Occurrence and in situ physiology of uncultured putative alphaproteobacterial glycogen-accumulating organisms in full-scale enhanced biological phosphorus removal wastewater treatment plants
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Published in:
Microbiology

DOI (link to publication from Publisher):
10.1099/mic.0.2006/001032-0

Publication date:
2007

Document Version
Publisher's PDF, also known as Version of record

Link to publication from Aalborg University

Citation for published version (APA):

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Abundance and ecophysiology of *Defluviicoccus* spp., glycogen-accumulating organisms in full-scale wastewater treatment processes

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The activity of glycogen-accumulating organisms (GAOs) in enhanced biological phosphorus removal (EBPR) wastewater treatment plants has been proposed as one cause of deterioration of EBPR. Putative GAOs from the *Alphaproteobacteria*, *Defluviicoccus* spp. (including *D. vanus*), were studied in full-scale EBPR plants to determine their distribution, abundance and ecophysiology. Fluorescence in situ hybridization (FISH) demonstrated that *Defluviicoccus* spp. were generally low in abundance; however, in one plant surveyed, Cluster 2 *Defluviicoccus* constituted 9% of all *Bacteria*. FISH combined with microautoradiography revealed that both Cluster 1 and Cluster 2 *Defluviicoccus* were capable of taking up a narrow range of substrates including acetate, propionate, pyruvate and glucose under anaerobic and aerobic conditions. Formate, butyrate, ethanol and several other substrates were not taken up. Cluster 2 *Defluviicoccus* demonstrated a phenotype consistent with the current metabolic model for GAOs – anaerobic assimilation of acetate and reduction to polyhydroxyalkanoates (PHA) using the glycolytic pathway, and aerobic consumption of PHA. Polyphosphate-accumulating organisms (PAOs, ‘*Candidatus Accumulibacter phosphatis*’) and other putative GAOs (‘*Candidatus Competibacter phosphatis*’) co-existed in two plants with Cluster 2 *Defluviicoccus*, but in both plants, the latter organisms were more abundant. Thus Cluster 2 *Defluviicoccus* can be relatively abundant and could be carbon competitors of PAOs and other GAOs in EBPR plants.

INTRODUCTION

Wastewater treatment plants operating for enhanced biological phosphorus removal (EBPR) reduce inorganic phosphorus (P$_i$) from point-source discharges that may otherwise cause eutrophication. EBPR is achieved by cycling wastewater together with an indigenous mixed microbial community through alternating anaerobic and aerobic conditions, in which organic carbon is available only under anaerobic conditions. Polyphosphate-accumulating organisms (PAOs) anaerobically assimilate organic carbon (e.g. volatile fatty acids, VFAs) as a storage polymer (e.g. polyhydroxyalkanoates, PHAs) and release P$_i$. During the subsequent aerobic phase, the stored carbon is utilized for growth, and excessive amounts of P$_i$ are taken up and accumulated as polyphosphate. The PAOs are removed from the bulk liquid to enable a very low discharge of P$_i$ to the environment (Mino *et al.*, 1998; van Loosdrecht *et al.*, 1997). However, the anaerobic–aerobic and feast–famine selective pressures used in EBPR plants may also favour growth of a different phenotypic group of organisms known as glycogen-accumulating organisms (GAOs) (Liu *et al.*, 1996; Satoh *et al.*, 1992). GAOs also take up and store organic carbon under anaerobic conditions but do not cycle P$_i$ (anaerobic release and aerobic uptake), and their selective enrichment over PAOs via competition for sparingly available VFAs in EBPR plants may also favour growth of a different phenotypic group of organisms known as glycogen-accumulating organisms (GAOs) (Liu *et al.*, 1996; Satoh *et al.*, 1992). GAOs also take up and store organic carbon under anaerobic conditions but do not cycle P$_i$ (anaerobic release and aerobic uptake), and their selective enrichment over PAOs via competition for sparingly available VFAs in EBPR plants leads to excessive P$_i$ in plant effluents (Liu *et al.*, 1996; Mino *et al.*, 1995). Lab-scale (Bond *et al.*, 1999; Oehmen *et al.*, 2005) and full-scale (Kong *et al.*, 2004, 2006; Saunders *et al.*, 2003) EBPR studies showed this PAO–GAO competition for VFAs. Pure cultures that display the PAO or GAO phenotype as described above have not yet been acquired (Blackall *et al.*, 2002; Seviour *et al.*, 2003).

