A study of selected characteristics of *Acinetobacter* spp. isolated from activated sludge in anaerobic/anoxic/aerobic and aerobic systems

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Abstract

This study was undertaken to investigate differences in carbon utilization, carbon storage as polyhydroxybutyrate (PHB) and phosphorus storage as polyphosphate (poly-P) in *Acinetobacter* spp. isolated from anaerobic/anoxic/aerobic and from completely aerobic activated sludge systems. Isolates from all systems, when cultured aerobically (in synthetic media), grew on acetate and glucose and exhibited PHB and poly-P accumulation. It was concluded that introducing anaerobic/aerobic sequencing into activated sludge systems does not promote the selection of a specific *Acinetobacter* strain capable of PHB and poly-P accumulation, but rather stimulates accumulation in strains already present in the system.

Introduction

The presence of *Acinetobacter* spp. in anaerobic/aerobic and anaerobic/anoxic/aerobic biological excess phosphorus removal activated sludge systems is widely accepted (*inter alia* Buchan, 1983; Lötter and Murphy, 1985). Not so well accepted is the fact that *Acinetobacter* spp. can be present in completely aerobic activated sludge systems. Lötter *et al.* (1986) cite data on aerobic systems where, in terms of their methods of bacterial identification, *Acinetobacter* spp. formed 40 per cent or more of the bacterial population. Whereas the anaerobic/aerobic systems exhibited biological excess phosphorus (P) removal, the aerobic systems did not.

In anaerobic/aerobic systems, *Acinetobacter* spp. mediate excess phosphorus (P) removal through the accumulation of P as polyphosphate in metachromic granules which may occupy up to 60 per cent of the cell volume (Buchan, 1983; Cloete, 1984). In aerobic systems the metabolic response tends to indicate that the metabolic nutrient requirements of *Acinetobacter* spp., in particular phosphorus, are about the same as those of other heterotrophs – Lötter *et al.* (1986) found that, despite the large fraction of *Acinetobacter* spp. in the sludge from the completely mixed aerobic single reactor system, the metabolic phosphorus requirement of this sludge was about the same as that normally observed in sludges. Comparing the behaviour of these organisms in anaerobic/aerobic and in completely aerobic systems, raises the question as to whether the *Acinetobacter* strains in the two systems are different, i.e. whether the one group has the propensity for phosphorus accumulation as polyphosphate and the other not, or, whether both groups have the propensity to store polyphosphate, the propensity being invoked only if appropriate conditions are imposed.

Acinetobacter spp. grow well on simple mineral media containing a single carbon compound, this compound serving both as a carbon and as an energy source for anabolic processes (Juni, 1978). They can be cultured equally well on liquid and on solid media (Warskow and Juni, 1972; Henriksen, 1973; Juni, 1978). The ability of *Acinetobacter* spp. to use oxygen as external electron acceptor is well documented (Juni, 1978); more recently it has been shown that some strains can also utilize nitrate as external electron acceptor when oxygen is absent (Henriksen, 1976, Lötter, 1985; van Groenestijn and Deinema, 1985). Of particular interest is carbon metabolism in *Acinetobacter* spp.: All *Acinetobacter* strains grow aerobically on acetate (Baumann *et al.*, 1968); some strains can utilize glucose but do so exclusively via the Entner-Doudoroff pathway (McDonald and Juni, 1973), a pathway inoperative under anaerobic conditions. (By anaerobic is meant the absence of both nitrate and oxygen, and no input of these.) Furthermore, none of the strains appear to possess the glycolytic (Embden-Meyerhof) pathway. In consequence glucose, and similar compounds, can be metabolized by strains possessing the Entner-Doudoroff pathway only where an external electron acceptor (nitrate or oxygen) is available; strains not possessing the Entner-Doudoroff pathway cannot metabolize such compounds at all. The tricarboxylic acid cycle functions in *Acinetobacter* spp., as evidenced by the detection of the key enzymes of that cycle in cell-free extracts (Juni, 1978). Key enzymes of the anaplerotic glyoxylate cycle also have been identified; in fact these two cycles appear to function simultaneously in *Acinetobacter* spp. (Sturm *et al.*, 1970; Juni, 1978). PHB accumulation has been demonstrated also in *Acinetobacter* spp. (Lawson and Tonhazy, 1980), but the pathways have not been identified.

