The effect of acetate on polyphosphate formation and degradation in activated sludge with particular reference to *Acinetobacter calcoaceticus*: a microscopic study

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Abstract

An *Acinetobacter calcoaceticus* strain which was isolated from the aerobic zone of a five-stage Bardenpho activated sludge plant, was subjected to phosphate starvation and treatment with excess acetate or succinate prior to resuspension in a medium containing phosphate. Samples of the cells were examined by light and electron microscopy at various stages of the experiment. Similar studies were carried out with samples of mixed liquor from the aerobic zone of the same plant.

Acetate treatment resulted in increased polyphosphate formation after aeration, while phosphate starvation and succinate treatment resulted in polyphosphate accumulation at similar levels to the untreated samples.

Introduction

The phenomenon of enhanced phosphorus removal by activated sludge systems has been well documented (Srinath *et al.*, 1959; Nicholls and Osborn, 1979). However, attempts to elucidate the exact mechanism have not yet been completely successful.

At the microbiological level, the identification of the dominant bacteria responsible for phosphorus removal has been achieved (Buchan, 1983; Kerdachi and Roberts, 1983; Lötter, 1984). It has however, also been shown that the mere presence of this bacterial species, namely *Acinetobacter calcoaceticus* is not sufficient to guarantee good phosphorus removal (Lötter, 1984).

Research on the molecular level has revealed the importance of substrate composition and in particular, the role of acetate in enhanced phosphorus removal (Fuhs and Chen, 1975; Wentzel *et al.*, 1984).

It has become necessary to attempt to link these effects to a particular state in *Acinetobacter calcoaceticus,* which stimulates its ability to accumulate polyphosphate. Polyphosphate accumulation has been reported for a variety of micro-organisms (Harold, 1963; Harold and Sylvan, 1963; Deinema *et al.*, 1980), and in general, occurs in the log phase. It is consumed in the rapid growth phase and accumulates again in the stationary phase (Harold, 1963; Deinema *et al.*, 1980). Accumulation also occurs when growth is inhibited by the lack of an essential ingredient e.g., nitrogen or sulpur or when previously phosphate starved cells are grown in a phosphate rich medium (Harold and Sylvan, 1963; Lawson and Tonhazy, 1980; Deinema *et al.*, 1980; du Preez *et al.*, 1981).

Phosphorus is released on addition of acetate to a mixed liquor sample from the aerobic zone of a plant (Wentzel *et al.*, 1984; Lötter, 1984). The amount of phosphorus released on addition of acetate is considerably higher than on the addition of succinate, which unlike acetate, requires energy to enter the cell (Lötter, 1984).

In this study an attempt was made to compare the effect of acetate and succinate treatment with phosphate starvation on polyphosphate accumulation in *Acinetobacter calcoaceticus.*

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Materials and methods

Activated sludge samples

Mixed liquor samples were taken from the aerobic zone of the Johannesburg Northern Works five-stage activated sludge plant.

The volatile suspended solids content of the mixed liquor was determined by the ignition at 550 °C of the residue retained on a glass fibre filter dried at 105 °C (American Public Health Association, 1981).

Five hundred millilitre aliquots of mixed liquor were used for each experiment. The sample treatments included the addition of acetic acid to a final concentration of 100 mg/ℓ, and resuspension of the solids in acetate/sewage medium (Fuhs and Chen, 1975), modified by the omission of phosphate and fermented raw sewage. The samples were stirred at room temperature for the duration of the experiment. Untreated samples were run simultaneously and all experiments were carried out in duplicate. The samples were subjected to acetate treatment or phosphate starvation for a period of 160 min, after which each sample was centrifuged at 5 000 g for 5 min and the residue resuspended in 250 mℓ modified acetate/sewage medium (Fuhs and Chen, 1975) and aerated at room temperature for a further 160 min. Samples were taken for microscopy at the beginning and end of the treatment period and at the end of each aeration period.

Acinetobacter calcoaceticus cultures

An isolate from the Northern Works plant, which had shown high polyphosphate accumulation and had been maintained by weekly subculture onto GCY agar (Pike *et al.*, 1972) and once monthly subculture onto *Acinetobacter* agar (Fuhs and Chen, 1975), was used for the experiment.

Thirty plates of the culture of *Acinetobacter calcoaceticus* were inoculated into 100 mℓ acetate/sewage medium (Fuhs and Chen, 1975). This culture was incubated at 35 °C with stirring and the volume in the flask doubled by the daily addition of acetate/sewage medium, until a 5 ℓ volume of culture with an optical density between 1 and 2, was obtained. Some of this culture was drawn off for each experiment and new medium added to maintain the culture for subsequent experiments.

