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REVIEW PAPER

MICROBIOLOGY AND BIOCHEMISTRY OF THE ENHANCED BIOLOGICAL PHOSPHATE REMOVAL PROCESS

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Abstract—This paper reviews microbiological and biochemical aspects of the enhanced biological phosphate removal (EBPR) process. The discussion includes: microorganisms responsible for EBPR, isolation of polyphosphate accumulating organisms (PAOs), microbial diversity of the EBPR sludge, biochemical metabolisms of PAOs, energy budget in PAOs metabolism, denitrification by PAOs, glycogen accumulating non-poly-P organisms (GAOs), etc. Since pure cultures which possess complete characteristics of PAOs have not been isolated yet, the biochemical mechanism cannot be definitively described. The criteria to obtain a pure culture isolate are proposed. Based on the review, essential characteristics of PAOs are summarized in a table and directions for future research are identified. © 1998 Elsevier Science Ltd. All rights reserved

Key words—enhanced biological phosphate removal (EBPR), glycogen, glycogen accumulating organisms (GAOs), polyhydroxy alkanoates (PHA), polyphosphate accumulating organisms (PAOs), succinate–propionate pathway, TCA cycle

INTRODUCTION

Biological phosphate removal from wastewater can be achieved in two ways: stoichiometric coupling to microbial growth or enhanced storage in the biomass as polyphosphate (poly-P). The latter was formerly called "luxury uptake" (Levin and Shapiro, 1965) and is the key mechanism in the enhanced biological phosphate removal (EBPR) process. The EBPR process is primarily characterized by circulation of activated sludge through anaerobic and aerobic phases, coupled with the introduction of influent wastewater into the anaerobic phase (Barnard, 1975). By this anaerobic aerobic configuration, microorganisms which accumulate poly-P and thus have a high phosphorus content are selected and grow to dominance in the process. High phosphate removal efficiency can be achieved by withdrawing the excess sludge with high phosphorus content.

Predominance of polyphosphate accumulating organisms (PAOs) in the anaerobic–aerobic configuration can be explained as follows: if an anaerobic phase is introduced in which activated sludge is mixed with the influent wastewater, microorganisms capable of anaerobically taking up carbon sources from the influent are favored. PAOs can do this because they are able to hydrolyze stored poly-P in order to supply energy for the anaerobic uptake of the carbon sources. Thus, in the anaerobic phase, PAOs take up the carbon sources and store them in the form of polyhydroxyalkanoates (PHA) accompanied by degradation of poly-P and consequent release of orthophosphate. In the subsequent aerobic phase, PAOs grow aerobically and take up orthophosphate to recover the poly-P level by using the stored PHA as the carbon and energy source. Since PHA is a reduced polymer, its synthesis requires reducing power. Wentzel *et al.* (1991) pointed out two possible biochemical models to explain the source of the reducing power, the Mino model and the Comeau–Wentzel model. In the Mino model (Mino and Matsuo, 1984; Mino *et al.*, 1987; Arun *et al.*, 1988a), the reducing power is considered to be derived from degradation of intracellularly stored glycogen, whereas in the Comeau–Wentzel model (Matsuo, 1985; Comeau *et al.*, 1986; Wentzel *et al.*, 1986) partial oxidation of acetyl-CoA through the TCA cycle is assumed to produce the required reducing power.

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Microbiological aspects of the EBPR process were reviewed by Jenkins and Tandoi (1991). At that time the common understanding was that *Acinetobacter* spp. is responsible for EBPR (Fuhs and Chen, 1975; Buchan, 1983; Lotter, 1985). Nevertheless, the conclusion was that no pure cultures of *Acinetobacter* studied have shown all the typical characteristics of biologically phosphate removing EBPR sludges. This situation has remained unchanged until now. Still no single pure cultures have been proved to be one of the predominant bacteria in the EBPR process. In other words, microorganisms responsible for EBPR have never been isolated or identified, therefore, very little enzymological and genotypical aspects of PAOs have been studied so far. The EBPR process is well established in practice. Many full scale EBPR plants are in operation. It is surprising to note that a technically established biological process like the EBPR process still lacks fundamental understanding from microbiological and biochemical points of views.

Following the reviews by Jenkins and Tandoi (1991) from a microbiological view point and by Wentzel *et al.* (1991) from a biochemical view point, some of the questions have been answered but many new aspects have arisen. It is, therefore, an appropriate time to review what has been clarified and what remains unclear about the EBPR process. The present paper reviews microbiological and biochemical aspects of the EBPR process, and proposes possible future research areas to further improve understanding of the process.

MICROBIOLOGICAL ASPECTS

*Is **Acinetobacter** responsible for EBPR?*

It has been demonstrated now that *Acinetobacter* is NOT primarily responsible for EBPR. The experimental evidences reported so far that indicate this are as follows:

(1) A fluorescent antibody staining technique for *Acinetobacter* revealed (Cloete and Steyn, 1987) that the number of *Acinetobacter* in the EBPR processes studied was less than 10% of total bacteria, and could not account for the EBPR observed.

(2) The dominant respiratory quinones in PAO-enriched sludges were quinone-8 (Q-8) and menaquinone-8(H_4) (MK-8(H_4)), whereas *Acinetobacter* has Q-9 (Hiraishi *et al.*, 1989; Hiraishi and Morishita, 1990; I Made *et al.*, 1998). Quinones are one of electron carriers in the respiratory chain. The type of quinone is species specific, and therefore, by analyzing the quinone profile of a microbial community the dominant populations can be identified.

(3) Auling *et al.* (1991) used a polyamine, diaminopropane (DAP), as a biomarker for *Acinetobacter* and showed that EBPR plants eliminating phosphate very efficiently had nearly no DAP in the polyamine pattern.

(4) Application of a 16s-rRNA targeted oligonucleotide probe specific for *Acinetobacter* spp. showed that *Acinetobacter* was less than 10% of total bacteria and not dominant in the EBPR processes studied (Wagner *et al.*, 1994; Bond *et al.*, 1995; Kampfer *et al.*, 1996; I Made *et al.*, 1998).

Acinetobacter spp. was first identified as the bacterium responsible for EBPR by Fuhs and Chen (1975). Subsequently many researchers reported its predominance in EBPR processes based on culture dependent identification methods such as the API system (Buchan, 1983; Lotter, 1985; Wentzel *et al.*, 1988). In these methods, only those bacteria which are culturable on the artificial media used under the defined conditions can be isolated and identified. It is likely that only a minor portion of bacteria in activated sludges can grow under such conditions and thus can be detected (Wagner *et al.*, 1993; Kampfer *et al.*, 1996). In fact, the gene probe technique has revealed that the classical culture-dependent methods for bacterial counting are strongly selective for *Acinetobacter* spp. (Wagner *et al.*, 1994); the above mentioned evidences against *Acinetobacter* were all obtained through non-culture-dependent methods. Further, very intensive research has demonstrated that no pure cultures of *Acinetobacter* have shown the typical characteristics of EBPR sludges with high P removal capability (for details, see Jenkins and Tandoi, 1991; Van Loosdrecht *et al.*, 1997). Accordingly *Acinetobacter* spp. need no longer be considered as the principle organisms responsible for the EBPR process.