**Abbreviations**: CLSM, confocal laser scanning microscope; EBPR, enhanced biological phosphorus removal; FISH, fluorescence *in situ* hybridization; GAO, glycogen-accumulating organism; MAR, microautoradiography; PAO, polyphosphate-accumulating organism; PHA, polyhydroxyalkanoates; PolyP, polyphosphate; P$_i$, inorganic phosphorus; TCA, tricarboxylic acid; VFA, volatile fatty acid.
While numerous investigations of putative GAOs have been reported (Seviour et al., 2000, 2003), only ‘Candidatus Competibacter phosphatis’ (hereafter called Competibacter spp.) has been demonstrated to be important in full-scale plants (Croce et al., 2002; Kong et al., 2006; Saunders et al., 2003). However, some lab-scale investigations have suggested that uncultured organisms belonging to the Alphaproteobacteria also play a role in breakdown of stable EBPR. These organisms have been phylogenetically identified as closely related to the pure culture Defluviicoccus vanus (Meyer et al., 2006; Wong et al., 2004) and Sphingomonas spp. (Beer et al., 2004). Due to the nature of the lab-scale studies carried out by enrichment of microorganisms on sole organic carbon substrates, key aspects of their ecophysiology in full-scale plants are not known.

The aim of this investigation was to determine the role, if any, of the putative GAOs Defluviicoccus spp. and Sphingomonas spp. in full-scale EBPR processes. Traits of their ecophysiology including substrate uptake patterns, fate of assimilated substrates, anaerobic activity of particular central metabolic pathways and capability to denitrify were investigated. Factors of relevance to the competition between Defluviicoccus spp., PAOs and other putative GAOs in EBPR systems are discussed.

METHODS

Sampling and EBPR plant description. Biomass obtained from nine Danish plants and one Swedish plant was screened for microorganisms closely related to Defluviicoccus spp. and Sphingomonas spp. to determine their abundance in these plants. All plants were being operated for biological N- and P-removal (except for Skovlund, which was operated for N-removal only). The plant influent characteristics are detailed in Table 1.

Of the ten plants studied, two (Bjerringbro and Skagen) contained relatively high numbers of Defluviicoccus spp. (Table 1), and in these two, the abundance of other important EBPR micro-organisms (i.e. Accumulibacter spp. and Competibacter spp.) was determined.

Ecophysiological investigations of Defluviicoccus spp. using FISH-microautoradiography (FISH-MAR) and post-FISH chemical staining techniques were only carried out in plants that contained a relatively high abundance of Defluviicoccus spp. Two plants (Aalborg West and Helsingborg) were chosen to study Cluster 1 Defluviicoccus. Four plants (Aalborg West, Skagen, Bjerringbro and Helsingborg) were chosen to study Cluster 2 Defluviicoccus. For each ecophysiological investigation, fresh biomass was sampled from the aerobic zone and transferred to the laboratory within 2–4 h.

FISH. FISH was done according to the method of Amann (1995). FISH probes used in this study were NON338 for non-specific binding (Wallner et al., 1993), EUBMIX for Bacteria (Amann et al., 1990; Daims et al., 1999), DF1MIX (TFO_DF218 plus TFO_DF6F18) for the Alphaproteobacteria from Cluster 1 Defluviicoccus spp. (Wong et al., 2004), DF2MIX (DF988, DF1020 plus helper probes H966 and H1038) for Cluster 2 Defluviicoccus spp. (Meyer et al., 2006), SBR9-1a for Alphaproteobacteria Sphingomonas spp. (Beer et al., 2004), PAOMIX (Croce et al., 2000) for the Betaproteobacteria Accumulibacter spp., and GAOMIX (equal amounts of GAQQ89 and GB_G2 (Croce et al., 2002; Kong et al., 2002) for the Gammaproteobacteria Competibacter spp. All microscopic examinations were carried out using a Zeiss LSM510 Meta confocal laser scanning microscope (CLSM).

