# Changes in Morphology and Phosphate-Uptake Patterns of *Acinetobacter calcoaceticus* strains

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## Abstract

When sewage isolates, identified as *Acinetobacter calcoaceticus* strains, were grown under limited oxygen availability, they changed from typical coccobacilli to large pleomorphic rods. Even where a tendency towards filament production was exhibited under normal conditions, marked differences in appearance were found. The bacteria had an increased capacity for luxury phosphate uptake after they had changed into large rod-shaped cells.

## Introduction

Problems associated with the contribution of phosphorus compounds to pollution in rivers and impoundments are well known. Recognition of these problems and the economics involved in chemically removing phosphates from wastewater effluents led to research on the feasibility of biological phosphate removal in sewage treatment plants.

The biological removal of phosphates in activated sludge systems was observed to occur in a number of sewage treatment installations in various areas in America (Barnard, 1976). Yall *et al.* (1972) found that this was due to the presence of large numbers of volutin, or polyphosphate,-containing bacteria. According to Fuhs and Chen (1975) members of the bacterial genus *Acinetobacter* were mainly responsible for phosphate removal by activated sludge and pure cultures of their isolates were capable of phosphate uptake under aerobic conditions. Anaerobic conditions resulted in phosphate release from the cells.

Polyphosphate accumulation in bacteria is promoted by growth limitation (Harold, 1966). This physiological response was described as luxury phosphate uptake (Fuhs & Chen, 1975), which typically occurred when growth was arrested by lack of a nutrient other than phosphate in the presence of certain organic compounds that could serve as a source of carbon and energy.

Fuhs & Chen (1975) also concluded that wastewater treatment plants should incorporate anaerobiosis to favour facultative anaerobes which produced compounds such as ethanol, acetate and succinate for utilization by *Acinetobacter*, in this way enriching for biological removal of phosphorus by activated sludge. Without the correct anaerobiosis, the assemblage of microorganisms developed would not allow for suitable substrates to be present. They further stated that acinetobacters, which could not attack sugars or polysaccharides, would then be subjected to heavy competition for the supply of amino acids and thus fail to develop.

Using this information and that suggested by other authors (as reviewed by Barnard, 1976), Nicholls (1975) showed how experimentation on design of waste disposal plants in Johannesburg, incorporated the process of biological removal of phosphorus from sludge effluents. To facilitate the design and running of new waste disposal plants, a pilot plant was built at the Johannesburg Northern Works. The design of the pilot plant was described by Osborn & Nicholls (1978). Essentially this incorporated an anaerobic zone to produce carbon substrates utilizable by *Acinetobacter*. Also, to induce luxury uptake of phosphate, all the nitrogen in the effluent was removed by complete nitrification of the nitrogenous wastes. The nitrates were converted to gaseous nitrogen compounds in an "anoxic" zone where, due to absence of oxygen, nitrates acted as electron acceptors. Finally, phosphate removal took place in a zone of aeration, presumably by *Acinetobacter* strains.

Since there were occasions when the amount of phosphate removed in the pilot plant was exceptionally high, it was decided that the microbial population, particularly the acinetobacters, appearing during those periods and the factors promoting their presence should be investigated.

## Methods

### Strains

*Acinetobacter calcoaceticus* bacteria were isolated from the Northern Sewage Works pilot plant, Johannesburg, in a zone showing high phosphate removal. This was done with the enrichment technique described by Fuhs & Chen (1975). After liquid enrichment, plating of serial dilutions was carried out on acetate enrichment agar. Isolated colonies were investigated microscopically and those composed of gram-negative coccobacilli were subcultured and purified as potential *Acinetobacter* strains.

Of the numerous isolates obtained, eight were selected for further characterization according to the methods advocated by Bøvre & Henriksen (1976). Four strains, (SW1b, SW5a, SW8a and SW9b), were chosen for an investigation of their ability to accumulate phosphate.

### Media

1. Growth medium, or peptone-acetate-phosphate broth, had the following composition: Bacteriological Peptone, Oxoid L37, 10 g; NaCl, 2 g; KCl, 1 g; sodium acetate, 5 g; $KH_2PO_4$, 0,5 g; tap water, 1 ℓ. The final pH was adjusted to 7,0.