What microorganisms are responsible for EBPR?

Interesting isolates. Morphological characteristics of PAOs were first described by Fuhs and Chen (1975) based on microscopic observations of PAO-enriched sludge as follows: they are non-motile rods or cocci, usually exist in clusters, are PHB staining positive, and contain Neisser positive granules in the cell. They were believed to be Gram negative bacteria, but later a possibility has arisen that they are Gram positive (Wagner *et al.*, 1994; Liu, 1995). Many attempts have been made to isolate PAOs responsible for EBPR, but they have all failed. Formerly the target bacteria were *Acinetobacter* spp., which were subsequently shown not to be responsible for EBPR (see above). Many other pure cultures were isolated from EBPR processes, but none of them have exhibited all the characteristics which EBPR sludges should possess. In many cases, the key characteristics which are lacking in these isolates are the anaerobic acetate metabolisms (acetate uptake and its conversion to PHA for storage coupled with hydrolysis of stored poly-P and consequent release of orthophosphate under anaerobic conditions) (Jenkins and Tandoi, 1991).

Nakamura *et al.* (1991, 1995) isolated a polyphosphate accumulating bacterium from a laboratory scale EBPR process and named it *Microlunatus phosphovorus* strain NM-1. This bacterium accumulates polyphosphate under aerobic conditions, which is then used as the energy source for the anaerobic uptake of carbon sources like glucose and casamino acids, but not acetate. Ubukata and Takii (1994) independently isolated a similar bacterium and demonstrated that the bacterium exhibited the anaerobic utilization and aerobic accumulation of poly-P only after alternating anaerobic and aerobic conditions were applied, which implies that the enzyme system for the poly-P metabolism is not constitutive but inducible. NM-1 is physiologically close to PAOs, and phylogenetically belongs to Gram positive bacteria with a high G + C content which PAOs are suspected to belong to (Wagner *et al.*, 1994). However, it may not be one of the predominant bacteria in the EBPR process, because (1) it does not convert acetate to PHA under anaerobic conditions and (2) it contains Q-9 (Nakamura *et al.*, 1995), whereas PAO enriched sludges should have Q-8 or MK-8(H_4) as the major quinones (Hiraishi and Morishita, 1990).

Liu (1995) and Liu *et al.* (1997a) isolated several PHA, glycogen and /or poly-P accumulating bacteria from acetate fed anaerobic aerobic SBRs. They belong to Gram positive bacteria with a high G + C content and many of them contain MK-8(H_4) as the major quinone. Some of them could grow on acetate with production of PHA under aerobic conditions, but none of them have shown anaerobic conversion of acetate to PHA accompanied by poly-P utilization.

Stante *et al.* (1996) isolated a PHB storing strain from an SBR designed for EBPR and identified it as *Lampropedia* spp. It is Neisser staining positive, indicating poly-P storage, and has a capability to take up acetate and store it as PHA under anaerobic conditions. Functionally this isolate resembles PAOs, but morphologically it has a very unique sheet-like cell arrangement which is not common in EBPR processes.

Application of molecular techniques for microbial populations of EBPR process. Recently, it was found through fluorescence *in situ* hybridization (FISH), a molecular technique using oligonucleotide probes, that sludges from EBPR processes contained relatively high number of Gram positive bacteria with a high G + C content (Wagner *et al.*, 1994; Kampfer *et al.*, 1996). This group is suspected to play an important role in the EBPR process. However, Bond *et al.* (1995) applied the clone library approach and reported that only few Gram positive bacteria with a high G + C content were found in the EBPR reactor examined and that a critical difference in the microbial structure between EBPR and non-EBPR processes existed in the beta subclass of proteobacteria, suggesting that this group may have a specific role in the EBPR process. High occurrence of the beta subclass of proteobacteria was also reported by Wagner *et al.* (1994).

Conventionally, it has been assumed that EBPR sludges with high P removal capability would be enriched with a single dominant group of microorganisms. However, there is evidence which implies that the microbial community of the EBPR process is diverse: Liu (1995) has reported that a sludge with very high P removal capacity (phosphorus content: 8–12.5% based on VSS) contained at least three dominating microorganisms which are morphologically distinguishable. The gene probe techniques indicate that the EBPR sludges phylogenetically consist of several different microbial populations (Wagner *et al.*, 1994; Bond *et al.*, 1995; Kampfer *et al.*, 1996). The denaturing gel gradient electrophoresis (DGGE) technique (Brdjanovic *et al.*, 1997b; Liu *et al.*, 1997c) and the restriction fragment length polymorphism (RFLP) (Liu *et al.*, 1997b) have revealed that the 16s-rDNAs extracted from EBPR sludges contain several different DNA fragments implying that the sludges are not dominated by a single bacteria but composed of a few dominant bacterial strains. In addition, past experimental observations indicate that the EBPR community may not be identical but can change from time to time and from place to place: e.g., morphology of PAOs reported in the literature (e.g., Fuhs and Chen, 1975; Buchan, 1983; Streichan *et al.*, 1990; Matsuo, 1994) has been inconsistent. Based on these recently available results, it would appear that PAOs do not consist of one single dominant bacterium but consist of several different bacterial groups. In addition, diverse organisms performing functions other than EBPR should influence the relative number of PAOs in EBPR processes. At this stage, available data are so limited that no definite conclusions can be drawn about the microbial community structure of the EBPR process. Since the molecular techniques are very promising in characterizing microbial community structure, they should be further applied to solve the “microbiological puzzle” (Bond *et al.*, 1995) of the EBPR process.

It is strongly recommend that, when molecular characterizations are performed on a particular EBPR sludge, an acetate-fed pH-controlled anaerobic aerobic batch experiment with measurements of essential parameters like PHA, glycogen, acetate and orthophosphate as well as phosphorus content of the sludge should be done simultaneously. Such an experiment can indicate if the examined sludge performs “typical” metabolism of the EBPR sludge with high P removal capability, and to what extent the examined sludge biomass has similarity to and deviations from PAOs. Without such an experiment it is difficult to interpret the molecular data properly in the context of EBPR. Recently, microautoradiography has been performed together with *in situ* hybridization with rRNA targeted oligonucleotide probes to study *in situ* physiological functions of certain target microorganisms (Andreasen and Nielsen, 1997; Nielsen *et al.*, 1997). In this technique, the probing method can tell who are in the community and autoradiography can indicate what metabolism they are doing. Therefore, this technique may be used successfully instead of the above proposed batch experiment to see the metabolic function of certain microorganisms in the EBPR sludge.

What is necessary for successful isolation of PAOs?