Having described above the known key characteristics of *Acinetobacter* spp., it is possible to speculate on the interaction between these characteristics and the environment, in completely aerobic and in anaerobic/aerobic systems.

Completely aerobic systems

Acinetobacter strains with an Entner-Doudoroff pathway can utilize sugars, e.g. glucose, and can thus grow aerobically in competition with other heterotrophs. *Acinetobacter* strains that do not possess the Entner-Doudoroff pathway can only utilize short chain fatty acids, e.g. acetate, and thus would be at a severe disadvantage as many waste waters contain relatively small fractions of such substrates (Pitman and Lötter, 1986). In consequence *Acinetobacter* strains with an Entner-Doudoroff pathway can be expected to be the prevalent strains of the genus in aerobic systems receiving municipal waste waters.

Anaerobic/aerobic systems:

When an anaerobic phase is introduced into an activated sludge

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Received 6 May 1986.

system there is no information on how "aerobic" *Acinetobacter* strains, i.e. with an Entner-Doudoroff pathway, will react. If short chain acids are added to the anaerobic phase, *Acinetobacter* which have a polyphosphate pool will utilize the polyphosphate as an energy source to take up the acids and store these as polyhydroxybutyrate (PHB); subsequently in anoxic and aerobic zones the stored PHB is utilized for growth and for storage of phosphorus as polyphosphate, (Wentzel *et al.*, 1986). If a substrate like glucose is added to the anaerobic phase, then facultative organisms, capable of fermentation, will break down the glucose to short chain fatty acids; the acids are discharged to the medium and become available for sequestration by *Acinetobacter* with a polyphosphate pool (Brodisch, 1985; Wentzel *et al.*, 1985). *Acinetobacter* spp. which lack a polyphosphate pool will not be able to sequester substrate in the anaerobic phase. Hence under anaerobic/aerobic sequencing, the conditions favour *Acinetobacter* spp. possessing a polyphosphate pool.

From the above, if an aerobic system is changed to an anaerobic/aerobic one, then one of two behaviour options exist – either the "aerobic" strain (with the Entner-Doudoroff pathway) has the propensity to store polyphosphate, invokes it, and then sequesters acetate under anaerobic conditions, or, a new strain of *Acinetobacter* able to store polyphosphate develops, perhaps without the Entner-Doudoroff pathway. From the literature it is not possible to find an answer to this question.

Fuhs and Chen (1975), when studying the nutritional spectrum of an *Acinetobacter* strain isolated from an anaerobic/aerobic excess phosphorus removal system, found that the isolate grew on short chain fatty acids but could not use glucose as substrate, which implies that the isolate did not possess the Entner-Doudoroff pathway. Thus one might conclude that *Acinetobacter* strains found in completely aerobic systems differ from the strains found in anaerobic/aerobic systems in that "aerobic" strains possess the Entner-Doudoroff pathway whereas "anaerobic/aerobic" strains do not. The work of Rensink (1981) similarly does not supply an answer. Rensink operated a twelve-in-series reactor aerobic activated sludge plant which did not exhibit phosphorus release, uptake or excess phosphorus removal. The first six reactors in the series were converted to operate without aeration. Immediately after the change, on batch augmenting the feed with acetate, virtually no acetate disappeared in passing through the first six anaerobic reactors. Over a six-week period with similar batch augmentation tests, the rate of disappearance of the acetate increased, until finally, virtually all the acetate disappeared in the first two reactors. Concomitant with the acetate disappearance, phosphorus release increased in the anaerobic reactors, as did the phosphorus uptake in the aerobic reactors and phosphorus removal by the system. Clearly the phosphorus release, uptake and removal developed over a matter of weeks. Assuming that, during the initial aerobic operation, *Acinetobacter* spp. were present in similar proportion to that found by Lötter *et al.* (1986), namely 40 to 60 per cent in aerobic systems, it is not clear whether the long period for the development of P removal was required for a new *Acinetobacter* strain to become established in the system, this new strain possessing the propensity for poly-P accumulation, or, whether the strains present in the aerobic system required this long period to invoke a latent propensity for poly-P accumulation via development of the appropriate enzyme systems resulting in a progressive increase in poly-P and PHB.