2. Phosphate-uptake medium, a modified Fuhs & Chen broth consisted of the following: sodium acetate, 5 g; $MgSO_4.7\ H_2O$, 0,5 g; $KH_2PO_4$, 0,25 g; $CaCl_2.6\ H_2O$, 0,01 g; tap water,

200 mℓ; and distilled water, 800 mℓ. Here, too, the final pH was adjusted to 7,0.

No source of nitrogen was provided in order to induce maximum phosphate uptake. It has been reported that polyphosphate accumulation occurs under stress conditions (Harold & Sylvan, 1963) such as nitrogen starvation (Harold, 1963, 1966).

### Growth conditions

Each strain, in growth medium, was cultured for 22 h atr 30 °C. Since the bacteria were obligate aerobes, oxygen was introduced into the medium. This was done in two ways:

(a) 200 mℓ quantities of broth cultures in 500 mℓ Erlenmeyers were incubated in a Gallenkamp Orbital Incubator at a shaking speed of 100 r/min.

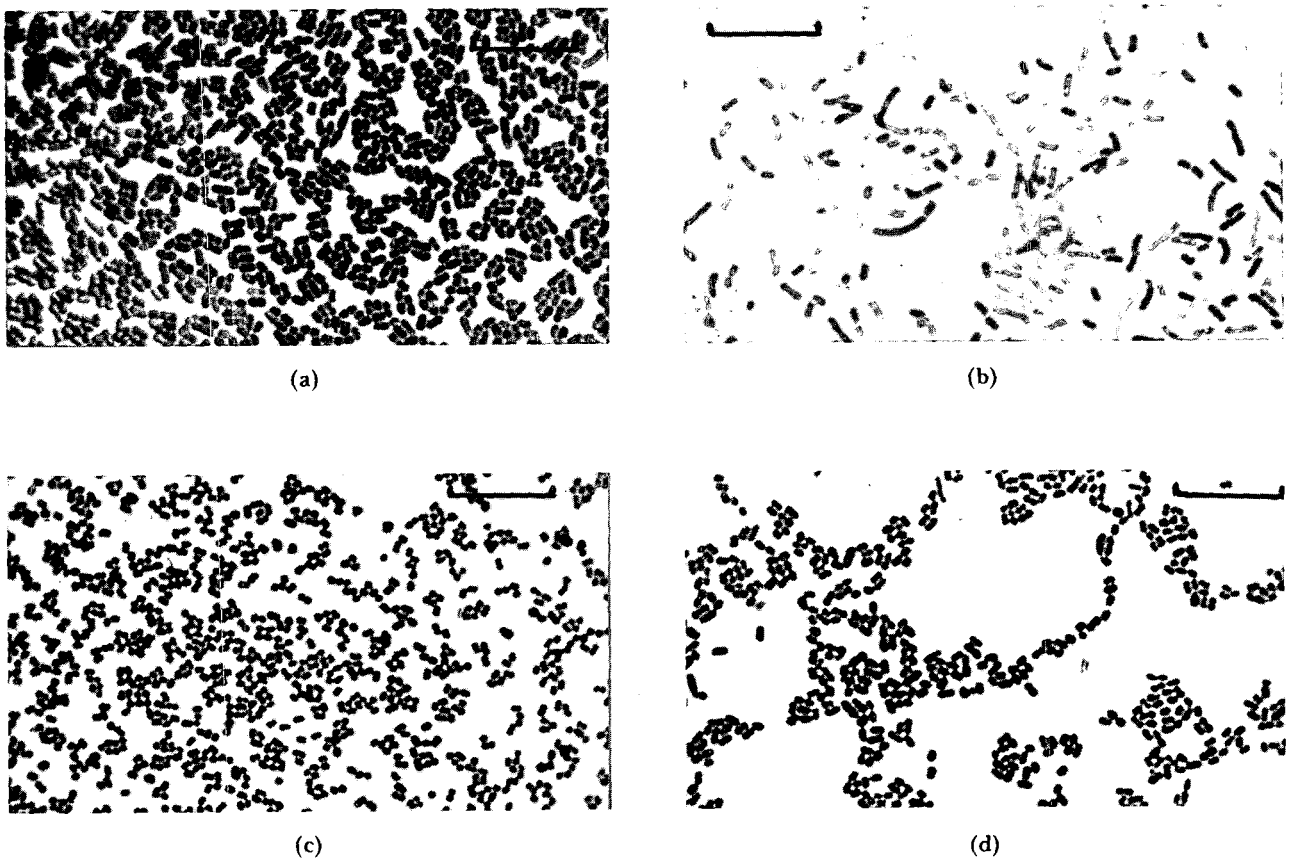
(b) 1 000 mℓ quantities of broth cultures in 2 ℓ Erlenmeyers had air bubbled into them at a rate of 80 to 120 mℓ $min^{-1}$, as measured with a rotameter. This was the maximum possible, otherwise excessive foaming of the medium occurred.

