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Role of Cations in Accumulation and Release of Phosphate by *Acinetobacter* Strain 210A

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Cells of the strictly aerobic *Acinetobacter* strain 210A, containing aerobically large amounts of polyphosphate (100 mg of phosphorus per g [dry weight] of biomass), released in the absence of oxygen 1.49 mmol of P_i, 0.77 meq of Mg^{2+}, 0.48 meq of K^+, 0.02 meq of Ca^{2+}, and 0.14 meq of NH_4^+ per g (dry weight) of biomass. The drop in pH during this anaerobic phase was caused by the release of 1.8 protons per PO_4^{3-} molecule. Cells of *Acinetobacter* strain 132, which do not accumulate polyphosphate aerobically, released only 0.33 mmol of P_i and 0.13 meq of Mg^{2+} per g (dry weight) of biomass but released K^+ in amounts comparable to those released by strain 210A. Stationary-phase cultures of *Acinetobacter* strain 210A, in which polyphosphate could not be detected by Neisser staining, aerobically took up phosphate simultaneously with Mg^{2+}, the most important counterion in polyphosphate. In the absence of dissolved phosphate in the medium, no Mg^{2+} was taken up. Cells containing polyphosphate granules were able to grow in a Mg-free medium, whereas cells without these granules were not. Mg^{2+} was not essential as a counterion because it could be replaced by Ca^{2+}. The presence of small amounts of K^+ was essential for polyphosphate formation in cells of strain 210A. During continuous cultivation under K^+ limitation, cells of *Acinetobacter* strain 210A contained only 14 mg of phosphorus per g (dry weight) of biomass, whereas this element was accumulated in amounts of 59 mg/g under substrate limitation and 41 mg/g under Mg^{2+} limitation. For phosphate uptake in activated sludge, the presence of K^+ seemed to be crucial.

Enhanced biological phosphate removal from wastewater, a biological alternative to chemical phosphate precipitation, is based on the enrichment of activated sludge with polyphosphate-accumulating bacteria (13). *Acinetobacter* is a very important genus in this process (5), and some studies on polyphosphate accumulation and degradation by *Acinetobacter* spp. have been carried out recently in pure cultures (9, 13, 23, 28). As is the case with activated sludge, in which biological phosphate removal has been observed, *Acinetobacter* spp. take up phosphate under aerobic conditions and release it anaerobically (10). Polyphosphate in *Acinetobacter* spp. is present in granules (13), and the negative charge of this polyanion is neutralized by cations (5, 27). As a result, simultaneous uptake and release of phosphate and of cations can be expected. In wastewater treatment plants with biological phosphate removal, this effect has already been observed. In the anaerobic areas of these plants, phosphate is released together with Mg^{2+} and K^+ (4, 7, 20, 29); under aerobic conditions, phosphate is taken up again simultaneously with Mg^{2+} and K^+ and small amounts of Ca^{2+} (7, 29). Kainrath et al. (16) analyzed the dry matter of sludge produced by the biological phosphate removal process and found that there was a correlation of total P content with Mg^{2+} and K^+ content but not with Ca^{2+}, Fe^{3+}, and Al^{3+} content. From these observations, it was concluded that the enhanced biological phosphorus removal from wastewater was indeed mainly a biological process, with chemical precipitation playing a minor role. However, it is possible that with an influent of another composition, such as water with high calcium concentrations, chemical precipitation is more important. An example of combined chemical and biological phosphorus removal has been reported by Arvin and Kristensen (4). They found that phosphate release from sludge under anaerobic conditions was always accompanied by a release of K^+ and Mg^{2+} and a decrease in Ca^{2+} concentration. In the two plants investigated, 50 to 60% of the phosphate in the sludge was precipitated and the dominating counterions in the precipitate were Ca^{2+} (high amounts) and Mg^{2+} (lower amounts). From these observations, it might be concluded that only Mg^{2+} and K^+ are important counterions in biological phosphate removal; however, this conclusion is at variance with the findings of Buchan (5). By means of energy-dispersive analyses with X rays of the electron-dense granules in sludge, Buchan proved that calcium plays a predominant role in the stabilization of polyphosphate ions and that a calcium requirement for enhanced phosphorus uptake does not necessarily point to chemical precipitation.

To distinguish between biological and chemical removal of phosphate and cations, we undertook a study of pure cultures of *Acinetobacter* strains 210A and 132, both isolated from activated sludge. This paper reports the results of experiments on the effects of K^+, Mg^{2+}, and Ca^{2+} on polyphosphate formation and degradation as well as on the role of polyphosphate as a possible cation (Mg^{2+}) reserve.

MATERIALS AND METHODS

Organisms. *Acinetobacter* strains 210A and 132 were isolated from activated sludge by the method described by Deinema et al. (9). Strain 210A is able to accumulate large amounts of polyphosphate, whereas strain 132 is not. The organisms were maintained on yeast extract agar slants (5 g of glucose, 2.5 g of yeast extract, and 12 g of agar per liter of tap water, pH 7), subcultured every 2 months, and stored at 4°C.

Media. Medium for batch cultivation containing butyrate

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as the carbon and energy source was prepared according to van Groenestijn et al. (28). Medium for batch cultivation containing acetate as the carbon and energy source was identical to the medium with butyrate except that sodium butyrate was replaced by 5.67 g of $NaOAc \cdot 3H_2O$. For optimal growth and polyphosphate accumulation, the acetate and butyrate media, respectively, were used (27).