In order to find an answer to the questions raised above it was decided to isolate *Acinetobacter* strains from an anaerobic/anoxic/aerobic system exhibiting excess P removal *and* from two aerobic systems not exhibiting excess P removal. The *Acinetobacter* strains isolated were to be used to determine:

- the relative proportion of *Acinetobacter* spp. in each system;

The strains then were to be tested for the ability to:

- reduce nitrate;
- accumulate polyphosphate and polyhydroxybutyrate; and
- utilize acetate and glucose as substrate.

By comparing the responses of the *Acinetobacter* isolates from the different systems it should be possible to come to some conclusion.

Materials and methods

The following three laboratory-scale activated sludge systems were operated:

- A system comprising a single completely mixed aerobic reactor (15 ℓ).
- A system comprising two-in-series completely mixed aerobic reactors, the first a "selector" (0,3 ℓ), the second the main reactor (14,7 ℓ); both systems received settled municipal waste water.
- A modified Bardenpho system receiving a mixture of unsettled municipal waste water and acetate.

The configuration details are given in Fig. 1 and the design/operational details in Table 1. Note that all systems were operated at 20 days sludge age. The single reactor system served as a control. The aerobic "selector" system and the Bardenpho system merit the following additional comments.

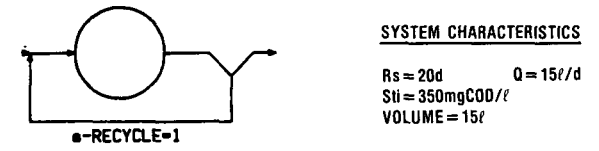
The "selector" reactor, with a volume of about 2 per cent of the volume of the main reactor, is a configuration that has been devised to control and inhibit the growth of certain filamentous organisms that cause bulking. According to Jenkins *et al.* (1985) and Still *et al.* (1986), the "selector" provides a region of high substrate/active mass ratio and appears to select for organisms with high maximum specific growth rates – maximum specific growth rates in the selector system have been measured to be 2,0 to 2,5 times those in the single aerobic reactor system (Still *et al.*, 1986). The relative volumetric size of the "selector" is determined from the requirement that all the readily biodegradable COD in the influent will be utilized in the "selector".

In the Bardenpho system the objective was to study the increase in P removal on supplementing the influent with acetate; acetate can be directly "utilized" by *Acinetobacter* spp., by sequestration in the anaerobic reactor, so that the increased substrate available to these organisms could be expected to favour their proliferation and to give rise to increased P removal. Indeed P removal did increase approximately proportionately to the increase in acetate fraction of the influent.

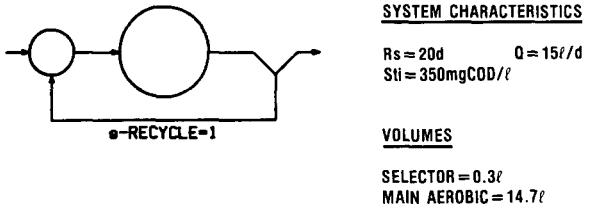
Mixed liquor samples from the aerobic zone of the Bardenpho system which exhibited excess P removal and from the two completely aerobic systems both not exhibiting excess P removal (see Table 1), were subjected to the isolation and identification procedure described by Lötter and Murphy (1985). Twenty-five

Acinetobacter isolates from each system were retained for the following studies:

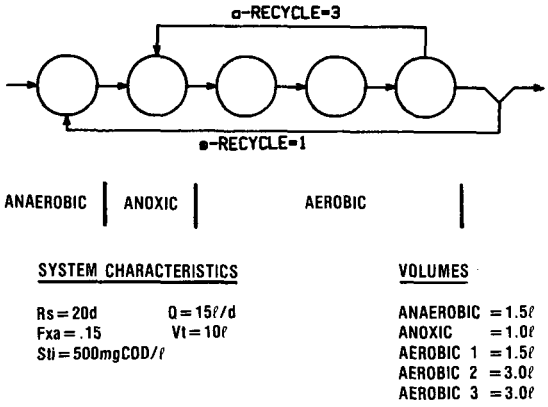
- The nitrate reducing propensity of each isolate was tested by growth in nitrate agar and subsequent determination of nitrogen and nitrite (Difco, 1957).