### Determination of dissolved oxygen

Measurements were made according to the Alsterberg (azide) modification of the Winkler method (American Public Health Association, 1960). Because of high altitude, atmospheric pressures in Johannesburg average about 625 mm Hg. The amount of oxygen dissolved in the water was therefore fairly low.

### Determination of luxury phosphate uptake by Acinetobacter strains

The cells from growth medium were harvested by centrifugation. These cells were washed twice with distilled water and then suspended in 50 mℓ quantities of phosphate-uptake medium. Since this had no nitrogen source, the cells removed phosphate under conditions of non-growth. Each experiment, always carried out in duplicate, was performed with cell suspensions of ap-




*Bar represents 10 μm*

*Figure 1*
*Gram-stained smears of* Acinetobacter *isolates grown on nutrient agar slants. (a) Strain SW1b. (b) Strain SW5a. (c) Strain SW8a. (d) Strain SW9b*

proximately the same turbidity (Absorbancy of 0,35–0,40 at 520 nm). Dry mass of the cells was obtained by the method of Meynell & Meynell (1970).

The ability of the bacteria to remove phosphate from the medium was examined over a period of 6 h under aerobic conditions obtained by shaking the suspensions in a reciprocal shaker at 80 oscillations/min at 30 °C.

Phosphate uptake was monitored hourly on 0,2 mℓ samples, diluted to 5 mℓ with distilled water, using the aminonaphtholsulfonic acid colorimetric method for the determination of orthophosphate (American Public Health Association, 1960). This technique determined the amount of phosphate in the medium and the decrease in phosphate concentration was therefore considered to be due to uptake by the cells. The results were calculated accordingly. The cells were not removed from the sample used for determinations because the "blank" prepared in exactly the same manner, except for absence of ammonium molybdate (which is necessary for producing the coloured phosphate complex) cancelled out turbidity variations. Cellular polyphosphate was considered not to contribute to orthophosphate concentrations under these conditions. The colour intensity developed after 5 min was read at 690 nm.

### Determination of phosphate uptake during growth

The procedure was similar to the above description, with samples taken after 8 h and 22 h growth.

## Results

### Characterization of bacteria

The coccobacilli, isolated from sewage, varied from short rods to spheres (Fig. 1). Although they were gram-negative there was a tendency towards gram-positiveness in the larger cells. In some isolates (e.g. SW5a) filaments were also present. The bacteria were found to be non-motile oxidase-negative obligate aerobes.

TABLE 1
CHARACTERIZATION OF EIGHT ISOLATES USING THE METHODS DESCRIBED BY BØVRE & HENRIKSEN (1976)

