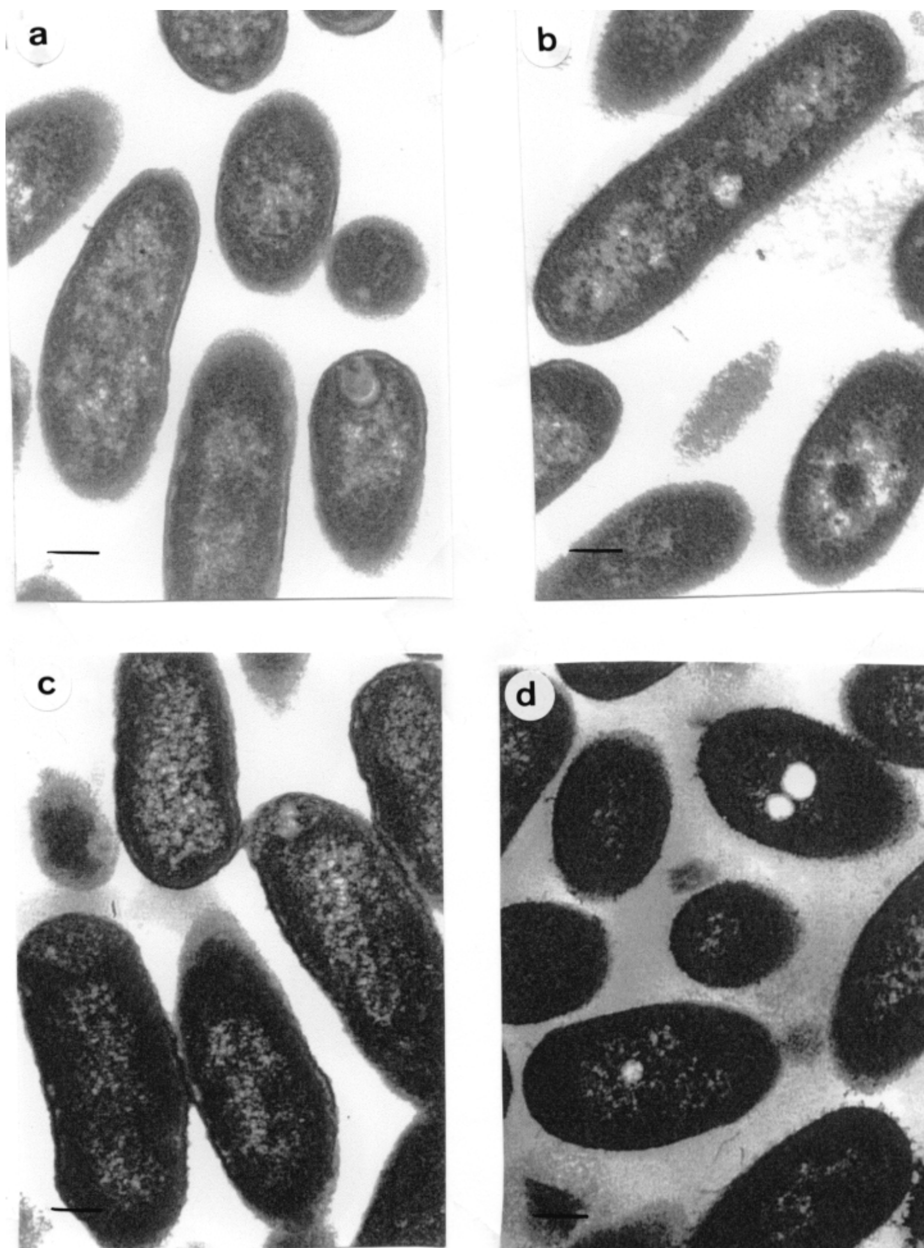

Figure 5 Transmission electron microscopy of *A. johnsonii* M45 cells taken from three-stage aerobic–anaerobic, cycling bioreactor. Samples were taken as follows: I: 48 h (end of first flow-through phase), (a) aerobic vessel, (b) anaerobic vessel. II: 96 h (middle of recycle phase), (c) aerobic vessel, (d) anaerobic vessel. Bar is 200 nm.

populations at 80 h (Figure 4A: recycle mode), four populations at 168 h (Figure 4C: end of recycle mode), and three at 216 h (Figure 4E: flow-through mode). In the latter the third populations contained little lipid. When samples from the anaerobic influents and effluents (cells before and after the anaerobic phase) were combined, three populations were detected at 80 h (Figure 4), four at 168 h (Figure 4D), and four at 216 h (Figure 4F) with respect to red fluorescence. Only in the mixed sample from 216 h (second flow-through period following recycle period: Figure 4F) was a population (open arrow) observed that was not present in the corresponding sample before the exposure and which had greater red fluorescence with respect to the major population (filled arrow).