(a): Single completely mixed aerobic reactor system.



(b): Completely mixed aerobic system with selector.



(c): Modified Bardenpho system.

Figure 1
Configurational details of the three laboratory-scale systems operated to produce mixed liquor for the investigation.

TABLE 1
DESIGN/OPERATIONAL DETAILS OF THE THREE LABORATORY-SCALE SYSTEMS FROM WHICH MIXED LIQUOR SAMPLES WERE TAKEN FOR ISOLATION OF *ACINETOBACTER* SPP.

System parameter	Completely aerobic systems		Bardenpho system
	single reactor	with selector	
Sludge age (d)	20	20	20
Substrate	settled municipal waste water	settled municipal waste water	Raw municipal waste water + acetate supplement
Influent COD (mg COD/ℓ)	350	350	200 waste water 300 acetate
Influent TKN (mg N/ℓ)	50	50	25
Flow rate (ℓ/d)	15	15	15
P removal (mg P/ℓ)	2	2	30

- Polyhydroxybutyrate accumulation by the *Acinetobacter* isolates was determined by growth on nutrient agar augmented with betahydroxybutyrate (Bovre *et al.*, 1972) and then examining cells microscopically for PHB inclusions after Sudan Black staining, as described by Gurr (1973).

- Growth and substrate utilization with acetate and glucose were determined as follows: Each isolate was grown in Fuhs and Chen (1975) nutrient medium. Solutions (250 mℓ) of the medium, containing only acetate or glucose as substrate at a theoretical COD of 4 000 mg/ℓ, were inoculated with 1,0 mℓ of a cell suspension with an optical density of 1,0 (Å = 520) and incubated aerobically for five days at 20°C. Thereafter the cell suspensions were centrifuged in tared centrifuge tubes at 20 000 g. The supernatant was retained for the determination of COD by the dichromate oxidation method as described in Standard Methods (1985). The concentration of cells was determined by massing the dried cells.

- Polyphosphate accumulation was determined by making a slide of each cell suspension after the incubation period above, and staining the slides with a Neisser stain (Lötter and Murphy, 1985).

Results and discussion

Presence of *Acinetobacter* spp.

The fractions of *Acinetobacter* spp. in the sludges are given in Table 2. Evidently the aerobic systems each developed an appreciable fraction of *Acinetobacter* spp. (Lötter *et al.*, 1986).

TABLE 2
PERCENTAGES OF TOTAL VIABLE BACTERIAL COLONIES THAT ARE *ACINETOBACTER* SPP. IN SLUDGES OF THREE SYSTEMS

System	Percentage *Acinetobacter* spp.
Completely mixed single aerobic reactor	40
Aerobic series with selector	60
Bardenpho	90

Nitrate reduction

The results of the nitrate reducing capacity of *Acinetobacter* isolates are given in Table 3. It is evident from Table 3 that a number of *Acinetobacter* isolates were capable of nitrate reduction. This is in conformity with the findings of previous workers on the capacity for nitrate reduction by *Acinetobacter* spp. (Lötter, 1985; van Groenestijn and Deinema, 1985). Also it is evident that in their capacity for nitrate reduction, there was little difference between isolates from the anaerobic/anoxic/aerobic and the aerobic systems. Thus the propensity for nitrate reduction did not appear to be induced by environmental conditions, that is, the inclusion of an anoxic zone in the activated sludge system did not augment the capacity for nitrate reduction in *Acinetobacter* spp. in the system. The majority of the *Acinetobacter* isolates able to reduce nitrate, reduced the nitrate to nitrite; only a minority of isolates reduced nitrate to nitrogen.

TABLE 3
PERCENTAGE OF *ACINETOBACTER* ISOLATES CAPABLE OF NITRATE REDUCTION

Isolate source	Nitrate reduction to nitrite Percentage of total (25 samples/system)	to nitrogen Percentage of total (25 samples/system)
Aerobic zone Bardenpho unit	32	6
Continuous aerobic unit	43	5
Continuous aerobic unit with selector	42	12

Polyphosphate and polyhydroxybutyrate accumulation

The slides of the isolates stained for the presence of polyphosphate and polyhydroxybutyrate were analysed as follows: For each isolate the slide was categorised by assigning to the slide a value of 0 to 4 according to the number of cells containing inclusions as follows. If none of the cells contain inclusions – 0; about one quarter of the cells – 1; one half of the cells – 2; three quarters of the cells – 3; and all the cells – 4. For each system the number of isolates in each category was expressed as a percentage of the total number of isolates. These results are depicted graphically in Figs. 2 (a, b and c).