Five hundred millilitre aliquots were used for each treatment with sampling and resuspension in acetate/sewage medium, as described for the mixed liqour. In addition to the treatments

already described, succinate at 100 mg/ℓ was added to one sample.

Light microscopy

A few drops of each sample were transferred to glass slides and allowed to dry. Duplicate slides were stained with methylene blue (Fuhs and Chen, 1975) and a modified Neisser stain (Society of American Bacteriologists, 1957), using Bismark brown as counterstain and viewed under a light microscope at 1 250 x magnification.

Electron microscopy

Five millilitre aliquots of each sample were centrifuged at 10 000 g. The pellet was fixed in glutaraldehyde solution and then post-fixed in osmium tetroxide. After each fixation step, the pellets were washed with three changes of sodium cacodylate buffer. The pellets were then dehydrated in a graded alcohol series and 100 % propylene oxide added. This was replaced by a 1:1 mixture of propylene oxide: Epon/Araldite resin which was allowed to infiltrate for 30 min. The pellets were transferred to Beem capsules overlaid by Epon/Araldite resin and polymerised in an oven at 60 °C for at least 48 h.

Sections of 0,4 to 0,6 μm were cut (Buchan, 1983), using an ultramicrotome and transferred onto copper grids. The grids were stained with uranyl acetate and lead citrate (Buchan, 1983). The most effective times were found to be 5 min with uranyl acetate and 3 min with lead citrate. The stained sections were examined on a JEM-100 S transmission electron microscope at 80 keV.

Results

Light microscopy

Comparison of the two stains for polyphosphate inclusions showed that the polyphosphate granules were more clearly distinguishable from other cellular material when stained with the Neisser stain, than with methylene blue. The Neisser stain was therefore used throughout the experiments. A degree of declumping was observed in all samples after resuspension in the acetate/sewage medium.

A large variation in cell size was observed in the *Acinetobacter* culture throughout the experiment; about 50% of the cells contained polyphosphate granules at the start of the experiment. No changes in cell size or number of cells containing polyphosphate granules was observed in the control or succinate treated cells during the experiment. The succinate grown cells were however, smaller than the others.

Acetate treatment resulted in a decrease in cell size and a large reduction in the number of cells containing polyphosphate granules. After the aeration period about 80% of these cells contained polyphosphate granules but did not increase to the size prevailing before treatment. After phosphate starvation, as in the case of acetate, a decrease in cell size was observed and only a few cells contained polyphosphate granules. At the end of the aeration period, a slight increase in the number of cells containing granules was noted.

At the start of the activated sludge mixed liquor experiment, about 70% of the *Acinetobacter* cells contained polyphosphate granules. The average cell size was greater than in the pure culture. As in the case of the pure culture experment, no change was observed in the control sludge throughout the experiment.

At the end of the acetate treatment, a large percentage of the cells were empty and again, as observed in pure culture, a decrease in cell size occurred. After aeration, the cells had increased in size and about 90% of the cells contained small polyphosphate granules. Unlike in the pure culture, a number of dividing cells was observed.

Not much release was observed at the end of phosphorus starvation, although some additional empty cells were present. After aeration, a slight increase in cell size was observed but no increase in size or number of polyphosphate granules was detected.

Electron microscopy

As observed by light microscopy, the electron microscope examination showed that about 50% of the cells of the pure culture contained electron dense polyphosphate granules at the start of the experiment. A number of cells showed areas where granules had been spliced out. Again, no change in cell size or number of cells containing polyphosphate was observed during the experiment. Electron microscopy also revealed the smaller size of the succinate-treated cells (see Plates 1 to 3).

About 50% of the succinate-grown cells contained electron dense areas (PP), identified as granules and a number of the sections contained electron lucent areas where the granules had been spliced out (S). Throughout the succinate treatment, no change in the number or size of the granules was evident and the cell size remained consistently small.

Electron microscopy confirmed the decrease in cell size observed by light microscopy after acetate treatment. Most of the cells contained no electron dense bodies. After aeration, about 80 % of the cells contained large diffuse areas of electron density. The density was however, not as high as in the smaller electron dense areas observed in the cells at the start of the experiment. The electron microscope examination of the cells at the start of the phosphate starvation experiment revealed the presence of developing granules (DE), in addition to completely formed granules (PP). After phosphate starvation, a few cells still contained polyphosphate granules of both types. After aeration, well formed granules appeared in a number of cells (see Plates 4 to 6).