Medium for cultivation under phosphorus limitation contained (per liter of demineralized water) 7 g of $NaOAc \cdot 3H_2O$, 0.1 g of $Na_2S_2O_3 \cdot 2H_2O$, 1 g of NH_4Cl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 40 mg of KH_2PO_4, 0.06 g of $CaCl_2 \cdot 2H_2O$, 2 ml of trace elements (28), and 12 g of Tris. The pH was adjusted to 7.0 with 6 N HCl.

Phosphate release medium contained (per liter of demineralized water) 2.5 g of acetic acid and 4 g of Tris. The pH was adjusted to 7.5 with 6 N HCl.

Unbuffered phosphate release medium (used in the experiments in which the decrease in pH caused by phosphate release was studied) contained (per liter of demineralized water) 20 mmol of butanol and 6 g of NaCl. The pH was adjusted to 7.5 with 0.1 N NaOH. Butanol, also a substrate of *Acinetobacter* strain 210A, was chosen because it does not buffer the medium as acetate or butyrate would do.

Phosphate uptake medium for the *Acinetobacter* cultures contained (per liter of demineralized water) 136 mg of KH_2PO_4, 11 mg of NH_4Cl, 44 mg of $CaCl_2 \cdot 2H_2O$, 61 mg of $MgCl_2 \cdot 6H_2O$, 12 mg of NaCl, 300 mg of acetic acid, 6 g of Tris, and 200 mg of streptomycin sulfate. The pH was adjusted to 7.0 with 6 N HCl. Experiments with various concentrations of K^+ were carried out by replacing KH_2PO_4 with equimolar amounts of $NaH_2PO_4 \cdot 2H_2O$. K^+ was added as KCl.

Phosphate uptake medium for activated sludge contained (per liter of demineralized water) 0.6 g of $MgSO_4 \cdot 7H_2O$, 0.25 g of $NaH_2PO_4 \cdot 2H_2O$, 1 g of NH_4Cl, 0.07 g of $CaCl_2 \cdot 2H_2O$, 1.5 g of Tris, and various amounts of KCl. The pH was adjusted to 7.0 with 6 N HCl.

Medium without Mg^{2+} (used in the Mg reserve batch experiments) contained (per liter of demineralized water) 5.67 g $NaOAc \cdot 3H_2O$, 1 g of NH_4Cl, 0.44 g of KH_2PO_4, 0.1 g of $Na_2S_2O_3 \cdot 5H_2O$, 0.07 g of $CaCl_2$, and 2 ml of a trace mineral solution (28). The pH was brought to 7.0 with 6 N HCl.

Continuous cultivation under substrate and cation limitation was carried out with three media. (i) Substrate limitation medium was basic medium containing (per liter of demineralized water) 5.67 g of $NaOAc \cdot 3H_2O$, 1 g of NH_4Cl, 0.6 g of $MgSO_4 \cdot 7H_2O$, 0.44 g of KH_2PO_4, 0.1 g of $Na_2S_2O_3 \cdot 5H_2O$, 0.07 g of $CaCl_2$, and 2 ml of a trace mineral solution containing 50 g of EDTA, 5 g of $FeSO_4 \cdot 7H_2O$, 1.6 g of $CuSO_4 \cdot 5H_2O$, 5 g of $MnCl_2 \cdot 4H_2O$, 1.1 g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 2.2 g of $ZnSO_4 \cdot 7H_2O$, 50 mg of H_3BO_3, 10 mg of KI, and 50 mg of $CoCl_2 \cdot 6H_2O$ per liter of demineralized water. The pH was brought to 7.0 with 6 N HCl. The trace mineral solution used in this experiment differed from that used in the batch experiments for the practical reason that no precipitation was formed after extended sterilization of the complete medium in 10-liter carboys. (ii) Mg^{2+} limitation medium was basic medium containing only 80 mg of $MgSO_4 \cdot 7H_2O$ per liter of demineralized water. (iii) K^+ limitation medium was basic medium in which KH_2PO_4 was replaced by equimolar amounts of $NaH_2PO_4 \cdot 2H_2O$; 15 mg of KCl was added.

Phosphate release experiments. *Acinetobacter* strains 210A and 132 were grown at 15°C in shaken Erlenmeyer flasks in medium containing butyrate as the carbon and energy source. Cultures from the log phase of strain 210A, containing 100 mg of phosphorus per g (dry weight) of biomass, and of strain 132, containing only 26 mg of phosphorus per g (dry weight) of biomass, were centrifuged and washed with demineralized water. The pellets were suspended in 100-ml serum vials containing 100 ml of phosphate release medium. The vials were made free of oxygen by flushing them with a gas mixture of N_2-CO_2 (99%:1%, vol/vol). Before incubation, samples were taken with a syringe for determination of biomass content. The serum vials were placed in a shaker (to keep the biomass in suspension) at 30°C. At various time intervals, 12-ml portions were taken and centrifuged. The supernatant was analyzed for K^+, Ca^{2+}, Mg^{2+}, NH_4^+, Na^+, and P_i.