Electron microscopy

Samples for electron microscopy were taken during bioreactor operation (open arrows, Figure 2). Cells were examined for morphological and ultrastructural changes resulting from exposure to aerobic–anaerobic cycling. Transmission electron micrographs showing representative fields of view are shown in Figure 5. During the first 96 h, the anaerobic samples had large electron-transparent inclusions that were not apparent in the aerobic samples (Figure 5b, d; cf. Figure 5a, c), continuing up to 216 h; electron-transparent inclusions increased in both size and number during this time in accordance with the flow cytometric data (Figure 5; cf. Figure 4). In addition, electron-opaque particles (diameter approx. 25–50 nm), containing phosphorus as identified by energy dispersive X-

ray microanalysis, were observed from samples taken from both the aerobic and the anaerobic vessel during the second flow-through phase (not shown) but not the initial flow-through phase (Figure 5). A more detailed description of localization of metal in the granules (where Cd^{2+} was granule-associated but UO_2^{2+} was not) and cellular polyphosphate (using ^{31}P NMR as a confirmatory probe) was reported previously [6].

Discussion

At the beginning of recycle the biomass protein declined as a result of carbon/energy limitation (acetate was supplied only to the anaerobic vessel during recycle mode), reaching a new steady state at approximately 48 h (Figure 2A). The settling stage allowed the process to proceed at a lower growth rate whilst the dilution rate remained constant, the new level of biomass representing the difference between loss of biomass from the system (attributable to inefficiency in the settling vessel whereby not all of the biomass was returned to the aerobic vessel (data not shown), a proportion being removed to waste) and continued aerobic cell division. An increase in biomass upon resumption of flow-through mode from the level observed during recycle mode was as expected because acetate was again supplied during the aerobic phase. The higher level of biomass attained during the second flow-through phase may be attributable to adaptation to C-limited conditions whereby on return to a higher level of exogenous carbon/energy source, the substrate was more efficiently assimilated and converted into biomass (protein). Limitation of the carbon/energy source required for Pi uptake and polyP formation is also likely to have resulted in the decline in aerobic phosphate uptake (Figure 2B) upon switching to recycle. Subsequent stabilization of aerobic phosphate uptake after 72 h may represent an adaptation of cells to aerobic–anaerobic cycling, possibly by using carbon stored in the form of PHAs as an endogenous energy source for subsequent phosphate uptake [8,24,29].

Exposure of *A. johnsonii* to repeated aerobic–anaerobic cycles (recycle operation), with acetate supplied to the anaerobic vessel only, appeared to stimulate anaerobic acetate uptake (Figure 2D). Conditions within the three-phase system probably produced selective pressure favouring cells that could use carbon source stored (as PHA) under anaerobic conditions to support growth in the following aerobic phase. Under the conditions of the present study, Pi released and acetate taken up was correlated but only in flow-through mode and more strongly during the second flow-through period, following the recycle interlude (Table 1). At two points during recycle, high levels of Pi release (at approx. 72 and 144 h, Figure 2C) corresponded to low levels of acetate uptake (Figure 2D). Some authors [8,29] have suggested a positive correlation between anaerobic acetate uptake and Pi release using mixed cultures, proposing a requirement for polyP degradation/Pi release to supply energy for PHB formation. However, in batch experiments with pure cultures, Rustrian *et al* [26] found that anaerobic Pi release was influenced by the organic substrate (acetic, propionic, or butyric acid), growth phase, and the *Acinetobacter* strain used. In similar experiments, Ohtake *et al* [22] found that Pi release in a strain of *Acinetobacter calcoaceticus* was unaffected by the organic substrate because acetate was not taken up during anaerobic Pi release. We suggest that batch data may be influenced by the previous "history" of the strain; the apparently conflicting observations between published (batch-culture) data and the present study could be attributed to the underlying biochemical processes and their control, such that in a given system a range of responses is possible.