Isolation of PAOs remains essential, because experiments with pure cultures will provide substantial information about the microbiological and biochemical aspects of the EBPR process. For future attempts to isolate PAOs, the following is recommended:

(1) PAOs are considered to be relatively slow growing (Nakamura and Dazai, 1986; Smolders *et al.*, 1994b). Adequate cultivation time has to be allowed before harvesting cells to enable PAOs to grow to a substantial amount. Nakamura *et al.* (1991) and Liu (1995) successfully isolated slow growing bacteria with poly-P or PHA storing capability by using colony forming methods. They picked up colonies after incubating the cells for 1–3 weeks. At least one week or more cultivation time is recommended.

(2) Ubukata and Takii (1994) reported that the enzymes for the anaerobic substrate uptake and polyphosphate utilization appear to be inducible and that alternating anaerobic aerobic conditions are necessary to induce the EBPR metabolism in their isolate. The alternating anaerobic aerobic conditions may be necessary even during the isolation period. They can be achieved, for example, by the floating filter technique (de Bruyn *et al.*, 1990) with some modifications.

(3) Presence of phosphate and carbon sources at the same time under aerobic or anoxic conditions has negative effects on P uptake (Wentzel *et al.*, 1988; Smolders *et al.*, 1994b; Kuba *et al.*, 1994; Brdjanovic *et al.*, 1998). Carbon sources available under these conditions will be primarily used for PHA formation. Only when the external carbon sources are exhausted, P uptake occurs. This implies that simultaneous presence of carbon sources and an electron acceptor (either oxygen or nitrate/nitrite) should be prevented.

(4) Various kinds of selective culturing techniques may be applied. For example, if the target is limited to Gram positive bacteria with high G + C content, treatment with an alkali may be applicable to suppress the growth of Gram negative bacteria. With such a treatment, Gram positive bacteria, which have cell walls thicker and less sensitive to alkali than Gram negative bacteria, may grow exclusively.

Past unsuccessful attempts to isolate PAOs have all violated points 2 and 3. So, special emphasis should be given to these two points.

A possibility is that PAOs cannot grow as a single pure culture, but that some kinds of interspecies relations between different groups of microorganisms are essential. Several isolates from EBPR processes did not grow when put on an agar plate as a single cell (Liu, personal communication). However, no experimental evidence has been reported to clearly indicate that the presence of multiple populations is essential for PAO's growth. In fact, the ecological aspects of the EBPR process have been very little investigated. The limited available information includes: (1) fermentative bacteria are beneficial to PAOs because they supply short chain fatty acids (Brodisch and Joyner, 1983), and (2) a conceptual model of the ecological relations among sulfate reducers, sulfate oxidizers, denitrifiers and PAOs has been proposed (Yamamoto-Ikemoto *et al.*, 1994). It may be important in the isolation work to consider the interspecies relations between PAOs and other microbial populations. The ecological aspects (symbiotic or competitive relations between species) should receive attention in the future.

BIOCHEMICAL ASPECTS

Which pathway is used for the generation of reducing power necessary for anaerobic PHA synthesis – the degradation of glycogen or the TCA cycle?

The conversion of acetate, a favorable substrate for EBPR, to PHA requires reducing power, because PHA is a more reduced compound than acetate. The idea that the TCA cycle functions under anaerobic conditions to oxidize a part of the acetate to CO_2 and to generate reducing power in the form of NADH was first proposed by Matsuo (1985) and later by Comeau *et al.* (1986) and Wentzel *et al.* (1986). An alternative idea was proposed by Mino and Matsuo (1984), Mino *et al.* (1987) and Arun *et al.* (1988a) in which anaerobic degradation of intracellularly stored glycogen to acetyl-CoA as well as its partial oxidation to CO_2 is hypothesized to generate reducing power for PHA synthesis. Wentzel *et al.* (1991) referred to the former as "the Comeau–Wentzel model" and the latter as "the Mino model".

There are several experimental evidences which strongly support the Mino model:

(1) The theoretically developed stoichiometry for the Mino model explains very well the experimentally observed stoichiometry of anaerobic acetate uptake, PHA formation, glycogen utilization and CO_2 production by PAO-enriched sludges (Satoh *et al.*, 1992, 1996; Smolders *et al.*, 1994a).

(2) Bordacs and Chiesa (1989) used radioactively labeled acetate as the carbon source for an EBPR

sludge. Their results showed that only a very small portion of the radioactivity was found in the CO_2 generated under anaerobic conditions, which indicates that the acetate taken up anaerobically was not oxidized to CO_2, and thus not metabolized through the TCA cycle.

(3) Satoh *et al.* (1992) showed in a ^{13}C tracer experiment using NMR that, in the anaerobic uptake of propionate, the acetyl-CoA necessary for PHA production was not derived from the external substrate, but from somewhere else, implying that there should be an additional mechanism to supply acetyl-CoA. The utilization of glycogen was proposed as this mechanism.

(4) Smolders *et al.* (1994a,c) found that under low pH conditions the phosphate release was less than the amount theoretically necessary for the formation of acetyl-CoA, indicating that there is an additional mechanism to supply energy (ATP) under anaerobic conditions. This could be achieved through degradation of glycogen.

(5) Pereira *et al.* (1996) demonstrated using ^{13}C NMR that acetate anaerobically taken up by an EBPR sludge is converted to PHA, which, in the subsequent aerobic phase, is converted to glycogen, which further supplies the carbon source for PHA formation and CO_2 generation in the next anaerobic phase. Maurer (1997) also showed using ^{13}C NMR that glycogen is involved in the anaerobic metabolisms of EBPR sludges.

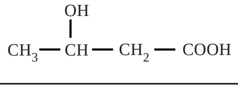
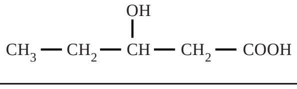
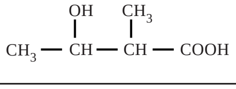
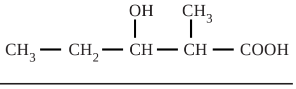
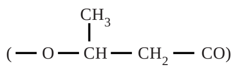
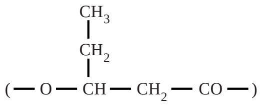
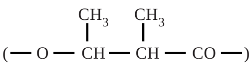
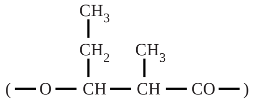
From the above, it can be reasonably concluded that the reducing power required for anaerobic PHA synthesis is primarily supplied by the degradation of stored glycogen and that the Mino model is likely to be correct.