Phosphate uptake experiments. Cells of *Acinetobacter* strain 210A were precultured under P limitation at 25°C in shaken Erlenmeyer flasks until growth stopped. At that time, no polyphosphate granules could be seen in strain 210A after staining by the method of Neisser (14). Grown cultures were centrifuged and washed twice with a Tris hydrochloride buffer, and the pellets were suspended in phosphate uptake medium to obtain a biomass concentration of 0.5 g (dry weight) per liter. The acetate in this medium served as the energy source for the uptake processes, while streptomycin was added to prevent growth of the cells. During the experiments, the dry-weight content of the biomass increased by about 8% as a result of phosphate and cation uptake. After the phosphate was taken up, polyphosphate granules were visible by Neisser staining. Experiments were also carried out in phosphate uptake medium from which K^+, Mg^{2+}, Ca^{2+}, or P_i was omitted. In addition, the ATP content of cells exposed to various K^+ concentrations was measured after 0.5 h of incubation.

Uptake of phosphate by activated sludge in mineral media with various K^+ concentrations was investigated as well. Sludge was obtained from an intermittently aerated laboratory-scale pilot plant which biologically removed phosphate from synthetic wastewater (3). When the sludge sample was taken at the end of the anaerobic period, phosphate had been released from the cells. To remove adsorbed K^+, the sludge was centrifuged and washed twice at 4°C with a solution of 9 g of NaCl per liter of demineralized water. The pellet was suspended in mineral phosphate uptake medium. All experiments with sludge were carried out in Erlenmeyer flasks at 25°C on a rotary shaker (200 rpm). At various time intervals, samples were taken to measure P_i content and dry weight.

Continuous cultivation. *Acinetobacter* strain 210A was continuously cultivated under substrate, K^+, or Mg^{2+} limitation in a 2-liter bioreactor (Applikon, Schiedam, The Netherlands) with automatically controlled pH (7.0) and temperature (25°C). When cultures had reached a steady state, samples were taken and centrifuged. The supernatant was analyzed for K^+, Mg^{2+}, P_i, and acetate; the pellet was then washed with demineralized water and used for determination of dry-weight content.

Mg reserve experiments. Cells of *Acinetobacter* strain 210A containing 75 mg of P per g (dry weight) of biomass were cultivated in batch in a medium containing acetate as the carbon and energy source, harvested, washed with demineralized water, and centrifuged as described for the phosphate release experiments. Phosphorus-poor cells (those with only 20 mg of P per g [dry weight] of biomass) were obtained as described for the phosphate uptake experiments. All precultures were grown at 15°C. The pellets were suspended in medium without Mg^{2+} and with acetate as the carbon and energy source. The cell suspensions were shaken

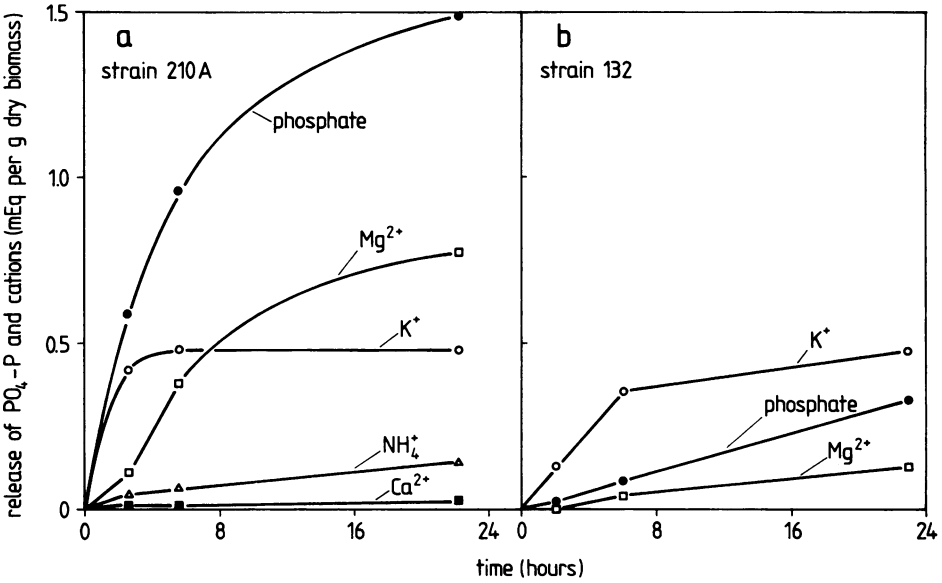

FIG. 1. Release of P_i (1 meq = 1 mmol), Mg^{2+}, K^+, NH_4^+, and Ca^{2+} by *Acinetobacter* strains 210A and 132 under anaerobic conditions at 30°C and pH 7.5. The data were determined in one experiment and are representative of results obtained in five (strain 210A) or three (strain 132) other experiments.

in Erlenmeyer flasks at 25°C. At various time intervals, samples were taken for estimation of biomass dry weight.

Reproducibility. Although rates and quantities varied considerably among experiments (the most extreme cases differed by 30%), the stoichiometry between phosphate and the various cations and among the cations themselves remained constant in each experiment.

Chemicals. Streptomycin sulfate was obtained from Sigma Chemical Co., St. Louis, Mo. Peroxidase and the ATP bioluminescence constant light signal test combination including luciferase-luciferin reagent and disodium ATP were obtained from Boehringer GmbH, Mannheim, Federal Republic of Germany.