In accordance with published work [24] the detection of electron-transparent inclusions using electron microscopy provided evidence for the formation of lipid material in the anaerobic cells (Figure 5). The recently developed method of Nile red staining in conjunction with bacterial flow cytometry [12,21] revealed discrete subpopulations with respect to lipid content and showed that the proportions of cells within these populations can vary depending on the conditions (aerobic or anaerobic) to which the cells have previously been subjected. The presence of a population of cells with greater red fluorescence following anaerobic exposure to acetate (Figure 4E and F) indicates that the lipid content of these cells increased during this phase, consistent with the postulated increase in PHB synthesis (see above). The presence of further discrete subpopulations within samples, compared to a continuous distribution, is of interest because it may represent the divergence of metabolic types under selective pressure.

Variation in polyP metabolism has been observed between *Acinetobacter* isolates from wastewater treatment plants operating EBPR [4,14] and it has been found [14] that there was no correlation between the point in the treatment plant from which *Acinetobacter* spp. were isolated, their taxonomic group, and the regulation of their polyP metabolism. Thus, it appears that metabolic variation can exist between closely related *Acinetobacter* isolates and that, furthermore, different metabolic strategies may arise within aerobic/anaerobic cycling systems. The close relationship between phosphate and PHB metabolism in *Acinetobacter* [19,27] suggests that the above observations regarding polyP metabolism are likely to extend also to PHB metabolism.

Preliminary studies [10] showed that La^{3+} readily forms insoluble phosphate deposits attached to *Acinetobacter* cell surfaces. In the present study the ability of the system to remove La^{3+} continuously from solution was demonstrated. Pi was released by the biomass in the anaerobic and settling vessels before addition of La^{3+} (Figure 3). The concentration of free phosphate entering the anaerobic vessel was negligible and lanthanum was accumulated by precipitation with Pi released by the biomass during the anaerobic and settling stages, as shown previously using temporally separated aerobic and anaerobic periods [10]. Although it has been proposed that the principal mechanism of metal removal in activated sludge systems is through sorption processes [15], it appears likely that where EBPR processes are used, precipitation of metals as phosphates will contribute significantly to the total metal removal. Evidence that the presence of heavy metal ions promotes polyP degradation has been described [31], whereas Buchan [7] has suggested that metal ions may contribute to the regulation of the phosphate uptake/release mechanism in EBPR. Indeed, removal of phosphate from free solution by precipitation with heavy metal ions may lead to an increased, externally directed, phosphate gradient, promoting polyP degradation and phosphate release.

This model study demonstrated that a pure culture of *A. johnsonii* can take up and release phosphate in a continuously cycling aerobic–anaerobic system in which the biomass concentration is limited by the level of carbon source (acetate). Subjecting this organism to such conditions promoted anaerobic acetate uptake and resulted in biomass in which, when returned to flow-through conditions, the coupling between anaerobic acetate uptake and phosphate release was increased relative to that observed before recycle operation. It is proposed that cells acclimated to acetate-limited conditions form increased amounts of PHA when returned

to medium with excess acetate. The three-phase system also allowed spatial separation of Pi uptake and release, and coupling of the latter to the continuous removal of heavy metal ions from solution. Further work would determine the maximum metal loading of the biomass, and hence the possibility of commercial exploitation of the process for bioremediation of dilute heavy metal solutions: the nonspecificity of *Acinetobacter*-based heavy metal accumulation systems toward Cd^{2+}, La^{3+}, and UO_2^{2+} and the stoichiometry of Pi release with metal uptake in a discontinuous system was demonstrated previously [10]. Although phosphate release and metal removal were similar between *Acinetobacter* spp. and activated sludge (C.D. Boswell, R.E. Dick and L.E. Macaskie, unpublished) *Acinetobacter* spp. probably comprise a small proportion of the EBPR microflora (see the Introduction) and further studies would expand this approach to the study of mixed EBPR populations.

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