Although the experimental evidence favors the Mino model, the possibility of partial functioning of the TCA cycle cannot be totally excluded. In fact, Pereira *et al.* (1996) incubated ^{13}C labeled acetate with a PAO-enriched sludge under anaerobic conditions (with no nitrite or nitrate) and found that a small fraction of the labeled carbon in acetate was released as CO_2. Also based on a redox balance considerations, they concluded that the reducing power generated in the observed degradation of glycogen was insufficient to account for the PHA production. These are strong indications that a small fraction of acetate is metabolized through the TCA cycle under anaerobic conditions supplying a minor part (30%) of the reducing power for PHA formation. So far, this is the only experimental result indicating the possible functioning of the TCA cycle in the anaerobic phase of the EBPR process. Usually the TCA cycle is linked with respiration and operates only under aerobic or anoxic conditions. The oxidation of succinate to fumarate in the TCA cycle requires a terminal electron acceptor with a redox potential ($E^{0\prime}$) more positive than that of fumarate/succinate couple (+32 mV). Only O_2 (O_2/H_2O, $E^{0\prime} = +818$ mV), NO_3^- (NO_3^-/NO_2^-, $E^{0\prime} = +433$ mV) and NO_2^- (NO_2^-/N_2^-, $E^{0\prime} = +970$ mV) appear to meet these conditions (Thauer, 1988). A hypothetical pathway is postulated to explain the conversion of acetate to CO_2, in which the glyoxylate cycle plays the key role and acetate is metabolized to CO_2 or 3HV (Mino *et al.*, 1998). Therefore, precise measurements of the produced CO_2 and the 3HB/3HV ratio will be useful for the identification of the pathway. For more definitive conclusions, further tracer experiments are recommended as well as enzymological studies. Clearly, a pure culture is desirable in this respect.

What is the intrinsic role of glycogen in the PAOs metabolism?

Satoh *et al.* (1992) found that, when lactate was taken up anaerobically by a PAO-enriched sludge, significant amounts of 3-hydroxyvalerate (3HV, see Table 1) were found in the PHA produced. A 3HV unit of PHA is made from an acetyl-CoA molecule and a propionyl-CoA molecule. Therefore, this experimental results suggests that there should be a mechanism to produce propionyl-CoA in addition

Table 1. Monometric units of PHA found in the EBPR sludge

	3-hydroxybutyrate (3HB)	3-hydroxyvalerate (3HV)	3-hydroxy-2-methylbutyrate (3H2MB)	3-hydroxy-2-methylvalerate (3H2MV)
Free Acid	 $CH_3-\overset{OH}{\overset{\vert}{CH}}-CH_2-COOH$	 $CH_3-CH_2-\overset{OH}{\overset{\vert}{CH}}-CH_2-COOH$	 $CH_3-\overset{OH}{\overset{\vert}{CH}}-\overset{CH_3}{\overset{\vert}{CH}}-COOH$	 $CH_3-CH_2-\overset{OH}{\overset{\vert}{CH}}-\overset{CH_3}{\overset{\vert}{CH}}-COOH$
In PHA	 $(-O-\overset{CH_3}{\overset{\vert}{CH}}-CH_2-CO)$	 $(-O-\overset{CH_3}{\overset{\vert}{\overset{CH_2}{\overset{\vert}{CH}}}}-CH_2-CO-)$	 $(-O-\overset{CH_3}{\overset{\vert}{CH}}-\overset{CH_3}{\overset{\vert}{CH}}-CO-)$	 $(-O-\overset{CH_3}{\overset{\vert}{\overset{CH_2}{\overset{\vert}{CH}}}}-\overset{CH_3}{\overset{\vert}{CH}}-CO-)$
Precursors	2 acetyl-CoA	1 acetyl-CoA 1 propionyl-CoA	1 acetyl-CoA 1 propionyl-CoA	2 propionyl--CoA

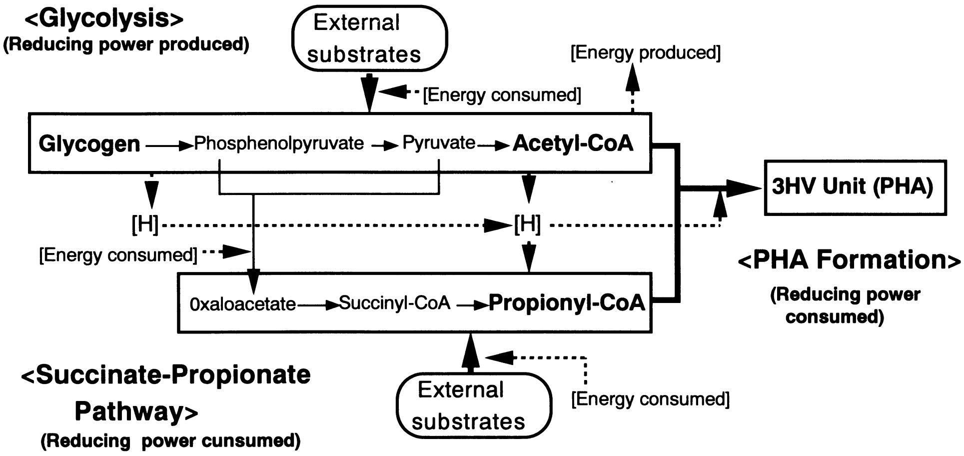


Fig. 1. A conceptual biochemical model for the anaerobic uptake of organic substrates and their conversion to PHA by PAOs.

to acetyl-CoA. Satoh *et al.* (1992) and Liu *et al.* (1994) assumed partial conversion of pyruvate to propionyl-CoA. There are two pathways known for this conversion in certain anaerobic bacteria: the succinate–propionate pathway and the acrylic acid pathway (Gottschalk, 1986). It is likely that the former is functioning, because compounds located in the succinate–propionate pathway can be well metabolized by PAO-enriched sludges (Matsuo and Miya, 1987; Arun *et al.*, 1988b; Satoh *et al.*, 1996). If pyruvate is metabolized through the succinate–propionate pathway, reducing power is consumed. By combining the ideas of the glycogen degradation for the supply of reducing power with acetyl-CoA production and the succinate–propionate pathway for the consumption of reducing power with propionyl-CoA production, an integrated biochemical model can be developed for anaerobic uptake of various carbon sources by PAOs (Satoh *et al.*, 1992; Mino *et al.*, 1994). The overall concept (Mino *et al.*, 1995b, 1996) of the developed model is graphically shown in Fig. 1. In terms of this model, glycogen stored in the cell functions as the regulator of the redox balance in the cell. Conversion of glycogen to acetyl-CoA and CO_2 generates reducing power, whereas conversion to propionyl-CoA via the succinate–propionate pathway consumes reducing power. In actual EBPR processes, PAOs have to be ready for the uptake of various kinds of reduced or oxidized organic substrates in the anaerobic phase without disturbing the redox balance in the cell. The function of stored glycogen to maintain the redox balance, therefore, appears to be essential for the anaerobic uptake of various organic substrates, and thus for the proliferation of PAOs in the EBPR process.

Both poly-P and glycogen are needed for the anaerobic uptake of organic substrates by PAOs, because the former supplies energy and the latter supplies reducing power as well as energy. Both can be the limiting substance for the anaerobic substrate uptake. Under normal conditions, neither poly-P nor glycogen are totally depleted at the end of the anaerobic phase (personal experiences of the authors, data not shown) and they appear to be stored to greater levels than needed for routine anaerobic metabolism. It has, however, been reported (Kuba *et al.*, 1996b; Brdjanovic *et al.*, 1997a) that, when excess acetate is fed, the anaerobic uptake of acetate by a PAO-enriched sludge stops not because of poly-P limitation or PHA saturation, but because of exhaustion of glycogen. This implies that glycogen can be the limiting substance in the anaerobic substrate uptake by PAOs under shock loading conditions. Brdjanovic *et al.* (1997a) further suggest that poly-P (energy source) would be limiting at high pH, since more energy is required for acetate transport through the membrane at high pH (Smolders *et al.*, 1994a,c).