Analyses. P_i content was determined spectrophotometrically as described by the American Public Health Association (2), using the ascorbic acid method. K^+, Mg^{2+}, Ca^{2+}, and Na^+ were quantified by using an atomic absorption spectrophotometer. NH_4^+ was analyzed with the Nessler reagent as described by the American Public Health Association (2). Acetate was measured after addition of Amberlite IR-120H ion exchanger (Rohm & Haas Co., Philadelphia, Pa.), using gas-liquid chromatography with a Varian (Palo Alto, Calif.) 2400 Aerograph (1). We determined bacterial dry weight after centrifuging a 50-ml sample of the culture, washing the pellet once with demineralized water, and drying the pellet at 100°C overnight. Dry weights were also estimated from optical density at 660 nm (i.e., optical density as a function of dry weight) by using a calibration curve. The phosphorus content of the biomass was calculated from the increase in biomass dry weight and the decrease in P_i concentration in the bulk during cultivation. The major part of the accumulated phosphorus was polyphosphate, and the rest (±8 mg/g [dry weight] of biomass) was present in other compounds (23). The ATP content of the culture was quantified as described by Pradét (24). Samples (0.5 ml) of culture were boiled for 4 min in 5 ml of a solution containing 50 mM Tris hydrochloride and 4 mM EDTA, pH 7.75, in order to extract ATP from the cells and stop all enzyme activity. Luciferase-luciferin reagent was added to a subsample of this solution, and the resulting light emission was measured with a luminescence meter (University of Georgia, Athens). Polyphosphate was stained by the Neisser method (14). The polyamines spermine, spermidine, and putrescine were measured by the method described by Nickerson et al. (22), which is based on thin-layer chromatography of dansylated polyamines. Cell extracts were obtained by sonicating cell suspensions in 0.1 M $MgCl_2$ for 10 min. A portion of the cell extracts was treated with 6% perchloric acid, and another portion was boiled for 20 min with 5% trichloroacetic acid to degrade polyphosphate and nucleic acids. The presence of putrescine was also tested by an enzymatic method (18, 19). Cell extracts of *Acinetobacter* strain 210A were heated for 5 min at 80°C to inactivate all enzymes. Putrescine in the extracts was oxidized with amine oxidase from *Trichosporon adeninovorans* CBS 8244 precultivated on putrescine. In the presence of peroxidase, the H_2O_2 produced oxidized 2,2'-azino-di-(3-ethylbenzthiazolinsulfonate) in a substance that absorbs light with a wavelength of 660 nm.

RESULTS

Release of phosphate and cations. The anaerobic release of phosphate, Mg^{2+}, K^+, Ca^{2+}, and NH_4^+ by cultures of *Acinetobacter* strain 210A containing 100 mg of phosphorus per g (dry weight) of biomass is presented in Fig. 1. Mg^{2+} and K^+ were the most important cations released under anaerobic conditions. For comparison, release of phosphate, Mg^{2+}, and K^+ by *Acinetobacter* strain 132, which is unable to accumulate polyphosphate, was also determined. Strain 132 released anaerobically the same amount of K^+ as did strain 210A but much less phosphate and Mg^{2+} (Fig. 1). Release of K^+ by strain 210A seemed to be independent of phosphate release, because release of K^+ was completed earlier than that of phosphate and Mg^{2+} (Fig. 1) and at low pH the release of K^+ was less inhibited than the release of phosphate (data not shown). At pH 5.9 and 30°C, 1 g of biomass released anaerobically within 3 h 0.13 mmol of K^+ but only 0.05 mmol of phosphate.

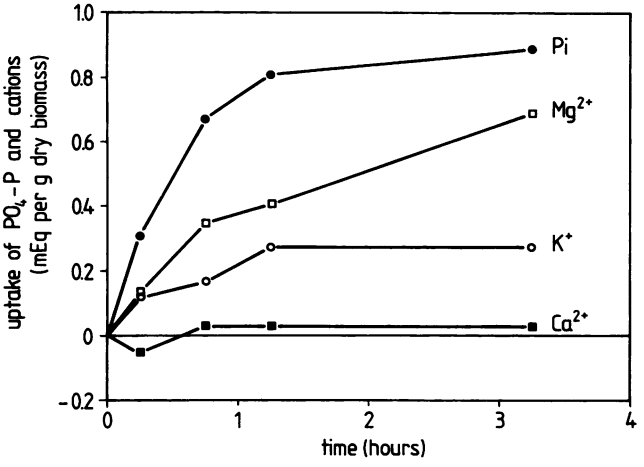

FIG. 2. Uptake of phosphate (1 meq = 1 mmol), Mg^{2+}, K^+, and Ca^{2+} by stationary-phase cultures of *Acinetobacter* strain 210A in medium with acetate and streptomycin (pH 7, 25°C). Data are from one experiment and are representative of results obtained in other experiments carried out by the same procedure.