| | SW1b | SW3a | SW4a | SW5a | SW7a | SW8a | SW9a | SW9b |
|---|---|---|---|---|---|---|---|---|
| Cellular morphology | Coccobacilli | Coccobacilli | Coccobacilli | Coccobacilli | Coccobacilli | Coccobacilli | Coccobacilli | Coccobacilli |
| Cellular size: | | | | | | | | |
| Length | 1,25 μm | 1,00 μm | 1,27 μm | 1,13 μm | 1,62 μm | 1,40 μm | 1,20 μm | 1,65 μm |
| Diameter | 0,95 μm | 0,95 μm | 0,79 μm | 1,00 μm | 0,92 μm | 1,00 μm | 0,97 μm | 0,92 μm |
| Gram's stain | – | – | – | – | – | – | – | – |
| Capsule | + | + | – | + | + | + | + | + |
| Growth temp. range | 4–37°C | 4–37°C | 4–37°C | 4–37°C | 4–37°C | 4–37°C | 4–37°C | 4–37°C |
| Growth temp. optimum | 30°C | 30°C | 30°C | 30°C | 30°C | 30°C | 30°C | 30°C |
| Motility (Hanging drop) | | – | – | – | – | – | – | – |
| Flagella (EM) | - | - | – | – | – | – | – | – |
| Pigment | Creamy | Creamy | Creamy | Creamy | Creamy | Creamy | Creamy | Creamy |
| Blood agar haemolysis | - | – | – | – | – | – | – | – |
| Colony size (48 h on N/A) | 2,5 mm | 2,5 mm | 2,5 mm | 4,0 mm | 2,8 mm | 3,0 mm | 2,5 mm | 3,0 mm |
| Colony morphology: | | | | | | | | |
| Shape | Round | Round | Round | Round | Round | Round | Round | Round |
| Height | Flat | Flat | Flat | Flat | Flat | Flat | Flat | Flat |
| Appearance | Glistening | Glistening | Dull | Glistening | Glistening | Glistening | Glistening | Glistening |
| Oxygen relation | All isolates are obligately aerobic. | | | | | | | |
| BIOCHEMICAL TESTS: | | | | | | | | |
| Oxidase reaction | – | – | – | – | – | – | – | – |
| Catalase reaction | + | + | + | + | + | + | + | + |
| Nitrite reduction | – | – | – | – | – | – | – | – |
| Nitrate reduction | – | – | – | – | – | – | – | – |
| Koser's citrate test | + | + | – | – | – | + | + | – |
| Triple Sugar Iron Agar: | | | | | | | | |
| Acid | + | – | – | – | + | – | + | + |
| $H_2S$ | – | – | – | – | – | – | – | – |
| Gas | – | – | – | – | – | – | – | – |
| Production of poly-β-hydroxybutyrate inclusions | + | + | – | – | + | – | + | + |
| Gelatin liquefaction | – | – | – | – | – | – | – | – |
| Indole production | – | – | – | – | – | – | – | – |
| Urease test | – | – | – | – | – | – | – | – |
| Deamination of: | | | | | | | | |
| Phenylalanine | – | – | – | – | – | – | – | – |
| Tryptophan | – | – | – | – | – | – | – | – |
| Resistance to 5 units of penicillin G | + | + | + | + | + | + | + | + |

That these were *Acinetobacter calcoaceticus* strains was confirmed using the API 20E system (1976 Analytab Products Inc., U.S.A.) for the identification of gram-negative bacteria.

Tables 1 and 2 show the results of further characterization of eight isolates, using the methods advocated by Bϕvre & Henriksen (1976).

Baumann, Doudoroff & Stanier (1968) and the eighth edition of Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons, 1974) state that *Acinetobacter* does not store poly-β-hydroxybutyrate. Yet, as can be seen from Table 1, a number of the isolates had the capacity to produce intracellular poly-β-hydroxybutyrate (PHB). In addition, these also produced acid from a number of carbohydrates not dissimilated by the other isolates. There thus appeared to be two distinct varieties of *Acinetobacter* in the sewage.

The division of *Acinetobacter* strains into group A, with the capacity to utilize a wide range of substrates, and group B, with narrow-range capacity, as proposed by Baumann, Doudoroff & Stanier (1968), was followed by Warskow & Elliot (1972) with the finding that only one of their many sewage isolates belonged to group A. The possibility therefore arose that the PHB-producing, or group A, strains were mainly responsible for luxury phosphate uptake in the sewage plant, particularly since Fuhs & Chen (1975) also characterized their strain, which vigorously accumulated phosphate, as storing PHB. This possibility was tested as follows:

Four strains were selected for an investigation of their ability to accumulate phosphate. The four chosen were two PHB-producing strains: SW1b, which could utilize citrate (Table 1) and was able to produce acid from melibiose (API 20E system); and SW9b, which was negative for the two characteristics just described. The other two strains, SW5a and SW8a, did not produce PHB.