The analytical methods for glycogen have been often questioned. Traditionally either colorimetric methods such as the anthrone method (e.g., Mino *et al.*, 1987) and the phenol method (e.g., Liu *et al.*, 1994) or HPLC analysis for glucose after acid digestion (e.g., Smolders *et al.*, 1994a) have been used for "glycogen" determination. These are all analytical methods for total carbohydrate or total glucose, and determine not only glycogen but also other carbohydrates, leading to possible overestimation of glycogen. Liu *et al.* (1994) proved enzymatically that the carbohydrate stored in the EBPR sludge is glycogen. Recently two innovative methods have been proposed for glycogen determination: Schulze *et al.* (1995) used an enzymatic method for glycogen determination after extraction from the cell. Brdjanovic *et al.* (1997a) have proposed a batch experiment in which the sludge is exposed to excess acetate feeding under anaerobic conditions and the maximum acetate uptake is measured; for glycogen determination, the stoichiometric relation between acetate uptake and glycogen consumption is applied assuming that glycogen is the limiting substance in the anaerobic acetate uptake. The former method measures total glycogen present, whereas the latter measures glycogen which is associated with anaerobic acetate uptake by PAOs only. For further detailed consideration on glycogen metabolism in the EBPR process or for characterization of the EBPR biomass for mathematical modeling purpose, these novel methods would be useful.

What are other essential metabolic characteristics of PAOs?

PHA structure. From a metabolic point of view, PAOs are characterized primarily by the capability to anaerobically take up organic substrates and store them by utilizing energy from the hydrolysis of stored poly-P without consuming any electron acceptors. When organic substrates are taken up anaerobically, they are usually converted to PHA and stored. In the early years of the EBPR research, "polyhydroxybutyrate (PHB)" was already recognized as a storage polymer in the anaerobic phase of the EBPR process by staining techniques followed by microscopic observation (e.g., Buchan, 1983). Later, Comeau *et al.* (1987) analytically verified that the PHB-like polymer contains 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) as monometric building units. Satoh *et al.* (1992) further revealed that the storage polymer consists of four monometric units as shown in Table 1. Thus, today the polymer is called polyhydroxyalkanoate (PHA) in general. PHA in PAO-enriched sludges have been verified to be co-polymers composed of these four units (Inoue *et al.*, 1996). When acetate is the only carbon source available in the anaerobic phase, the 3HB unit is the major unit in the PHA formed (Satoh *et al.*, 1992; Smolders *et al.*, 1994a), which can then be called PHB. The composition of PHA formed from some other carbon sources can be predicted theoretically based on the stoichiometries developed by Mino *et al.* (1994) and Satoh *et al.* (1996).

Favorable carbon sources for EBPR. It has been reported that PAOs thrive on short chain fatty acids (SCFAs) like acetate and propionate (e.g., Wentzel *et al.*, 1985) and that fermentation in the anaerobic zone is important for EBPR because it produces SCFAs (Munch and Koch, 1997). In practice, prefermentation of primary sludge has been successfully applied to enhance biological P removal by increasing the supply of SCFAs (Rabinowitz *et al.*, 1987; Barnard, 1992). Many laboratory scale EBPR reactors have been successfully operated with acetate as the major carbon source (e.g., Smolders *et al.*, 1994a). If the retention time of wastewater in the sewer pipes is long enough for fermentation, a significant portion of organic matters in the sewage has been fermented to SCFAs (mainly to acetic acid) before being transported to the treatment plant. In such cases (for example, in the Netherlands) acetate is the most important substrate for EBPR processes. A lot of research has been indeed dedicated to investigate the acetate metabolism in the EBPR process. Consequently, it is assumed when the EBPR process is mathematically modeled (Henze *et al.*, 1995; Smolders *et al.*, 1995a) that PAOs utilize only SCFAs. On the other hand, a wide range of organic matter including carboxylic acids, sugars and amino acids have been reported to be utilized anaerobically by PAO-enriched sludges (Matsuo and Miya, 1987; Arun *et al.*, 1988b,c, 1989; Satoh *et al.*, 1990, 1996). The importance of carbon sources other than SCFAs for proliferation of PAOs is not clear at the moment. More attention should be paid to the type of carbon source and its effects on EBPR, especially when a large portion of organic matters in the wastewater reaches the treatment plant before acid fermentation is completed (for example, in Japan). Further research will be needed in this respect.

The accumulation of PHA under the anaerobic conditions is a strategy adopted by PAOs to survive in the anaerobic–aerobic process. It is not surprising that under different conditions storage polymers other than PHA play the same role as PHA. Fukase *et al.* (1982) reported that a glucose fed EBPR sludge took up glucose and stored it as glycogen accompanied by P release in the anaerobic phase. Satoh *et al.* (1990) reported that, when glutamic acid was taken up anaerobically by a PAO-enriched sludge, a part of the carbon "disappeared" without releasing ammonium ions, strongly indicating that the glutamic acid was converted to nitrogenous storage compounds. Satoh *et al.* (1997) have further suspected the conversion of glutamic acid to a kind of polypeptide. From biochemical

Table 2. Ratios of carbon uptake to phosphorus release in the anaerobic metabolism of EBPR processes reported in the literature (modified from Liu *et al.* (1997a) and Smolders *et al.* (1994a))

Reference	P content (mg P/g TSS)	P release/acetate uptake (mol P/mol C)
Fukase *et al.*, 1982	7.6–8.1	0.45
Arvin and Kristensen, 1985	–	0.62–0.74
Fukase *et al.*, 1985	2.1	0
	9.4	0.42
Wentzel *et al.*, 1986	–	0.24
Comeau *et al.*, 1987	4.1	0.7–0.8
Mino *et al.*, 1987	3.3	0.15
	6.5	0.39
Arun *et al.*, 1988a,b,c	4.34.5	0.21–0.39
Wentzel *et al.*, 1988	14.4–15.6	0.52–0.57
Satoh *et al.*, 1992	63–7.1	0.44
Cech and Hartman, 1993	9.6	0.4
Liu *et al.*, 1994	< 2.0	0
Smolders *et al.*, 1994a	7.0–7.5	0.52–1.52
Matsuo, 1994	3.0	0
	9.4	0.55
Liu *et al.*, 1996b	8.0	0.30–0.73
	12.0	0.50–0.88
Brdjanovic *et al.*, 1997d	–	0.35–0.50

point of view, identification of storage polymers and the metabolic pathways for their synthesis from carbon sources other than SCFAs will be an interesting research topic for the future.