Referring to Figs. 2 (a and b), polyphosphate accumulation occurred in *Acinetobacter* isolates from both aerobic systems and from the anaerobic/anoxic/aerobic systems, on both glucose and acetate substrates. Although the Bardenpho system favoured the growth of *Acinetobacter* spp., indicated by the fact that 90 per cent of the organisms were *Acinetobacter* spp., the proportion of isolates that exhibited poly-P accumulation was not significantly higher than those from the aerobic systems. Indeed the Bardenpho isolates contained a higher proportion of isolates exhibiting zero presence of polyphosphates than the aerobic systems. It would appear that the *Acinetobacter* isolates from the completely

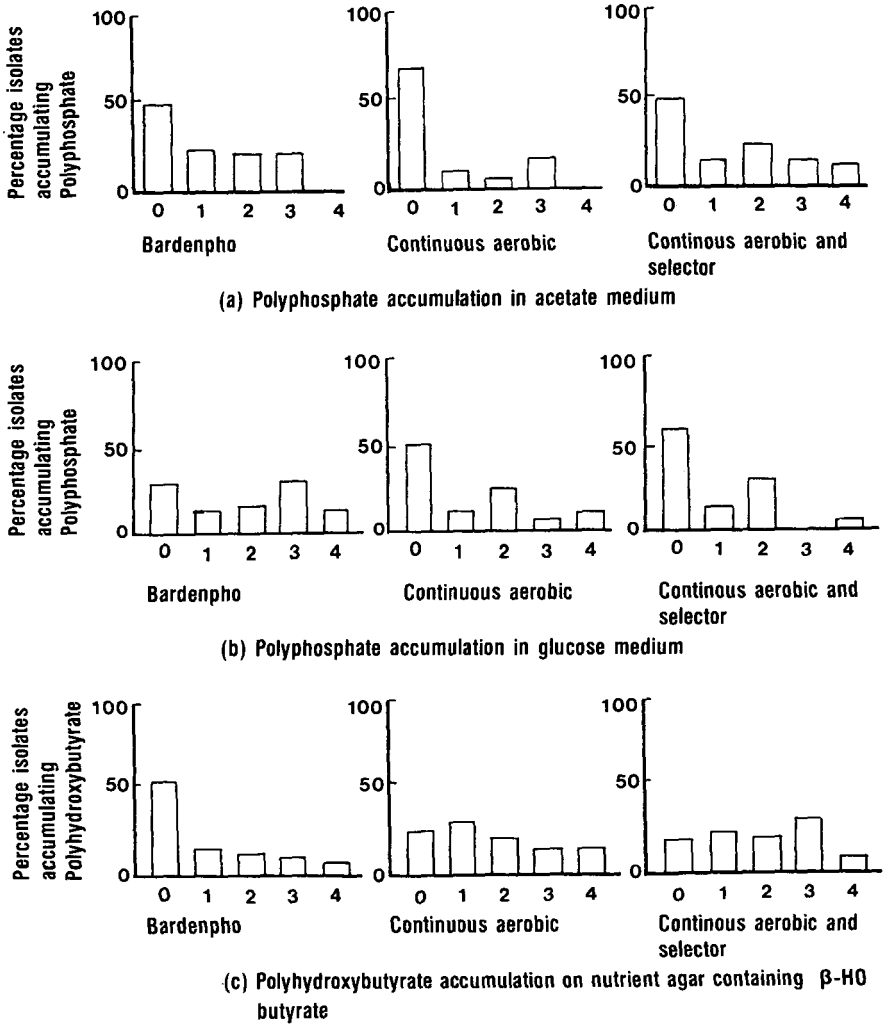


Figure 2
Distribution of isolates showing accumulation of polyphosphate with acetate and glucose, and of polyhydroxybutyrate with β-HO butyrate.
Key: 0 – No cells contain inclusions
1 – One quarter of cells contain inclusions
2 – Half cells contain inclusions
3 – Three quarters cells contain inclusions
4 – All cells contain inclusions.