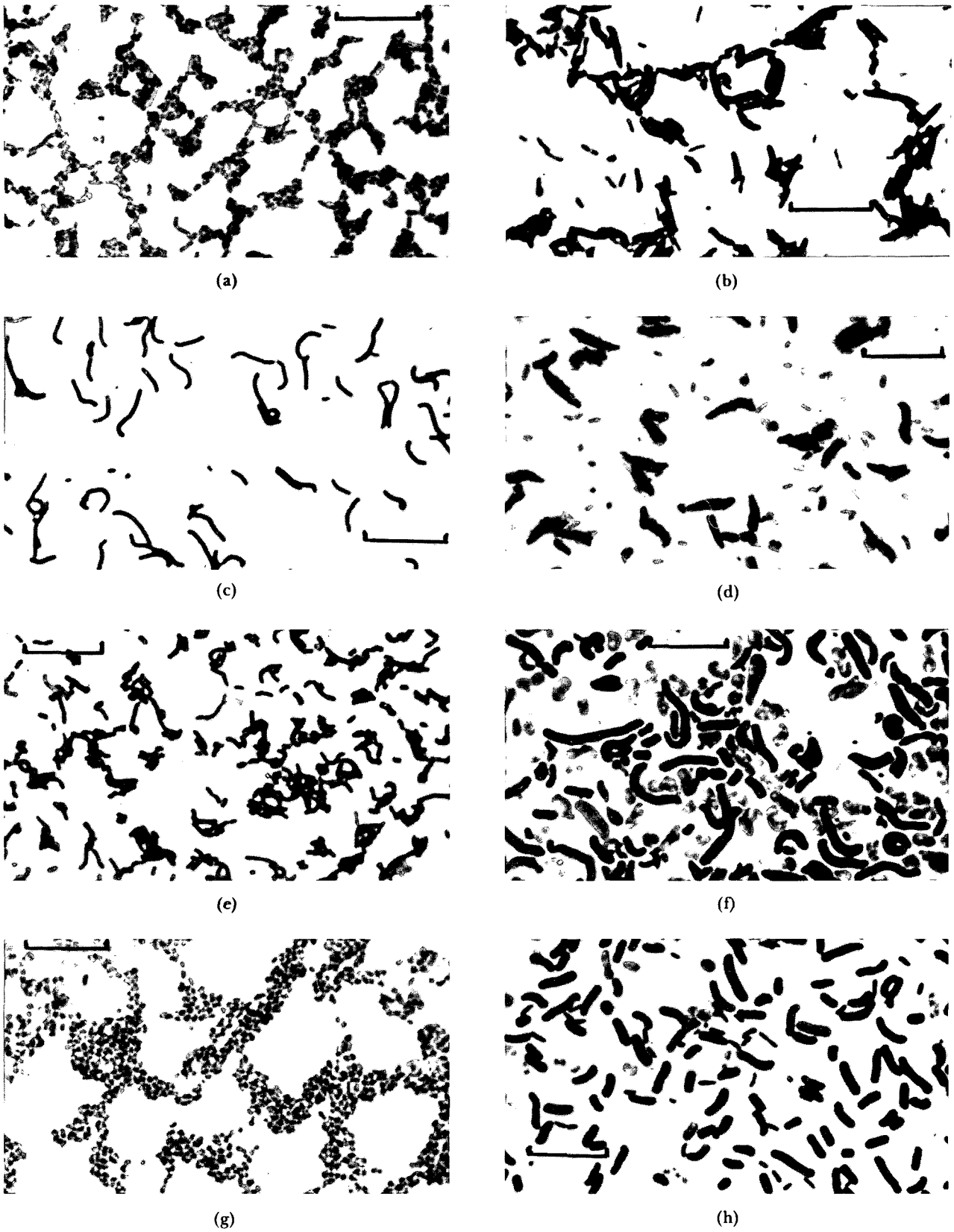
### Growth conditions for obtaining cell mass

When, first, cells were harvested from either shake or aerated cultures and then investigated for ability to take up phosphate under non-growth conditions, inconsistent results were obtained and seemed to depend on how oxygen had been introduced into the medium during growth. In addition, microscopic examination of the cells grown under the different growth conditions gave a first impression of completely different bacteria since morphologically they bore little resemblance to each other (compare a and b, c and d, e and f, g and h, Fig. 2). Checking for contamination ruled out this possibility.