In phosphate release medium without Tris hydrochloride buffer, the pH decreased from 7.4 to 6.4 within 6 h while 10.5 mg of P_i phosphorus per liter (0.34 mM) was released by cells of strain 210A. In a control experiment with the same medium but without biomass, the pH decreased from 7.4 to 6.4 after addition of 0.28 mM KH_2PO_4 plus 0.06 mM $KNaHPO_4$. From these results, it can be calculated that the drop in pH was caused by release of 1.8 protons per PO_4^{3-} molecule.

Uptake of phosphate and cations. Concomitantly with P_i, cations were taken up by a culture of *Acinetobacter* strain 210A devoid of polyphosphate (Fig. 2). This experiment was carried out in complete phosphate uptake medium. To prevent growth and thus interference between phosphate uptake for polyphosphate formation and phosphate incorporation in newly formed cells (biomass), streptomycin was added to the medium. To measure the relationship between phosphate and the different cations, individual cations were omitted from the complete uptake medium (Fig. 3). Simultaneously with uptake of P_i, K^+ and Mg^{2+} were incorporated into the cells. All three ions, if present, were absorbed quickly in the first hour (Fig. 2 and 3b through d). Later, the rate diminished steadily. The concentrations of Na^+ and NH_4^+ did not change much, and there was no correlation with phosphate uptake. The largest uptake or release of NH_4^+ and Na^+ occurred mostly in the first 15 min (results not shown). Mg^{2+} correlated best with the amount of phosphate that was accumulated; if no phosphate was taken up by the cells, Mg^{2+} uptake was reduced markedly (Fig. 3a). The equivalents of Mg^{2+} were 60 to 100% of the equivalents of phosphate taken up. Uptake of K^+ was independent of

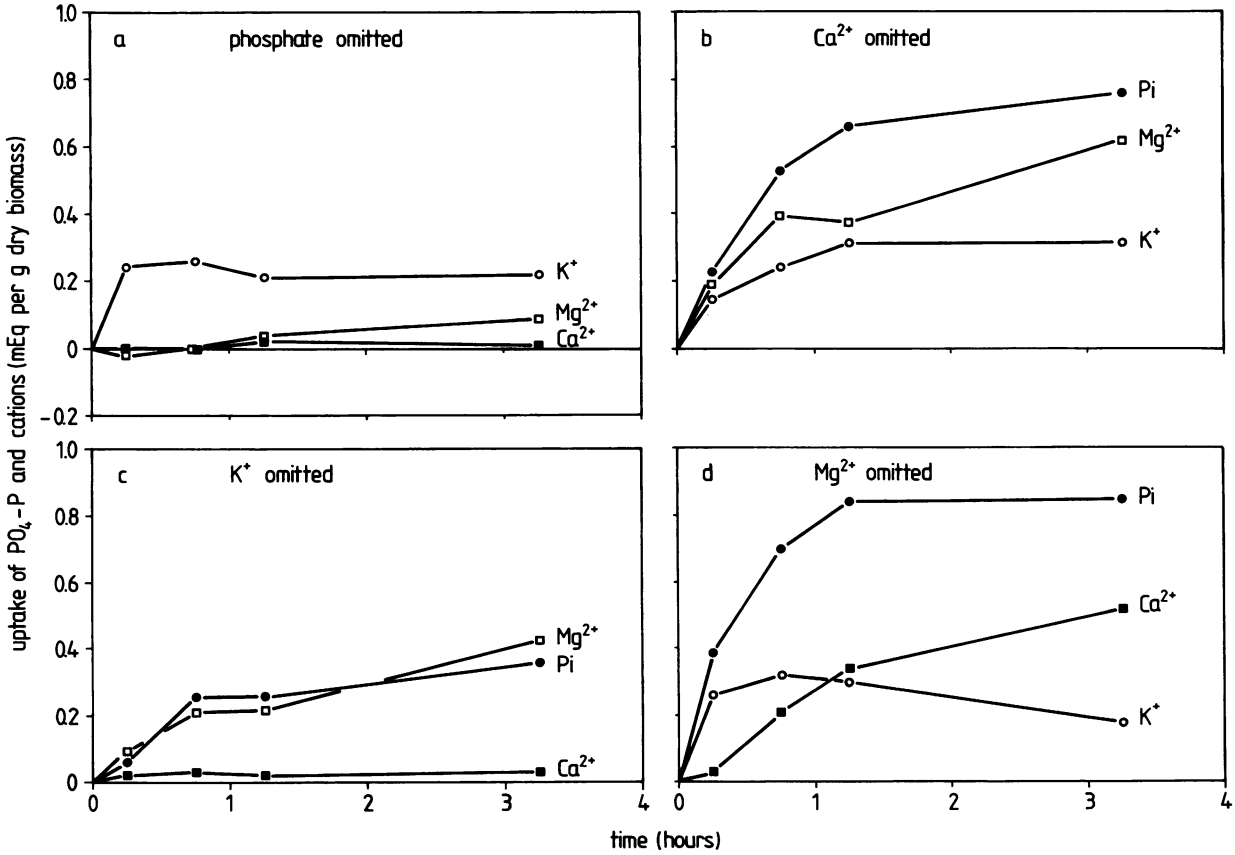

FIG. 3. Uptake of phosphate (1 meq = 1 mmol), Mg^{2+}, K^+, and Ca^{2+} by stationary-phase cultures of *Acinetobacter* strain 210A in medium with acetate and streptomycin (pH 7, 25°C) under the limitations shown. Data in panels a through c are from one experiment and are representative of results obtained in other experiments carried out by the same procedure. Data in panel d are mean values from five experiments in which three separately grown cultures were used that took up 0.85 ± 0.07 mmol of phosphate per g of biomass dry weight.