Quantitative FISH. FISH quantification was performed by digital image analysis of FISH images using ImageJ V1.35k (http://rsb.info.nih.gov/ij/). FISH images were captured using a Zeiss LSM510 Meta CLSM. Samples were hybridized with Cy3-labelled specific probes and FLUOS-labelled EUBMIX probes. A total of 45 images were taken from three separate hybridizations for quantification of a specific FISH-probe-defined organism in each sample. The pixel area of the cells positive for the Cy3-labelled specific probe was quantified as a percentage of the pixel area for cells positive with the FLUOS-labelled EUBMIX probe. The percentage abundance reported for a

Table 1. Occurrence of Cluster 1 and Cluster 2 Defluviicoccus in nutrient-removal wastewater treatment plant biomass from Denmark and Helsingborg in Sweden

<table>
<thead>
<tr>
<th>Plant name</th>
<th>Plant configuration</th>
<th>Influent type*</th>
<th>Equivalent persons</th>
<th>Abundance (%)†</th>
<th>Cluster 1</th>
<th>Cluster 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aalborg East</td>
<td>Biodenpho</td>
<td>M</td>
<td>100 000</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Aalborg West</td>
<td>Biodenpho</td>
<td>M</td>
<td>330 000</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Egaa</td>
<td>Biodenpho</td>
<td>M</td>
<td>100 000</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Helsingborg</td>
<td>Biodenpho</td>
<td>M</td>
<td>300 000</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Bjerringbro</td>
<td>Recirculation</td>
<td>M and I</td>
<td>80 000</td>
<td>ND</td>
<td>3 ± 1</td>
<td>ND</td>
</tr>
<tr>
<td>Bjerringbro‡</td>
<td>Recirculation</td>
<td>M</td>
<td>65 000</td>
<td>ND</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Assens</td>
<td>Recirculation</td>
<td>I</td>
<td>20 000</td>
<td>ND</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Harbourne</td>
<td>Biodenpho</td>
<td>I</td>
<td>60 000</td>
<td>ND</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Hoglund</td>
<td>Biodenpho</td>
<td>I</td>
<td>40 000</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Skagen</td>
<td>Biodenpho</td>
<td>I</td>
<td>280 000</td>
<td>ND</td>
<td>9 ± 2</td>
<td>ND</td>
</tr>
<tr>
<td>Skovlund</td>
<td>Biodenitro</td>
<td>I</td>
<td>25 000</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>

* M, municipal wastewater; I, industrial wastewater. All but Skovlund (N-removal only) were N- and P-removal processes.
† ND, None detected; < 1 indicates that FISH-probe positive cells were present in the entire probe-hybridized area, but were below the detection limit of the method.
‡ Defluviicoccus determined when Bjerringbro received only municipal wastewater.
The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase was inhibited using sodium iodoacetate (Bickis & Quastel, 1965) at final concentrations of 0.5, 1.0 or 2.0 mM, and aconitase, which catalyses conversion of citrate to isocitrate in the tricarboxylic acid (TCA) cycle, was inhibited using sodium fluorooacetate (Lotspeich et al., 1952) at final concentrations of 0.5, 1.0 or 2.0 mM.

**RESULTS**

**Distribution and abundance of *Defluviicoccus* spp. and *Sphingomonas* spp. in full-scale plants**

The occurrence of three different subgroups of putative GAOs belonging to the *Alphaproteobacteria* was investigated in 10 plants (Table 1). Cluster 1 and Cluster 2 *Defluviicoccus* cells (targeted by DF1MIX and DF2MIX, respectively) were all targeted by EUBMIX. The NON338 probe did not bind DF1MIX- or DF2MIX-positive cells. Cluster 2 *Defluviicoccus* (coccii in tetrads or clumps) were present in 7 of the 10 plants surveyed, with their abundance varying from <1% to 9% of *Bacteria*. Cluster 1 *Defluviicoccus* (also coccii in tetrads or clumps) were present in 3 of the 10 plants; however, they were always <1% of all *Bacteria*. *Sphingomonas* spp. (targeted by SBR9-1a) were not observed in any of the 10 plants surveyed. Biomass from plants Bjerringbro and Skagen had the highest abundance of Cluster 2 *Defluviicoccus* (3% and 9% of *Bacteria*, respectively) and also contained *Candidatus Accumulibacter phosphatis*, hereafter called *Accumulibacter* spp. (2% and 6% of *Bacteria*, respectively), and *Competibacter* spp. (1% and 6% of *Bacteria*, respectively).