In the activated sludge sample, great variation in cell and polyphosphate granular size was observed with the electron microscope. Again, about 70% of the *Acinetobacter* cells contained polyphosphate granules, mostly in the form of large central inclusions. No observable changes in the control sample occurred during the duration of the experiment (see Plates 7 to 9).

After phosphate starvation, a slight decrease in cell size was observed and a corresponding decrease in granule size, but no entire loss of granules was observed. After aeration, many dividing cells were observed and a number of the cells contained developing granules.

Acetate treatment resulted in most of the *Acinetobacter* cells being empty. In some cells, only ill-defined porous structures remained (arrow). After aeration, the average size of the cells was smaller, as observed light microscopically. Many of the cells contained three or four small granules dispersed throughout the cell rather than one large central granule. About 90% of the cells contained at least one small granule. A number of cells showed the light areas caused by scouring out the granules (S) and some contained developing granules (DE) (see Plates 10 to 12).

Discussion

While the use of a metachromatic stain in conjunction with light

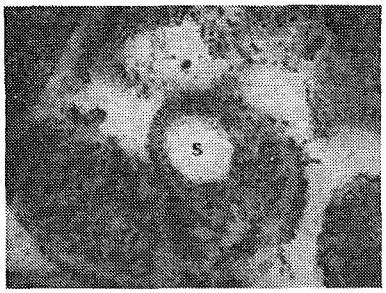

Plate 1.

Acinetobacter cells grown in succinate medium at the start of the experiment.

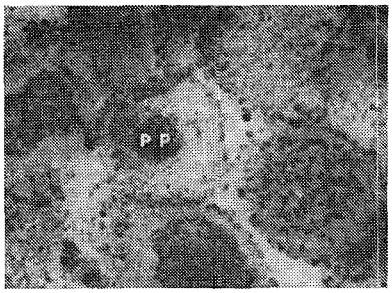

Plate 2.

Succinate grown Acinetobacter cells at the end of succinate treatment

Plate 3.

Succinate treated Acinetobacter cells at the end of the aeration period.

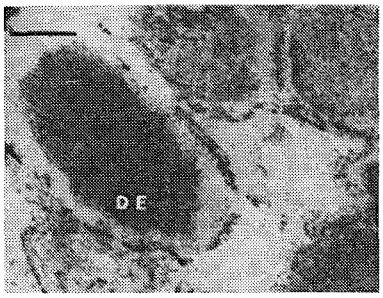

Plate 4.

Acinetobacter cells at start of phosphate starvation.

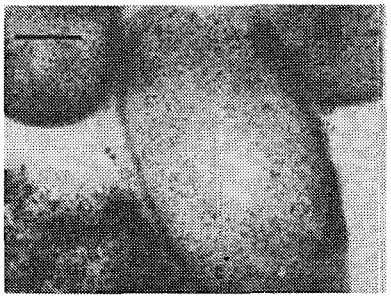

Plate 5.

Acinetobacter cells at the end of starvation period.

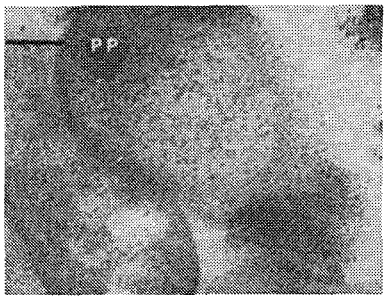

Plate 6.

Phosphate starved Acinetobacter cells at the end of aeration period.

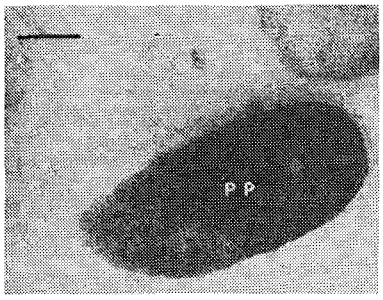

Plate 7.

Control activated sludge at the start of the experiment.

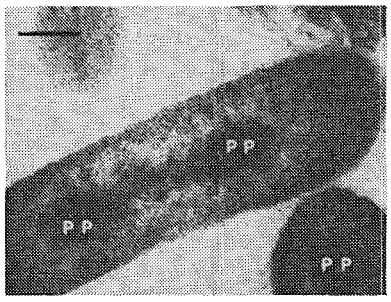

Plate 8.

Control activated sludge at the end of the treatment period.

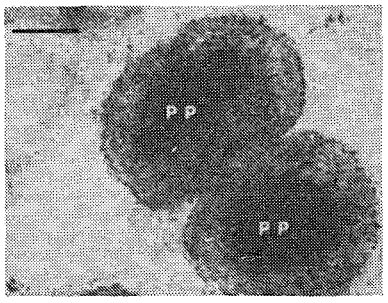

Plate 9.