Energy budget. Poly-P has been considered to be an energy storage polymer for anaerobic substrate uptake. Van Groenestijn *et al.* (1987, 1989) and van Groenestijn (1988) showed that the activity of AMP-phosphotransferase correlated with the EBPR capacity (expressed as P removal percentage) of several activated sludges. This enzyme catalyzes the reaction: $(\mathrm{Pi})_n + \mathrm{AMP} \rightarrow (\mathrm{Pi})_{n-1} + \mathrm{ADP}$, and appears to be responsible for the energy conservation in PAOs. One of the strange phenomena observed in the EBPR process is the inconsistent variation of the ratio of carbon source taken up to phosphate released. The reported acetate uptake/phosphorus release ratios are summarized in Table 2. This variation indicates that the dependency on poly-P as energy source can vary due to the balance between production and consumption of energy in the cell. In the early stages of the EBPR research, poly-P was considered to be the sole energy source for PAOs under anaerobic conditions. Now it is generally accepted that the utilization of stored glycogen also provides PAOs with energy during anaerobic substrate uptake. No other anaerobic energy generating mechanisms have been identified at present. Since the energy budget is one of the intrinsic parts of the regulation mechanism of anaerobic substrate uptake for PAOs as well as the redox balance, more attention should be paid to it.

Energy requiring processes under anaerobic conditions include: (1) transport of external substrates into the cell, (2) conversion of substrates to PHA and related metabolism, and (3) maintenance (endogenous respiration). Smolders *et al.* (1994a,c) discussed that the transport of acetate into the cell should be thermodynamically influenced by pH and experimentally showed that a lower pH gave a lower P-release/Acetate-uptake ratio with a variation of 0.25–0.75 P-mol/C-mol in a pH range of pH 5.5–8.5, which was also confirmed by Liu *et al.* (1996b). Thus, the pH can be a crucial factor affecting the energy budget of anaerobic substrate uptake by PAOs. Energy requirement for the PHA formation metabolism depends on the pathways used. It has been questioned which pathway is used for the conversion of glycogen to pyruvate, the Embden–Meyerhof (EM) pathway or the Entner–Doudoroff (ED) pathway. The former produces 3 mol of ATP per 1 mol of glycosyl unit (monometric unit of glycogen) degraded, whereas the latter produces 2 mol. Maurer (1997) has shown in their ^{13}C tracer study that the 3- and 4-carbons in glycogen are converted to the 4-carbon in PHB, which strongly indicates that glycogen is metabolized through the ED pathway. Based on this result, it can be tentatively concluded that the ED pathway is used in PAOs for anaerobic utilization of glycogen, which will have to be further subjected to enzymological confirmation. Smolders *et al.* (1995a) and Kuba *et al.* (1996b) estimated the energy requirement for the anaerobic maintenance based on phosphate release after external substrates were exhausted, and obtained a maintenance coefficient of $2.5*10^{-3}$ mol P/mol C h (equivalent to 3.2 mg P/mg PAO-VSS h).

In the aerobic phase, PAOs grow aerobically on the anaerobically stored PHA. They also utilize the stored PHA as the energy and carbon source to recover the glycogen and poly-P levels. Consequently, intracellular glycogen and poly-P increase, stored PHA decreases and soluble orthophosphate is taken up by the sludge. Under anoxic conditions where nitrate is available, the same carbon metabolism has been observed (Kuba *et al.*, 1997a). The cyclic storage and consumption of glycogen and poly-P lead to significant energy consumption in addition to the energy requirement for growth. Apparently PAOs have an energy wasting metabolism. Smolders *et al.* (1994b) estimated the yield value of PAOs to be 13% lower than that of usual heterotrophic biomass. Rapid uptake of substrates in the anaerobic phase is the key for PAOs to survive in the EBPR process. This can only be realized by the glycogen and the poly-P metabolisms. The recovery of poly-P and glycogen to sufficient levels during the aerobic phase may have a higher priority than growth in an ecological context. Microorganisms which are capable of quickly storing substrates and consume them in a more balanced way have a strong competitive advantage over microorganisms without the capability of substrate storage (Van Loosdrecht and Heijnen, 1997).

Denitrification by PAOs. A part of PAOs have been found to utilize nitrate as electron acceptor (Vlekke *et al.*, 1988; Kerrn-Jespersen and Henze, 1993; Kuba *et al.*, 1993, 1997b; Bortone *et al.*, 1994; Chuang *et al.*, 1997). Under anoxic conditions where no oxygen is present but nitrate is available, PAOs perform the same metabolism as the one under aerobic conditions, namely, recovery of intracellular glycogen and poly-P levels by consuming stored PHA and taking up external orthophosphate (Kuba *et al.*, 1993, 1996b). However, the energy production efficiency with nitrate expressed in terms of mol ATP/mol NADH is estimated to be 40% lower than that with oxygen (Kuba *et al.*, 1994). Consequently, a 20% lower cell yield value was reported for an anaerobic–anoxic EBPR process than for an anaerobic–aerobic process (calculated after Murnleitner *et al.*, 1997). Anoxic P uptake rates were lower than aerobic ones, but the overall P removal performance was good for both anaerobic–anoxic systems and anaerobic–aerobic systems (Kuba *et al.*, 1994). This may be partly because of the fact that the energy for poly-P formation is relatively small compared to the energy for growth or

PHA production (Mino *et al.*, 1987; Kuba *et al.*, 1996b); poly-P production may not be significantly affected by the decrease of available total energy. Another possible reason is that poly-P accumulation capacity of PAOs is usually higher than normal P loading; even a slower P uptake does not lead to overall P removal deterioration. In such a case, higher phosphorus content is expected in the anoxic P removal system. However, the complicated metabolism of PAOs, including the poly-P metabolism and the PHA-glycogen metabolism, can affect the P removal performance in many different ways, and more detailed consideration will be needed on this aspect.

In practice, the denitrifying capability of PAOs is important for two reasons: (1) in the mathematical modeling of the EBPR process, behavior of phosphate and nitrogenous compounds like ammonia, nitrate and nitrite can be predicted only by introducing denitrifying PAOs into the model (Kerrn-Jespersen and Henze, 1993; Mino *et al.*, 1995a; Kuba *et al.*, 1996b; Murnleitner *et al.*, 1997), and (2) the available amount of COD in the wastewater is a crucial limiting factor for both EBPR and denitrification; usage of anoxic phosphate removal can achieve EBPR and denitrification at the same time and save significant amounts of COD (Wanner *et al.*, 1992; Kuba *et al.*, 1996a). Kuba *et al.* (1996a) and Murnleitner *et al.* (1997) established an experimental method to measure the fraction of PAOs which have a capability to denitrify, in which anoxic and aerobic acetate uptake rates are compared. This method may be applicable for sludge characterization in monitoring or mathematical modeling of EBPR processes.