aerobic systems, while not accumulating polyphosphate in the system, did in fact possess a similar propensity to accumulate polyphosphate (under the aerobic culture conditions) as isolates from the anaerobic/anoxic/aerobic system where *Acinetobacter* strains did accumulate polyphosphate. Thus the environment of the system from which the *Acinetobacter* was isolated, namely anaerobic/anoxic/aerobic or aerobic only, did not appear to have a significant effect in selecting for strains that have the propensity to accumulate polyphosphate, but rather that the anaerobic/aerobic system invoked a latent propensity in the organism for poly-P accumulation.

With regard to storage of polyhydroxybutyrate, all the isolates, irrespective of their origin, appeared to exhibit similar propensities to accumulate polyhydroxybutyrate under the aerobic culture conditions (Fig. 2c), even though this accumulation has not been observed in completely aerobic activated sludge systems.

From the above it would appear that propensities to accumulate polyhydroxybutyrate and polyphosphate are inherent characteristics of some *Acinetobacter* strains regardless of the system from which they are isolated.

Glucose and acetate utilization

For each isolate the growth was measured over the 120 h incubation period, on glucose and acetate respectively, and the mean growth calculated for the isolates from each system (Table 4).

TABLE 4
MEAN GROWTH OF ISOLATES ON ACETATE AND GLUCOSE OVER 120 h

Source of isolates	Mass of cells produced (mg) (25 samples/system)	
	Acetate	Glucose
Bardenpho aerobic zone	159	168
Continuous aerobic unit	146	186
Continuous aerobic unit with selector	188	198

These results indicate that isolates from all the systems could utilize acetate and glucose as substrate under aerobic conditions. Over the test period, from the measurement of soluble COD remaining after 120 h, the substrate was practically depleted so that it was not possible to assess whether some systems produced isolates with higher growth rates than others. The data from Table 4 indicates that, contrary to the findings of Fuhs and Chen, *Acinetobacter* isolates from an anaerobic/anoxic/aerobic system can use glucose as substrate i.e. they possess the Entner-Doudoroff pathway. Furthermore, from Figs. 2 (a, b and c), under aerobic conditions, some of these isolates accumulated poly-P both with glucose and with acetate as substrates, and exhibited an ability to accumulate PHB.

In contrast with the behaviour of the cultures, the *Acinetobacter* spp. accumulated neither poly-P nor PHB in aerobic activated sludge systems. Hence it would appear that in aerobic systems and in aerobic cultures, the different conditions induce the organisms to respond in the different ways described above. These conditions are investigated in detail by Wentzel *et al.* (1986); their conclusion is that the key parameters controlling poly-P and PHB metabolism are the ATP/ADP and NADH/NAD ratios, which in turn are influenced by environmental conditions.

Conclusions

Acinetobacter strains can be present in appreciable concentration in both anaerobic/anoxic/aerobic systems that exhibit excess P removal and completely aerobic systems that do not exhibit excess P removal. There appear to be no significant differences in the *Acinetobacter* strains isolated from the anaerobic/anoxic/aerobic system and from the aerobic system despite the apparent different selective pressures created in the systems.

A number of the *Acinetobacter* strains isolated from anaerobic/anoxic/aerobic and from completely aerobic activated sludge systems can utilize glucose aerobically as substrate; such utilization most likely is via the Entner-Doudoroff pathway, a pathway which operates only with oxygen and/or nitrate present.

Acinetobacter strains isolated both from systems exhibiting excess phosphorus removal and from systems that do not, have the propensity to accumulate polyphosphate (poly-P) and polyhydroxybutyrate (PHB) under aerobic culture conditions, with acetate and with glucose as substrate.

Imposing conditions conducive to excess phosphorus removal in a system (by anaerobic/aerobic sequencing) does not appear to stimulate new *Acinetobacter* strains, but rather to stimulate the polyphospate and polyhydroxybutyrate accumulating propensities inherent in strains already present.

Acknowledgements

The research was supported by the Water Research Commission and this paper is published with their permission.

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