TABLE 1. Effect of extracellular K^+ concentration on uptake of phosphate by stationary-phase cultures of *Acinetobacter* strain 210A[a]

Time (h)	Phosphate uptake (mmol of PO_4-P per g [dry wt] of biomass) at K^+ concn (mg/liter) of:		
	0.5	10	100
0.1	0.16	0.21	0.13
0.5	0.40	0.55	0.63
1	0.51	0.72	0.82
2	0.65	0.96	1.11
4	0.74	1.04	1.12

[a] Cells were cultured in medium containing acetate and streptomycin (pH 7, 25°C). Data are from one experiment and are representative of results obtained in other experiments carried out by the same procedure.

phosphate absorption. In the absence of phosphate in the medium, K^+ uptake showed the same behavior as in the complete medium (Fig. 2 and 3a). The absence of Ca^{2+} did not affect the uptake of phosphate, Mg^{2+}, and K^+ (Fig. 3b), whereas less phosphate and Mg^{2+} were absorbed in the absence of K^+ (Fig. 3c). Omission of Mg^{2+} from the medium did not reduce the uptake of phosphate, but Ca^{2+} instead of Mg^{2+} was taken up in large amounts (Fig. 3d).

In a separate experiment, cells of *Acinetobacter* strain 210A containing no polyphosphate were suspended in phosphate uptake medium at various initial concentrations of K^+. This cation stimulated the rate of phosphate uptake and increased the final concentration of phosphate in the cells (Table 1). For maximum phosphate uptake, K^+ concentrations higher than 10 mg/liter were necessary. Concentrations of 1.0, 1.5, and 3.5 mg of K^+ per liter were tested as well, but the amounts of phosphate taken up at these concentrations did not differ much (±10%) from those at 0.5 mg of K^+ per liter. After 0.5 h of incubation, the ATP content of the cells was measured. ATP content decreased with increasing K^+ concentrations, from 7.2 to 2.6 and 3.0 μmol of ATP per g (dry weight) of biomass at K^+ concentrations of 0.5, 10, and 100 mg/liter, respectively. Phosphate uptake by activated sludge showed a similar dependence on the addition of K^+ (Fig. 4), but in these experiments a lower K^+ concentration (5 mg/liter) was sufficient for maximum phosphate uptake. Concentrations of 12, 25, 40, and 70 mg of K^+ per liter were also tested with the same sludge sample. Phosphate uptake did not differ more than 30% from that measured at 5 and 100 mg of K^+ per liter. A different sludge sample, taken from the same pilot plant 1 week earlier, accumulated 30% more phosphate in the presence of 100 mg K^+ per liter, whereas the same level of uptake as in the first sludge sample was found in the absence of K^+.

Polyphosphate accumulation in continuous cultures under various limitations. *Acinetobacter* strain 210A was continuously cultivated at different dilution rates under substrate, K^+, or Mg^{2+} limitation (Table 2). During cultivation with limited amounts of cations, a steady state was always reached in which acetate was still present in considerable amounts while the biomass concentration was not more than half that found in substrate-limited cultures. Cells cultivated under Mg^{2+} limitation were still able to accumulate considerable amounts of phosphate, whereas K^+ limitation had a strongly negative effect on this accumulation. At low dilution rates, at which the steady-state concentration was lowest, no polyphosphate was accumulated; the cells contained only small amounts of phosphorus and did not color upon Neisser staining.

Polyamines. Spermidine and spermine could not be detected in *Acinetobacter* strain 210A by thin-layer chromatography. However, *Acinetobacter* extracts showed clear spots on the thin-layer plates at the same place as dansylated putrescine. This spot occurred in all extracts from strain 210A, regardless of whether they were obtained from cells with polyphosphate or from cells cultivated under P limitation and thus without polyphosphate. Measurements of fluorimetric intensities revealed an unexpectedly high concentration of about 30 mg of putrescine per g of biomass. With the enzymatic method, however, putrescine could not be positively identified. Control solutions containing only putrescine or putrescine and cell extracts of strain 210A always showed positive reactions, but cell extracts of strain 210A alone were invariably negative.

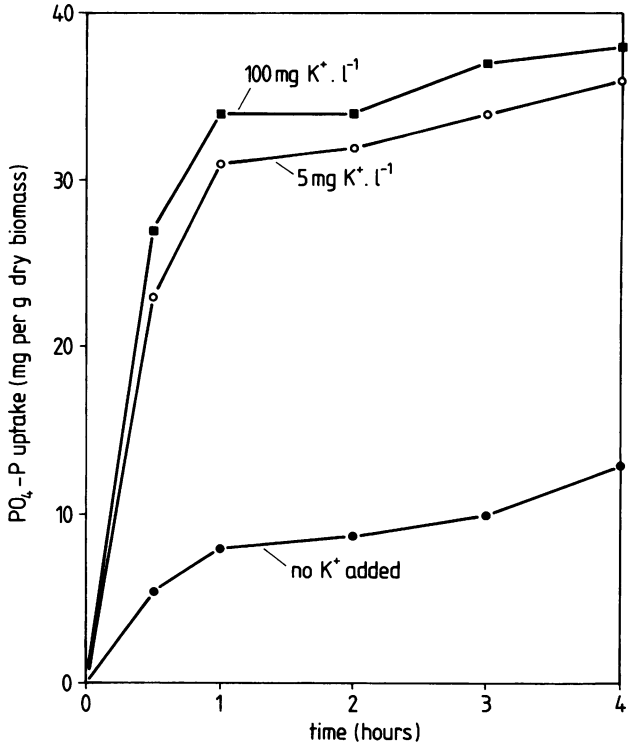

FIG. 4. Uptake of phosphate by activated sludge in an aerobic mineral medium with different initial K^+ concentrations.