Mathematical modeling of EBPR process. The above mentioned biochemical aspects of the EBPR process have significantly contributed to mathematical modeling of the process. A comprehensive structured model for EBPR was first proposed by Wentzel *et al.* (1989), and later re-structured as IAWQ Activated Sludge Model No. 2 (ASM 2) by Henze *et al.* (1995). In ASM 2, the denitrification capability of PAOs is not considered and glycogen is not introduced as a variable, because these aspects were considered too specific for a general mathematical model and relevant data available were so limited when the model was developed. However, the former is essential if anoxic behavior of PAOs is to be predicted and the latter is necessary if, for example, shock loading conditions are modeled where glycogen can be the limiting substance (see above). The most detailed mathematical model so far was developed by Smolders *et al.* (1994a,b, 1995a), in which most of the biochemical aspects discussed in this paper were considered including stoichiometries of the EBPR metabolism with glycogen, PHA and poly-P as intracellular storage polymers, energy budget under aerobic and anaerobic conditions, etc., but not directly including the effect of pH. This model was further extended to describe anaerobic–anoxic process for EBPR (Kuba *et al.*, 1996b; Murnleitner *et al.*, 1997). These models can describe rather well the behavior of laboratory scale EBPR processes enriched with PAOs (Smolders *et al.*, 1995b, 1996). But, if more precise modeling of the process is needed for academic purpose or for the purpose of application of the models to practical situations, kinetic information of PAOs is still needed and should be further investigated.

Table 3. Expected characteristics of PAOs

	Items	Characteristics of PAOs	Specific reference
Morphology	cell shape etc.	most probably short rod or cocci.	–
		frequently present in cluster.	–
	Gram stain	uncertain.	–
	Neisser stain	strongly positive, after aerobic growth.	–
	PHB staining	strongly positive, after anaerobic substrae uptake.	–
	internal structures	poly-P and PHA granules are visible.	–
Chemotaxonomy	quinone	Q-8 or MK-8(H_4)	Hiraishi and Morishita, 1990
	fatty acid	16:1 d9c, 16:0, or 18:1 d11c	Liu, 1995
Phylogenetics		suspected to belong to Gram positive bacteria with a high G + C content or beta subclass of protobacteria	Wagner *et al.*, 1994; Bond *et al.*, 1995; Kampfer *et al.*, 1996
Physiology	anaerobic metabolism	take up acetate and convert it to PHA accompanied by poly-P and glycogen degradation and phosphate release.	–
		take up wide variety of low molecular organic compounds like sugars, carboxylic acids and amino acids.	Matsuo and Miya, 1987; Arun *et al.*, 1988b,c
		enzymes for anaerobic metabolisms are inducible.	Ubukata and Takii, 1994
	aerobic/anoxic metabolism	convert internally stored PHA to glycogen, accumulate poly-P level and grow in the absence of external carbon sources.	
		presence of carbon source inhibits uptake of phosphate.	Wentzel *et al.*, 1988; Smolders *et al.*, 1994b
		some can utilize nitrate as electron acceptor.	–

Summary of expected characteristics of PAOs

Based on the above microbiological and biochemical observations, expected characteristics of PAOs are summarized in Table 3. Some additional important morphological, chemotaxonomic and physiological characteristics associated with PAOs are also given in this table. These characteristics can be used as criteria to evaluate the resemblance of a sludge to PAOs and to determine whether an isolated bacterial stain can be considered to represent PAOs or not.

Table 5. Comparison of metabolisms of PAOs and GAOs

Metabolism	PAOs	GAOs
In the anaerobic phase		
Uptake of external organic substrates	+	+
Consumption of intracellular glycogen	+	+
Accumulation of intracellular PHA	+	+
Consumption of intracellular polyphosphate and consequent release of orthophosphate	+	−
In the aerobic phase		
Recovery of intracellular glycogen	+	+
Consumption of stored PHA	+	+
Growth	+	+
Recovery of intracellular polyphosphate	+	−

GLYCOGEN ACCUMULATING NON-POLY-P ORGANISMS (GAOS)

Under what conditions do GAOs appear?

In practice, the EBPR process treating municipal wastewaters is relatively stable in terms of P removal unless there are external disturbances like excessive rain fall, too high loading (Fukase *et al.*, 1985), shortage of potassium (Brdjanovic *et al.*, 1996), excessive aeration (Brdjanovic *et al.*, 1998) and high nitrate loading to the anaerobic zone (e.g., Kuba *et al.*, 1994). However, in some laboratory scale EBPR reactors, deterioration of EBPR has been reported due to unknown reasons. In such cases, a particular type of microorganism often dominates which can take up organic substrates in the anaerobic phase without P release, indicating no involvement of poly-P in the anaerobic metabolism. Cech and Hartman (1990) found such a microorganism in a glucose fed reactor and named it "G-bacterium". Here, this type of organisms is named glycogen accumulating non-poly-P organisms (GAOs) based on their biochemical characteristics described in the next section of this paper. Table 4 shows cases in which deterioration of EBPR was encountered due to proliferation of GAOs. Several possible reasons for GAOs' proliferation have been reported as shown in Table 4, which include presence of glucose in the wastewater (Cech and Hartman, 1990, 1993), long SRT and HRT (Fukase *et al.*, 1985) and improper seeding (Matsuo *et al.*, 1982). However, definite conditions for the proliferation of GAOs have not been identified. The only successful consistent method to artificially control proliferation of GAOs has been developed by Mino *et al.* (1987) and Liu *et al.* (1994, 1996a, 1997a), in which phosphorus feeding is limited to the requirement for biomass synthesis. This strategy can be applied to obtain stable GAO-enriched cultures for future research purpose.

What are metabolic characteristics of GAOs?

The metabolisms of PAOs and GAOs are compared in Table 5, where it can be seen that GAOs' metabolism is apparently very similar to that of PAOs' except for the involvement of poly-P as energy source under anaerobic conditions. The anaerobic metabolism of organic substrates by PAOs given in Fig. 1 is qualitatively the same as that by GAOs. The difference is that, in case of GAOs, internally stored glycogen would provide energy as well as reducing power necessary for the anaerobic substrate uptake (Cech and Hartman, 1993; Mino *et al.*, 1994). Glycogen is, therefore, the key storage compound for GAOs, and for this reason these types of organisms are named glycogen accumulating non-poly-P organisms (GAOs). GAOs have to be able to produce energy through utilization of glycogen without disturbing the redox balance in the cell. When one molecule of glycosyl unit is consumed, two molecules of pyruvate should be consumed if glycogen is utilized through the ED pathway. If half of the pyruvate produced is metabolized to PHA through acetyl-CoA and the other

Table 4. Cases in which deterioration of EBPR due to GAO proliferation was reported

Reference	Carbon sources	Causative operation	Note
Matsuo *et al.* (1982)	diluted night soil	seed sludge from night soil treatment plant	when seeded by EBPR sludge, good P removal achieved.
Fukase *et al.* (1985)	Ac, Pep, YEx	long SRT and HRT (54 d)	PHB accumulated anaerobically.
Cech and Hartman (1990, 1993)	Ac, Glu	addition of glucose	PHB accumulated and glycogen consumed anaerobically.
Matsuo (1994)	Pep, Ac, Prop	unclear	P removal recovered by extending anaerobic retention time.
Liu *et al.* (1994)	Ac, Pep	limited phosphorus feeding	PHB accumulated and glycogen consumed anaerobically.
Satoh *et al.* (1994)	Ac, Prop, Pep, YEx	unclear	PHB accumulated and glycogen consumed anaerobically.

half through propionyl-CoA, 1 mol of ATP (energy) can be used from glycogen consumption without affecting the redox balance (Satoh *et al*., 1992). GAOs may utilize such a metabolism very efficiently. The stoichiometries for the anaerobic conversion of acetate and propionate to PHA have been theoretically developed (Mino *et al*., 1994; Liu *et al*., 1994; Satoh *et al*., 1994) based on the redox balance concept and the energy budget by assuming that either the EMP pathway or the ED pathway is used for glycogen utilization and that 1 mol of ATP equivalent is required for the transportation of the external substrates into the cell and their conversion to either acetyl-CoA or propionyl-CoA in the cell. The developed stoichiometries can explain the experimental behavior of GAOs when acetate or propionate is fed anaerobically.