**Substrate uptake patterns of *Defluviicoccus* spp.**

All the experiments carried out with either radiolabelled or unlabelled substrates were repeated at least twice (total of three separate trials) in four different plants for Cluster 2 *Defluviicoccus* or two plants for Cluster 1 *Defluviicoccus*. Under separate anaerobic or aerobic incubation conditions both Cluster 1 and Cluster 2 *Defluviicoccus* exhibited identical substrate uptake behaviour, utilizing acetate, propionate, pyruvate and glucose. Cluster 2 *Defluviicoccus* could also take up an amino acid mixture. Other substrates, including formate, butyrate, ethanol, mannose, oleic acid, aspartic acid, glutamic acid, leucine, glycine and thymidine were not taken up by *Defluviicoccus* cells (Table 2).

Cluster 2 *Defluviicoccus* were capable of simultaneous uptake of two different substrates (Table 3). Propionate, leucine and thymidine were separately taken up in the presence of acetate under anaerobic incubation conditions (i.e. no external electron acceptor). Butyrate and oleic acid were not taken up as sole substrates and neither could be taken up in the presence of acetate. The nitrogen-containing substrates tested, leucine and thymidine, were unable to be taken up as sole substrates but they were taken up in the presence of acetate.
The fate of substrates taken up by *Defluviicoccus* under different electron acceptor conditions was determined in order to establish the phenotype of these micro-organisms and their capability to behave similarly to GAOs as predicted by metabolic models. The positive and negative control incubations that were included for all four sludges evaluated were consistently positive and negative for acetate uptake respectively (Table 4). The results of the separate test incubations with four sludges under the various electron acceptor conditions (no electron acceptor, oxygen, nitrate or nitrite) and different durations of pre-incubation with unlabelled substrate (3, 6 or 8 h) are reported in Table 4. A 3 h anaerobic (no electron acceptor) pre-incubation with unlabelled acetate (1C in Table 4) resulted in only a fraction of Cluster 2 *Defluviicoccus* (25–90 % cells) being able to take up radiolabelled acetate anaerobically. However, when a 6 h anaerobic pre-incubation with unlabelled acetate was employed, no Cluster 2 *Defluviicoccus* cells were capable of radiolabelled acetate uptake (1D in Table 4). Under prolonged aerobic pre-incubation conditions (2A–2C in Table 4), Cluster 2 *Defluviicoccus* cells were capable of radiolabelled acetate uptake (1D in Table 4). Under prolonged aerobic pre-incubation conditions (2A–2C in Table 4), Cluster 2 *Defluviicoccus* spp. in all four sludges were capable of radiolabelled acetate uptake (1D in Table 4). Under prolonged aerobic pre-incubation conditions (2A–2C in Table 4), Cluster 2 *Defluviicoccus* spp. in all four sludges were capable of radiolabelled acetate uptake (1D in Table 4). Under prolonged aerobic pre-incubation conditions (2A–2C in Table 4), Cluster 2 *Defluviicoccus* spp. in all four sludges were capable of radiolabelled acetate uptake (1D in Table 4). 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Under prolonged aerobic pre-incubation conditions (2A–2C in Table 4), Cluster 2 *Defluviicoccus* spp. in all four sludges.
were positive (>90 % cells) for radiolabelled acetate uptake. Cluster 2 *Defluviicoccus* cells could not take up radiolabelled acetate under anoxic conditions (nitrate or nitrite provided as the only external electron acceptor) after a 6 h anoxic (nitrate or nitrite) pre-incubation with unlabelled acetate (3A and 3B, Table 4).