Polyphosphate granules as a Mg reserve. Two cultures of *Acinetobacter* strain 210A were suspended in a Mg^{2+}-free growth medium. One culture, containing 75 mg of phosphorus per g (dry weight) of biomass, was able to grow. The second, with only 20 mg of phosphorus per g (dry weight) of biomass, grew considerably less. Cells from this culture were able to grow at high rates only in medium to which magnesium was added (Fig. 5).

DISCUSSION

The experiments reported here indicate that Mg^{2+} is the most important counterion of polyphosphate in *Acinetobacter* strain 210A. In this strain, Mg^{2+} was always released and taken up simultaneously with phosphate (Fig. 1 and 3a through c); strain 132, which does not accumulate polyphosphate, released only small amounts of Mg^{2+} under the same conditions. In the phosphate uptake experiments, every millimole of phosphate was taken up with 0.6 to 1 meq of Mg^{2+}. This uptake is enough to counterbalance a major part

TABLE 2. Growth and P content of *Acinetobacter* strain 210A in continuous culture with acetate medium under substrate, K^+, or Mg^{2+} limitation[a]

Limiting nutrient	Dilution rate (per h)	Biomass (mg [dry wt] per liter)	Concn of:			P content in biomass (mg/g [dry wt])
			Acetate (mM)	K^+ (mg/liter)	Mg^{2+} (mg/liter)	
Acetate (carbon and energy source)	0.09	624	0.2	12	59	59
	0.18	844	0.3	14	53	66
Mg^{2+}	0.06	314	21	85	0.2	41
	0.11	290	27	89	0.3	55
K^+	0.08	362	4.8	1.4	80	14
	0.20	310	20	2.6	77	26

[a] Data were obtained under steady-state conditions at pH 7 and 25°C.

of the negative charge of polyphosphate, since each phosphate group in this molecule carries one negative charge. Release of phosphate by strain 210A was also accompanied by a decrease in pH of the medium, which was caused by the release of 1.8 mmol of H^+ per mmol of PO_4^{3-}, which approaches the number of protons per phosphate expected during the net hydrolysis of magnesium polyphosphate.

Spermidine and spermine, which could act as organic counterions in polyphosphate, were not detected in *Acinetobacter* strain 210A. The presence of putrescine was very doubtful. The occurrence of a compound comigrating with putrescine was independent of the polyphosphate content of the cells.

The uptake and release of K^+ did not correlate with phosphate uptake and release, since (i) strain 132 released under anaerobic conditions the same amount of K^+ as did strain 210A (Fig. 1); (ii) K^+ released by strain 210A reached the maximum concentration much earlier than did the released phosphate (Fig. 1); (iii) at low pH, release of phosphate was slower than release of K^+; and (iv) independent of the presence or absence of phosphate, strain 210A took up a smaller amount of K^+ than of phosphate (Fig. 2 and 3a, b, and d). Uptake and release of K^+ might not be directly related to the accumulation and degradation of polyphosphate but rather to a reestablishment or decline of a specific energetic state of the cells. As indicated by Mulder et al. (21), maintenance of a steep K^+ gradient across the cell membrane costs energy. Since *Acinetobacter* cells are strict aerobes, the energy supply under anaerobic conditions is restricted and release of K^+ can be expected. Although K^+ did not play a quantitatively important role as a polyphosphate counterion in *Acinetobacter* strain 210A, the presence of K^+ in the medium appeared to be crucial for phosphorus accumulation by this strain (Tables 1 and 2) and by samples of activated sludge (Fig. 4). There could be many reasons for this dependency: K^+ could play a role in phosphate transport (17), energy generation (21), or stimulation of enzyme activities (11), or it may be important for the structure of polyphosphate granules. The suboptimal polyphosphate accumulation during growth under K^+ limitation points in the direction of energy shortage, in accordance with the findings of Mulder et al. (21) and Sall et al. (25). Mulder et al. (21) reported that K^+ limitation caused energy limitation, and Sall et al. (25) found that K^+-limited cells of *Corynebacterium diphtheriae* consumed less oxygen and accumulated less phosphate. However, the batch experiments presented in Table 1, in which phosphate uptake and ATP content in *Acinetobacter* strain 210A were measured, show that a shortage of ATP may not be the reason for the inhibition of polyphosphate accumulation by K^+ limitation. At low K^+ concentrations, less phosphate was taken up and the uptake rate was slower, but the cellular ATP content was higher. As ATP is most probably the energy and phosphate donor in polyphosphate synthesis by *Acinetobacter* spp. (26), an effect of K^+ limitation on phosphate transport and ATP production cannot be the cause of decreased polyphosphate accumulation under these conditions. Therefore, K^+ must have another role in polyphosphate accumulation, such as maintenance of a membrane potential (established ultimately by chemiosmotic proton release), which may be required for transport and accumulation of other ions. Furthermore, K^+ may have a role in stimulating the activities of enzymes involved in polyphosphate synthesis or a structural role in the formation of polyphosphate granules. Further research is needed to elucidate the relationship between polyphosphate and K^+ in the genus *Acinetobacter*.