TABLE 2
CARBOHYDRATE DEGRADATIONS BY EIGHT ISOLATES USING THE METHODS DESCRIBED BY BϕVRE & HENRIKSEN (1976)

| | SW1b | SW3a | SW4a | SW5a | SW7a | SW8a | SW9a | SW9b |
|---|---|---|---|---|---|---|---|---|
| Peptone water-sugar medium: | | | | | | | | |
| Glucose | A | A | – | – | A | – | A | A |
| Maltose | – | – | – | – | – | – | – | – |
| Sucrose | – | – | – | – | – | – | – | – |
| Lactose | – | – | – | – | – | – | – | – |
| Mannitol | – | – | – | – | – | – | – | – |
| Arabinose | A | A | – | – | A | – | A | A |
| Xylose | A | A | – | – | A | – | A | A |
| Galactose | A | A | – | – | A | – | A | A |
| Rhamnose | A | – | – | – | A | – | A | A |
| Mannose | A | A | – | – | A | – | A | A |
| Fructose | – | – | – | – | – | – | – | – |
| Hugh & Leifson O–F medium: | | | | | | | | |
| Glucose | O | O | O | O | O | O | O | O |
| Maltose | – | – | – | – | – | – | – | – |
| Sucrose | – | – | – | – | – | – | – | – |
| Lactose | O | O | – | – | O | – | O | O |
| Mannitol | – | – | – | – | – | – | – | – |
| Arabinose | O | O | O | – | O | O | O | O |
| Xylose | ND | ND | ND | ND | ND | ND | ND | ND |
| Galactose | O | O | – | – | O | – | O | O |
| Rhamnose | ND | ND | ND | ND | ND | ND | ND | ND |
| Mannose | ND | ND | ND | ND | ND | ND | ND | ND |
| Fructose | O | O | – | – | O | – | O | O |

A, acid product; O, oxidative; F, fermentative; –, negative; ND, not determined.




*Bar represents 10 μm*

*Figure 2*

Acinetobacter *isolates grown in shake medium with dissolved oxygen content of 6,4 mg/ℓ (a,c,e,g) and in aerated medium with dissolved oxygen content of 5,9 mg/ℓ (b,d,f,h). The bacteria are stained with a 1:5 000 (w/v) aqueous solution of crystal violet for polyphosphate content. The dark cells are filled with volutin whereas the light cells are not. Note that oxygen content of aerated medium, in particular, was not uniform throughout, giving rise to a mixture of morphological types. Some of these are similar to those found at higher oxygen levels. (a,b) Strain SW1b, which can store poly-β-hydroxybutyrate (PHB); (c,d) strain SW5a, which cannot store PHB; (e,f) strain SW8a, which cannot store PHB; (g,h) strain SW9b, which can store PHB*

It then became apparent that the two methods for culturing the cells had not only altered their capacity for phosphate uptake but also their morphology.

That one type of culture technique provided less oxygen to the bacteria than the other type was verified by determining the oxygen content of distilled water, shaken or aerated in exactly the same manner as described for the broth cultures.

The possibility that the turbulence created by shake culture prevented the drastic morphological changes seen after culturing with aeration was ruled out by Tina (1978). She found that the same changes in morphology, from short rods to long thick filaments, could be obtained by increasing the volume of broth in the 500 mℓ Erlenmeyers, incubated at a shaking speed of 100 r/min. Increased volume decreased the surface area of liquid in contact with air and increased its depth. In this way less oxygen was again provided to the growing cells.

### Aeration differences in culture techniques

The oxygen content of aerated distilled water was found to be 5,9 mg/ℓ. It increased to 6,4 mg/ℓ in distilled water shaken at 100 r/min. Shake culturing, under the conditions described, therefore provided more oxygen to the cells than the aeration technique.