When biomass was incubated for 2 h anaerobically with acetate, PHA was observed inside Cluster 2 *Defluviicoccus* cells as determined by Nile blue A staining. This same biomass positive for PHA was subsequently subjected to a 2 h aerobic incubation without added acetate and little or no PHA was observed inside Cluster 2 *Defluviicoccus* cells. PolyP was not observed in Cluster 2 *Defluviicoccus* cells when examined directly from biomass sampled from the aerobic zone of full-scale plants or after a 2 h aerobic incubation. Cells other than *Defluviicoccus* spp. sampled from the aerobic zone were observed to contain polyP (data not shown).

Anaerobic incubation of biomass with acetate and the glycolysis inhibitor sodium iodoacetate (at 0.5, 1.0 or 2.0 mM) resulted in no PHA formation and no acetate uptake in Cluster 2 *Defluviicoccus* cells. However, PHA formation and acetate uptake were observed in Cluster 2 *Defluviicoccus* cells when biomass was incubated anaerobically with acetate and sodium fluorooacetate (at 0.5, 1.0 or 2.0 mM), an inhibitor of the TCA cycle enzyme aconitate.

**DISCUSSION**

Lab-scale reactors exhibiting deteriorated EBPR have enriched *Defluviicoccus* spp. (Meyer et al., 2006; Wong et al., 2004) and *Sphingomonas* spp. (Beer et al., 2004). We showed that *Defluviicoccus* spp. are present in low abundance in most full-scale nutrient removal plants (9 of the 10 were EBPR plants). However, in one EBPR plant receiving mainly industrial wastewater, Cluster 2 *Defluviicoccus* constituted 9 % of all *Bacteria*. A further investigation of the two plants with the highest abundance of Cluster 2 *Defluviicoccus* (Bjerringbro and Skagen) revealed that these bacteria co-existed with both *Accumulibacter* spp. and *Competibacter* spp. and indeed were present in higher abundance than either of the latter two. Meyer et al. (2006) observed Cluster 2 *Defluviicoccus* in two Australian full-scale plants and concluded that they might be industrially relevant. Our findings are in agreement, since we demonstrated that Cluster 2 *Defluviicoccus* are relatively abundant members of the microbial community in two full-scale EBPR plants. In our investigation they were more abundant in plants receiving mainly industrial sources of wastewater. The Bjerringbro plant recently stopped receiving wastewater from slaughterhouses and treats mainly municipal wastewater. Interestingly, Cluster 2 *Defluviicoccus* constituted 3 % of *Bacteria* in this plant when it received both municipal and slaughterhouse wastewater, but <1 % when the plant received only municipal wastewater (Table 1). Some components of the slaughterhouse wastewater might be responsible for the selection of Cluster 2 *Defluviicoccus*, leading to its relatively higher abundance (3 % of all *Bacteria*) in this plant. Cluster 1 *Defluviicoccus* always constituted <1 % of *Bacteria* in plants surveyed here. Previous surveys of full-scale plants from several countries (Wong et al., 2004, 2005) also demonstrated low numbers of Cluster 2 *Defluviicoccus.*, 0 % of cells MAR positive. Results for all four plants assessed were identical.

### Table 4. Uptake of radiolabelled acetate under different incubation conditions following pre-incubation with unlabelled acetate by Cluster 2 *Defluviicoccus*, as determined by MAR