Are PAOs and GAOs the same or different organisms?

Morphologically PAOs and GAOs are different. Cech and Hartman (1990, 1993)) found a dominant strain in their glucose fed EBPR reactor with deterioration of EBPR, which was thought to be a GAO. It was Gram-negative, appeared very similar in shape to *Methanosarcina* and existed in clusters. It was easily distinguishable from PAOs, because the GAO strain stained Neisser positive only on their cell walls whereas PAOs contained strongly Neisser positive granules inside the cell. Recently Liu (1995) and Liu *et al.* (1996a) described and compared the morphologies of a PAO-enriched sludge and a GAO-enriched sludge, and reported that GAOs differed from PAOs in that Neisser stain was negative, cells usually occurred in pair or tetrad and contained no intracellular granules before anaerobic substrate uptake.

Brdjanovic (personal communication) designed a batch experiment in which a PAO-enriched sludge, after nearly entire release of poly-P followed by an aerobic incubation in a phosphate free media to reestablish a high glycogen level, was subjected to acetate uptake under anaerobic conditions. The PAO-enriched sludge could not take up acetate after exhaustion of the residual poly-P level, even though glycogen was not limiting. This result implies that PAOs can not utilize glycogen as the sole energy source for anaerobic acetate uptake, although GAOs appears to be able to do so. It appears that PAOs and GAOs have similar metabolic pathways but that these are regulated by different mechanisms.

For the moment being, available observations seem to imply that PAOs and GAOs are different organisms. However, this remains to be definitively proved.

What are possible competition mechanisms between PAOs and GAOs?

If PAOs and GAOs are different organisms, they should compete with each other for organic substrates in the anaerobic phase of the EBPR process. A definite mechanism for such a competition still has to be clarified. PAOs and GAOs apparently have almost the same functional pathways. Both of them store PHA anaerobically. During the PHA formation, the redox balance is regulated by the degradation of glycogen most probably through the ED pathway and the succinate-propionate pathway. The major difference is the source of energy; PAOs generate energy very efficiently by degrading poly-P, whereas GAOs ferment glycogen to PHA and CO_2 to generate energy. The glycogen metabolism adopted by GAOs is much more complex and less efficient in terms of energy production than the poly-P metabolism of PAOs. This inefficiency could be a metabolic disadvantage for GAOs. In fact, PAO-enriched sludges take up acetate more and faster than GAO-enriched sludge (Liu *et al*., 1997a). The information available for the moment indicates that there are no possible reasons that GAOs should dominate over PAOs, which could lead to a conclusion that, if uptake of organic substrates by PAOs is slowed down by one reason or another and a part of the organic substrates remain available for GAOs at the end of the anaerobic phase, then there may be a niche for GAOs to grow (Liu *et al*., 1997a).

Sathasivan *et al.* (1993) performed an anaerobic batch experiment in which excess acetate was fed to a PAO-enriched sludge until the acetate uptake stopped, followed by addition of external glucose. The result showed that the glucose addition induced additional acetate uptake. Liu *et al.* (1996a) reported that the addition of glucose to a GAO-enriched sludge together with acetate reduced the use of internally stored glycogen for anaerobic acetate uptake. Both results strongly indicate that external glucose can replace intracellular glycogen and serve as the reducing power and energy source for anaerobic PHA formation, implying that the dependency on poly-P may be reduced. This could be a reason for the fact that presence of glucose in the influent often induces proliferation of GAOs. However, even when glucose is used as a major carbon source, good EBPR can be achieved occasionally (e.g., Arun *et al.*, 1989). This cannot be explained well.

From the point of view of microbial ecology, the competition between PAOs and GAOs is an interesting topic for further study. Closer examination of the metabolic and physiological aspects of GAO-enriched sludges, as well as analyses of microbial diversity and population dynamics of the EBPR community by means of molecular techniques, may lead to deeper understanding of this subject.

CONCLUSIONS AND FUTURE RESEARCH NEEDS

Major conclusions of the review are:

(1) *Acinetobacter* spp. are not the bacteria primarily responsible for EBPR.

(2) The microbial community of the EBPR process seems to be diverse and consists of several major groups of microorganisms.

(3) Reducing power needed for PHA formation is produced mainly through degradation of internally stored glycogen, and not through the TCA cycle. The possibility of partial contribution of the TCA cycle to generation of reducing power, however, cannot be excluded.

(4) Glycogen seems to be anaerobically metabolized through the Entner–Doudoroff pathway and the succinate–propionate pathway. The function of stored glycogen to maintain the redox balance in the cell appears to be essential for the anaerobic uptake of various organic substrates, and thus for the proliferation of PAOs in the EBPR process.

(5) A part of PAOs are capable of utilizing nitrate as electron acceptor. A batch experiment designed to measure the denitrifying fraction of PAOs is available.

(6) GAOs carry out a metabolism very similar to that of PAOs. The only apparent difference is that GAOs utilize internally stored glycogen as the only energy source for anaerobic substrate uptake.

(7) Available morphological and physiological information implies that PAOs and GAOs are different organisms.

Future research needs can be summarized as follows:

(1) Efforts to isolate PAOs should be continued.

(2) Molecular techniques are promising tools for characterization of the microbial communities of EBPR sludges. When they are applied, a simultaneous anaerobic aerobic batch experiment is recommended to evaluate the similarity of the examined biomass to PAOs.

(3) The possibility of a part of the TCA cycle or the glyoxylate cycle functioning under anaerobic conditions has to be further confirmed experimentally. Precise measurements of the produced CO_2 and the 3HB/3HV ratio as well as enzymological approaches will be useful for identification of the pathway.

(4) Not only the acetate metabolism but also metabolisms of other types of organic substrates should be investigated, because a wide range of organic matter other than short chain fatty acids can be utilized by PAOs and storage polymers other than PHA are found.

(5) Since the energy budget is one of the intrinsic parts of the regulation mechanism of anaerobic substrate uptake for PAOs as well as the redox balance, it should receive more attention.

(6) To clarify the mechanism of competition between PAOs and GAOs, the metabolic and physiological characteristics of GAO-enriched sludges, which can be obtained by limiting phosphate feeding, should be investigated.

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