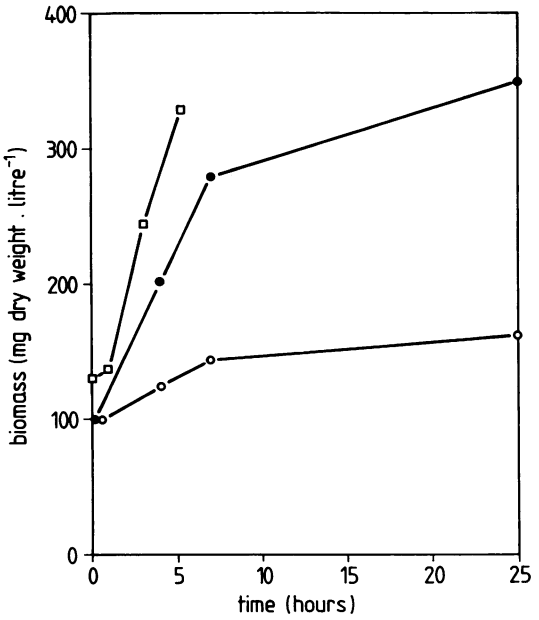

FIG. 5. Growth of cells of *Acinetobacter* strain 210A with polyphosphate (positive by Neisser staining; containing 75 mg of P per g [dry weight] of biomass) suspended in acetate medium without Mg^{2+} (●) and without polyphosphate (negative by Neisser staining; containing 15 mg of P per g [dry weight] of biomass suspended in a medium without (○) or with (□) Mg^{2+} (25°C, pH 7).

Although Mg^{2+} was preferred as a counterion, this cation was not essential for polyphosphate accumulation by *Acinetobacter* strain 210A. If Mg^{2+} was not available as a counterion, it was partly replaced by Ca^{2+} (Fig. 3d). Phosphate accumulation was still possible when the Mg^{2+} supply was

limited in continuous cultures of *Acinetobacter* strain 210A (Table 2).

From the results of the experiments on growth of cells with and without polyphosphate in a Mg^{2+}-free medium, it can be concluded that the polyphosphate granules can also act as a Mg^{2+} reserve. A function of polyphosphate as a cation reserve has always been hypothesized but has been difficult to prove because most microorganisms accumulating polyphosphate contain relatively small amounts of this compound. Since *Acinetobacter* strain 210A accumulates exceptionally large amounts of polyphosphate, the role of this compound as a cation reserve could clearly be demonstrated.

Many cations can serve as counterions in polyphosphate in various microorganisms. In *Micrococcus luteus* (formerly *lysodeikticus*) (12) and in *Desulfovibrio gigas* (15), Mg^{2+} was found to be the most important polyphosphate counterion. The major counterions for vacuolar polyphosphate in *Neurospora crassa* were basic amino acids and spermidine and, to a lesser degree, Mg^{2+} and Ca^{2+} (8). K^+ was found to be a polyphosphate counterion in a *Chlorella* sp. (6). The finding of Buchan (5) that Ca^{2+} is the predominant polyphosphate counterion in acinetobacters in activated sludge was not confirmed by our experiments. Since Buchan used energy-dispersive analysis with X rays, we have used the same technique to determine the quantitative composition of polyphosphate granules. Preliminary results indicate the presence of high amounts of Mg^{2+} and Ca^{2+} and minimal amounts of K^+ (unpublished data). One possible explanation for the differences between using energy-dispersive analysis with X rays and release experiments is that Ca^{2+} is a polyphosphate counterion in *Acinetobacter* spp. which is not released when the polyphosphate is broken down (Ca^{2+} may remain in the cells while P_i is released).

In conclusion, our findings with respect to the uptake and release of cations by pure cultures of *Acinetobacter* strain 210A are in accordance with the results obtained in experiments with activated sludge from wastewater treatment plants showing biological phosphate removal. Mg^{2+} has been reported as the most important counterion during phosphate uptake and release, followed by K^+, whereas Ca^{2+} has been found to play an insignificant role in this process (4, 7, 16, 20, 29). Arvin and Kristensen (4) concluded that Mg^{2+} is a typical counterion in polyphosphate formation in sludge, while Ca^{2+} is accumulated in sludge by means of chemical precipitation. Our results with *Acinetobacter* strain 210A confirm the importance of Mg^{2+} as a counterion of polyphosphate. Uptake and release of K^+ by activated sludge may have no relationship to phosphate uptake and release. This cation is concentrated aerobically inside the cells but not in polyphosphate granules and may be released when energy generation is limited or not possible (e.g., in the absence of oxygen). The importance of the presence of sufficient K^+ in wastewater for biological phosphate removal may be an interesting subject for further studies (Fig. 4).

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