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Pre-incubation*</th>
<th>Incubation*†</th>
<th>Uptake of radiolabelled acetate‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A. Anaerobic (positive control)</td>
<td>Not included</td>
<td>2 h anaerobic</td>
<td>+</td>
</tr>
<tr>
<td>1B. Anaerobic (pasteurized) (negative control)</td>
<td>Not included</td>
<td>2 h anaerobic</td>
<td>–</td>
</tr>
<tr>
<td>1C. Anaerobic</td>
<td>3 h anaerobic; 1.5 mM unlabelled acetate added at <em>t</em> = 0</td>
<td>2 h anaerobic</td>
<td>+/–</td>
</tr>
<tr>
<td>1D. Anaerobic</td>
<td>6 h anaerobic; 1.5 mM unlabelled acetate added at <em>t</em> = 0 and <em>t</em> = 3</td>
<td>2 h anaerobic</td>
<td>–</td>
</tr>
<tr>
<td>2A. Aerobic</td>
<td>3 h aerobic; 1.5 mM unlabelled acetate added at <em>t</em> = 0</td>
<td>2 h aerobic</td>
<td>+</td>
</tr>
<tr>
<td>2B. Aerobic</td>
<td>6 h aerobic; 1.5 mM unlabelled acetate added at <em>t</em> = 0 and <em>t</em> = 3</td>
<td>2 h aerobic</td>
<td>+</td>
</tr>
<tr>
<td>2C. Aerobic</td>
<td>8 h aerobic; 1.5 mM unlabelled acetate added at <em>t</em> = 0, <em>t</em> = 3 and <em>t</em> = 6</td>
<td>2 h aerobic</td>
<td>+</td>
</tr>
<tr>
<td>3A. Anoxic (nitrate)</td>
<td>6 h anoxic; 1.5 mM unlabelled acetate added at <em>t</em> = 0 and <em>t</em> = 3</td>
<td>2 h anoxic</td>
<td>–</td>
</tr>
<tr>
<td>3B. Anoxic (nitrite)</td>
<td>6 h anoxic; 1.5 mM unlabelled acetate added at <em>t</em> = 0 and <em>t</em> = 3</td>
<td>2 h anoxic</td>
<td>–</td>
</tr>
</tbody>
</table>

* Anaerobic, agitation with nitrogen gassing (no external electron acceptor provided); aerobic, agitation with air gassing (oxygen is external electron acceptor).† For all tests, 1.5 mM radiolabelled acetate was added at *t* = 0 h.‡ +, >90 % of cells MAR positive; +/-, 25–90 % of cells MAR positive; –, 0 % of cells MAR positive. Results for all four plants assessed were identical.
1 *Defluviicoccus*. Micro-organisms related to *Sphingomonas* spp. were not observed in any of the 10 plants, suggesting that they are not important. Previously, *Competibacter* spp. (putative GAOs) were found to be more widely distributed and typically in higher abundances than Cluster 1 or Cluster 2 *Defluviicoccus* (Kong et al., 2002, 2006; Saunders et al., 2003).

The physiology of *Defluviicoccus* spp. is relatively poorly understood and if they are GAOs, their detailed ecophysiology in full-scale EBPR plants is critical in understanding how the EBPR process deteriorates. It has been proposed that GAOs are capable of utilizing VFAs, carboxylic acids, sugars and amino acids (Mino et al., 1998; Seviour et al., 2003). However, only one study has investigated the capability of putative GAOs to take up this range of substrates (Kong et al., 2003). Here we demonstrated that *Defluviicoccus* spp. take up acetate, propionate, pyruvate, glucose and a mixture of amino acids under either anaerobic or aerobic conditions, but not formate, butyrate, ethanol and several other organic substrates. *Defluviicoccus* spp. clearly have many similarities to *Accumulibacter* spp. and *Competibacter* spp. in substrate uptake. A notable difference was the direct uptake of glucose by *Defluviicoccus* spp., a capability not shared by *Accumulibacter* spp. (Kong et al., 2004) or *Competibacter* spp. (Kong et al., 2006). Uptake of glucose and assimilation as PHA has previously been reported for the only extant pure culture of *Defluviicoccus*, *Defluviicoccus vanus* strain Ben114 (Maszenan et al., 2005).

Cluster 2 *Defluviicoccus* were capable of simultaneous uptake of two different organic carbon substrates. The nitrogen-containing compounds leucine and thymidine, which were not taken up as sole substrates, were taken up in the presence of acetate. *Accumulibacter* spp. (Kong et al., 2004) and *Competibacter* spp. (Kong et al., 2006) have been reported to have similar capabilities. Substrate uptake similarities between these three organisms relevant to EBPR (carbon uptake and simultaneous uptake of two different organic carbon substrates) make it difficult to design strategies that specifically select for the EBPR desired organism, *Accumulibacter*.