### Phosphate uptake during growth

From Table 3 it can be seen that the cells took up phosphate during growth but the amount removed increased with increasing oxygen concentration in the medium. That the amount of phosphate removed by SW8a, when grown under aeration, was so low could not be explained. Similarities in turbidities showed that all had grown to a comparable extent when the same conditions of cultivation were used.

TABLE 3
PHOSPHATE (mg/ℓ) TAKEN UP DURING GROWTH BY DIFFERENT ACINETOBACTER STRAINS

| | Aerated culture after | | Shake culture after | |
|---|---|---|---|---|
| Strain | 8 h | 22 h | 8 h | 22 h |
| SW1b | 0 | 46 | 35 | 54 |
| SW5a | 35 | 38 | 40 | 54 |
| SW8a | 4 | 5 | 50 | 54 |
| SW9b | 17 | 40 | 53 | 54 |

### Luxury phosphate uptake

Phosphate-uptake trends of the *Acinetobacter* strains, depicted by Figs. 3 and 4, were verified on different occasions by the authors and independently by Tina (1978). The two graphs (Figs. 3 and 4) show the quantities of phosphate taken up by the different strains over a period of 6 h under non-growth conditions. The cells, obtained after growth under reduced oxygen availability (Fig. 4) had a greater capacity to perform luxury phosphate uptake. Strain SW8a removed the most (50 mg/ℓ in 6 h) but this effect was probably a consequence of the small amount of phosphate it had accumulated during growth.

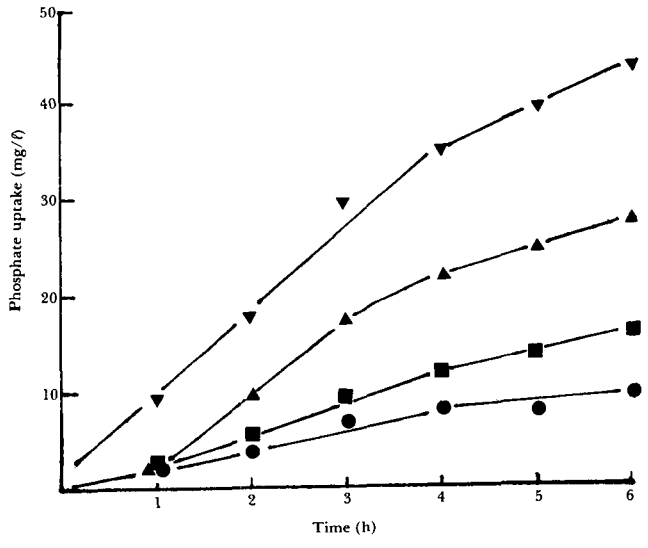



*Figure 3*
*Phosphate taken up by different* Acinetobacter *strains under non-growth conditions. The strains were: ●, SW5a; ■, SW8a; ▲, SW1b; ▼, SW9b. Biomass was obtained with shake culture and all the results were calculated to 50 mg dry weight per mℓ*

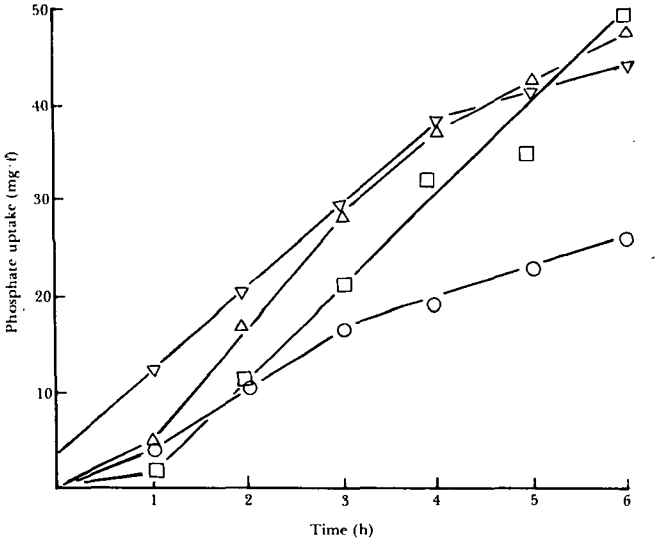



*Figure 4*
*Phosphate taken up by different* Acinetobacter *strains under non-growth conditions. The strains were: ○, SW5a; □, SW8a; △, SW1b; ▽, SW9b. Aeration was used during culture for biomass production and all the results were calculated to 50 mg dry weight per 100 mℓ*

If the cells were obtained from cultures grown under increased oxygenation (Fig. 3), those capable of poly-$\beta$-hydroxybutyrate (PHB) storage exhibited an increased rate of luxury phosphate uptake. However, this difference became less significant if more anaerobic growth conditions had been used. In addition, all four strains showed different and individual phosphate uptake patterns.