Control activated sludge at the end of the aeration period.

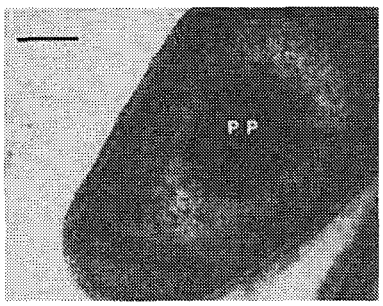

Plate 10.

Activated sludge at the start of the experiment.

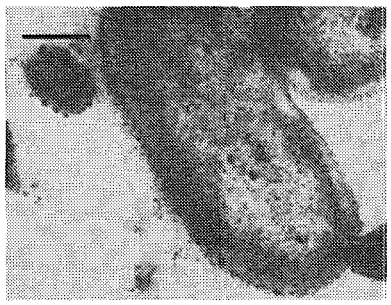

Plate 11.

Activated sludge after acetate treatment.

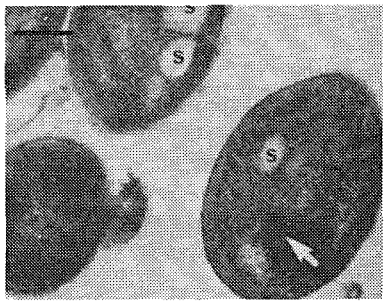

Plate 12.

Acetate treated activated sludge after aeration.

(All sections are 0,4 μ and bar represents 0,2 μ .)

microscopy, is a useful tool in screening samples for polyphosphate accumulaton, the finer details of polyphosphate granule formation and degradation are not observed with this technique.

The use of transmission electron microscopy on the other hand, allows the formation and degradation of polyphosphate granules to be studied in greater detail.

The decrease in cell size observed with light and electron microscopy, particularly after acetate treatment, indicates that the polyphosphate granules contribute to the size of the cell. The generally larger size of the *Acinetobacter* cells in the activated sludge, is probably due to commensalism between them and other sludge bacteria and the wider range of nutrients available in the activated sludge system.

The disappearance of the polyphosphate granules during acetate treatment implicates this phosphorus as the source of the orthophosphate released into the external medium, which has been observed under these conditions (Lötter, 1984; Wentzel *et al.*, 1984). The lighter areas of electron density observed under the electron microscope after this treatment, indicate that the polyphosphate granules are first broken up into smaller granules before disintegrating completely. This phenomenon was observed by Buchan (1983) in activated sludge samples under anaerobic conditions. The similarity between these two observations points to the presence of acetate in the anaerobic zone as being the cause of this disintegration, rather than lack of oygen or some other 'stress' condition.

The presence of one or more small granules in almost all the cells after the aeration period, rather than one large central granule as at the start of the experiment, indicates that the time period of the experiment was not long enough to allow complete formation of the granules. A minimum time of 92 h for the formation of a polyphosphate granule in *Plectonema boryanum* has been reported (Jensen, 1969).

The slight loss of polyphosphate granules during phosphate starvation, is almost certainly due to polyphosphate hydrolysis to replenish the orthophosphate pool after loss of soluble phosphate to the external medium by diffusion.

The treatment appeared to encourage the continued formation of polyphosphate granules, as evidenced by the higher number of completely formed granules, than at the start of the experiment.

The lack of effect on polyphosphate degradation and formation by succinate in comparison to acetate, is explained by the different membrane transport mechanisms for the two compounds. Acetate, which does not require energy to transverse the cell membrane, can still readily enter the cell under conditions of minimal energy supply as under anaerobic conditions, while succinate uptake which is an energy-requiring process (Ramos and Kaback, 1977), cannot take place. The uptake of acetate has been shown to cause dissipation of the proton motive force (Konings *et al.*, 1981). Phosphate is released by the cell to reinstate this essential force.

While acetate treatment and phosphate starvation both result in phosphorus release, similar to that observed in the anaerobic zones of activated sludge plants (Fuhs and Chen, 1975; Barnard, 1976; Buchan, 1983), acetate treatment stimulates polyphosphate formation to a far greater extent than phosphate starvation. It is clear from this that the stimulation of phosphorus release is not the only function of acetate in the enhanced removal of phosphorus from activated sludge. A link between the cellular metabolism of this compound and polyphosphate metabolism is probable and should be investigated. The formation and degradation of polyphosphate also needs to be followed chemically, for a more detailed picture of these reactions.

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