The currently accepted biochemical model for GAOs hypothesizes that acetate is taken up anaerobically and stored as PHA and that there is a finite amount of PHA that can be formed (Filipe et al., 2001; Zeng et al., 2003b). The stored PHA is metabolized aerobically as a carbon and energy source. Under anaerobic pre-incubation conditions, Cluster 2 *Defluviicoccus* progressively lost their ability to take up radiolabelled acetate (1A–1C, Table 4). We concluded that Cluster 2 *Defluviicoccus* saturated their PHA storage capacity during the pre-incubation, thus preventing them from further uptake of radiolabelled acetate. However, under aerobic conditions (2A–2C, Table 4), radiolabelled acetate was continuously taken up. We concluded that Cluster 2 *Defluviicoccus* are aerobically growing on acetate. FISH and post-FISH chemical staining revealed that acetate was anaerobically stored as PHA. By the same methods, aerobic PHA consumption occurred but polyP was not accumulated. The type of organic carbon cycling and absence of P transformations in these micro-organisms is in accordance with the GAO biochemical model (Filipe et al., 2001; Zeng et al., 2003b).

Some GAOs have the capacity to utilize nitrate/nitrite as an electron acceptor (instead of oxygen) in the phase following the anaerobic zone (Zeng et al., 2003c). Our experiments support the notion that Cluster 2 *Defluviicoccus* are unlikely to denitrify with acetate as a carbon source (3A and 3B, Table 4). However, we did not explore anaerobic–anoxic conditions in which the organisms could have anaerobically stored acetate as PHA that could have been used as a carbon and energy source anoxically. This latter phenotype has been demonstrated for enrichment cultures of *Accumulibacter* spp. (Zeng et al., 2003a) and enrichments of *Competibacter* spp. (Zeng et al., 2003c).

According to the GAO biochemical model (Filipe et al., 2001; Zeng et al., 2003b), glycolysis and the TCA cycle provide energy and reducing equivalents essential for anaerobic acetate assimilation and reduction to PHA. Incubation of Cluster 2 *Defluviicoccus* with iodoacetate (a glycolysis inhibitor) essentially stopped acetate uptake and PHA formation. However, inhibition of the TCA cycle had undetectable effects on acetate uptake and PHA formation. Thus under anaerobic conditions, Cluster 2 *Defluviicoccus* can take up acetate and reduce it to PHA even when the TCA cycle is limited. We conclude that the energy and reducing equivalents required for anaerobic acetate uptake and PHA formation can be generated solely via glycolysis in Cluster 2 *Defluviicoccus*. However, we cannot conclude that this organism does not use the TCA cycle when it is not restricted.

A comparison of the ecophysiological traits of Cluster 2 *Defluviicoccus* with *Accumulibacter* spp. (Kong et al., 2004) and *Competibacter* spp. (Kong et al., 2006) reveals a similar, shared physiology. To survive and proliferate under the anaerobic–aerobic cycling conditions applied in EBPR systems, it is critical for micro-organisms to take up and store carbon under carbon-rich, anaerobic conditions. The internally stored carbon is then utilized for growth in the subsequent aerobic, external-carbon-poor conditions. *Accumulibacter* spp., *Competibacter* spp. and *Defluviicoccus* spp. take up identical organic carbon compounds (typically VFAs such as acetate and propionate) and therefore, in full-scale EBPR plants, they likely compete for sparingly available VFAs. It could be that physiological factors, such as substrate uptake rates or substrate affinities for different organic carbon compounds, differ between *Accumulibacter* spp., *Competibacter* spp. and *Defluviicoccus* spp., facilitating selection for different microbial communities. Future research using techniques based on quantitative MAR in combination with FISH, as described by Nielsen et al. (2003), could be used to address these hypothetical physiological variations between these organisms.
ACKNOWLEDGEMENTS

The Danish Technical Research Council supported this study under the framework programme ‘Activity and Diversity in Complex Microbial Systems’. In Australia, the Queensland State Government – Growing the Smart State Initiative, the University of Queensland Graduate School Research Travel Award and the Environmental Biotechnology CRC, an initiative of Federal Government of Australia, are acknowledged by L. C. B. and L. L. B.

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Edited by: W. Liesack