Slope values of the graphs for luxury phosphate uptake, 1 to 3 h after start of the experiment, (Fig. 3) were approximately the same with the PHB-storing strains (8,6 mg phosphate/0,5 g dry biomass per hour for SW1b, 8,8 mg phosphate/0,5 g dry biomass per hour for SW9b) and the strains which did not accumulate PHB (3,8 for SW5a, 4,4 for SW8a) when the cells had been obtained by shake culture. There was, however, considerable variation in all of the slope values when the aerated system had been used for biomass production. This distribution was probably a consequence of the fact that the cells, growing in aerated broth, were not uniformly supplied with oxygen throughout the medium. The variation in cell kind found after this type of culturing (Fig. 2b, d, f, h) also showed that lack of homogeneity in growth conditions existed.

## Discussion

The similarity of slope values in graphs of luxury uptake of phosphate for strains having comparable physiological characteristics, strengthened the indication that the more nutritionally versatile strains of *Acinetobacter calcoaceticus* had an increased capacity for polyphosphate accumulation. However, growth under more anaerobic conditions altered this capacity to the extent that the physiological characteristics of the bacteria were no longer significant. Lack of oxygen during growth also strikingly changed the morphology of the cells.

That altered morphology is not the basic explanation for the increased luxury phosphate uptake is provided by strains SW9b and SW5a. Although the cells of SW9b had radically changed in appearance, their ability to accumulate phosphate under non-growth conditions did not increase significantly. Strain SW5a, on the other hand, already looked different from the normal "coccobacillus" of *Acinetobacter* under conditions of mild anaerobiosis but exhibited the lowest capacity for phosphate uptake. Of course it is not possible to be dogmatic about lack of effect of size on phosphate uptake since strain SW8a, which took up the most phosphate had the largest cells, completely filled with the phosphate (see Fig. 2f).

Under highly aerobic conditions the final electron acceptor of the respiratory chain in *Acinetobacter* has been shown to be cytochrome o (Jones, 1977). Under conditions of oxygen deprivation there is the replacement of cytochrome o by cytochrome oxidase d which has a substantially higher affinity for molecular oxygen (Jones, 1978). This enables the obligate aerobic cells to minimize the potentially deleterious effects of oxygen deprivation and maintain a high rate of respiration, thereby eliminating an excess of reducing power (e.g. NADH) and providing the necessary proton motive force to membrane transport.

As suggested by Meyer and Jones (1973), a low efficiency of energy conservation in the form of ATP may be the price paid for the possession of a terminal oxidase with a higher affinity for oxygen.

If, under conditions of oxygen deprivation, the demand for ATP for nucleic acid synthesis cannot be met, this would result in enzymatic degradation of stored polyphosphate according to the polyphosphate cycle proposed by Harold (1966). Phosphate leakage from the cells would occur. If, in addition, there is a halt in nucleic acid synthesis the usual cell division cycle would be impaired and result in abnormal cell morphology. Release from oxygen stress conditions would result in the "phosphate overplus phenomenon" referred to by Fuhs and Chen (1975) where uptake in excess of immediate need has been induced by phosphate starvation created by oxygen stress.

In conclusion anaerobiosis obviously plays a role in biological uptake of phosphorus in wastewater treatment plants but the role played is more complex than that suggested by Fuhs and Chen (1975) or McLaren and Wood (1976).

## Acknowledgements

We are indebted to J.S. Youngleson for characterizing the eight *Acinetobacter* strains. He provided the information on which Tables 1 and 2 are based. We also wish to thank D.W. Osborn and H.A. Nicholls for the access they gave us to the Northern Sewage Works Pilot Plant.

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