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

Burow, L. C.; Kong, Yunhong; Nielsen, Jeppe Lund; Blackall, L. L.; Nielsen, Per Halkjær

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

Microbiology (2007), **153**, 178–185 DOI 10.1099/mic.0.2006/001032-0

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

Luke C. Burow,[1] Yunhong Kong,[2] Jeppe L. Nielsen,[2] Linda L. Blackall[1] and Per H. Nielsen[2]

[1]Advanced Wastewater Management Centre, The University of Queensland, St Lucia, QLD 4072, Australia

[2]Section of Environmental Engineering, Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University, Sohngaardsholmsvej 57, DK-9000 Aalborg, Denmark

Correspondence
Per H. Nielsen
phn@bio.aau.dk

Received 7 August 2006
Revised 28 September 2006
Accepted 9 October 2006

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 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 (Crocetti *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 micro-organisms 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 micro-organisms 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_DF618) 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 (Crocetti *et al.*, 2000) for the *Betaproteobacteria Accumulibacter* spp., and GAOMIX (equal amounts of GAOQ989 and GB_G2 (Crocetti *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

Plant name	Plant configuration	Influent type*	Equivalent persons	Abundance (%)†	
				Cluster 1	Cluster 2
Aalborg East	Biodenpho	M	100 000	ND	ND
Aalborg West	Biodenpho	M	330 000	<1	<1
Egaa	Biodenpho	M	100 000	ND	ND
Helsingborg	Biodenpho	M	300 000	<1	<1
Bjerringbro	Recirculation	M and I	80 000	ND	3±1
Bjerringbro‡	Recirculation	M	65 000	ND	<1
Assens	Recirculation	I	20 000	ND	<1
Harboure	Biodenpho	I	60 000	ND	<1
Hogelund	Biodenpho	I	40 000	ND	ND
Skagen	Biodenpho	I	280 000	ND	9±2
Skovlund	Biodenitro	I	25 000	<1	<1

*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.

specific organism was expressed as the mean of three separate measurements, with each measurement derived from 15 images. A standard deviation for each mean measurement was calculated as the mean of the squared differences between each value and the mean value.

FISH-microautoradiography (FISH-MAR) and post-FISH chemical staining. FISH-MAR experiments were carried out using previously described methods (Kong *et al.*, 2004; Lee *et al.*, 1999) and different radiolabelled substrates (Kong *et al.*, 2004, 2005, 2006). The manipulations and incubation conditions of biomass samples for FISH-MAR batch tests were fully described by Kong *et al.* (2004). Briefly, biomass samples were incubated with a radiolabelled substrate under different electron acceptor conditions for various time periods before fixation with paraformaldehyde (4 %) in phosphate-buffered saline at 4 °C.

To investigate the fate of acetate taken up by *Defluviicoccus* spp. under different electron acceptor conditions, pre-incubations with unlabelled acetate were employed. All incubations were carried out on a shaker (Kikalabortechnik, Denmark) at 250 r.p.m. and kept at 20 ± 1 °C.

At least 100 MAR-positive and/or MAR-negative *Defluviicoccus* cells were counted. Uptake of a radiolabelled substrate was reported as positive (+) if > 90 % and negative (–) if 0 % of cells were visualized by silver grain formation. Typical exposure time was 4 days; however, cells incubated with radiolabelled propionate required up to 12 days' exposure time. Generally a clear positive or negative result was demonstrated. However, we always found that 25–90 % of *Defluviicoccus* cells took up glucose for all incubation times and for substrate uptake experiments with *Defluviicoccus* cells incubated in the absence of an electron acceptor pre-incubated with unlabelled acetate for 3 h. This was reported as a variable result (+/−). Negative MAR results (0 % of cells with silver grain formation) remained negative after extension of exposure time to 12 days, except in the case of radiolabelled propionate incubations, during which with extension of exposure time to 12 days, *Defluviicoccus* cells went from variable (25–90 % of cells with silver grain formation) to MAR-positive (> 90 % of cells with silver grain formation).

FISH and post-FISH chemical staining was carried out using methods fully described by Crocetti *et al.* (2000), with intracellular PHA and polyphosphate (polyP) determined by staining with Nile blue A (Ostle & Holt, 1982) and Neisser (Lindrea *et al.*, 1999), respectively. Briefly, FISH was carried out and images were captured. Then cells on the slide were stained for PHA or polyP, the fields of view captured in FISH were relocated and images from chemical staining were taken. Prior to carrying out FISH and chemical staining, biomass samples were subjected to the different incubation conditions described by Kong *et al.* (2004) with unlabelled acetate (final concentration of either 1.5 mM or 0.5 mM).

For visualization of PHA accumulation, biomass samples were incubated under anaerobic conditions (nitrogen gassing, no external electron acceptor provided) with unlabelled acetate at a final concentration of 1.5 mM for 2 h. To visualize PHA exhaustion (PAO and GAO properties) and polyP accumulation (an exclusive PAO property), biomass samples were first incubated under anaerobic conditions with unlabelled acetate at a final concentration of 0.5 mM for 2 h. A further 2 h aerobic incubation without added acetate followed the anaerobic incubation. A lower concentration of acetate was used for the PHA exhaustion and polyP accumulation experiments than for the PHA accumulation experiments to ensure that complete uptake of acetate had occurred prior to aerobic incubation. Accumulation of polyP was also determined in biomass sampled directly from the aerobic zone of full-scale plants.

Metabolic inhibitors. Some experiments used FISH-MAR or post-FISH chemical staining in combination with metabolic inhibitors. 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 fluoroacetate (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* (cocci 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 cocci 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.

Table 2. Uptake of carbon substrates by Cluster 1 and Cluster 2 *Defluviicoccus* under anaerobic (no electron acceptor) or aerobic (oxygen as the electron acceptor) conditions, as determined by MAR

Unlabelled substrate*	Radiolabelled substrate†	Substrate uptake by *Defluviicoccus*‡			
		Cluster 1		Cluster 2	
		Anaerobic	Aerobic	Anaerobic	Aerobic
Formic acid	[1-^{14}C]Formic acid	−	−	−	−
Acetate	[^{3}H]Acetate (Na)	+	+	+	+
Propionate	[1-^{14}C]Propionate (Na)	+	+	+	+
Butyrate	[2,3-^{3}H]Butyric acid	−	−	−	−
Pyruvate	[1-^{14}C]Pyruvate (Na)	+	+	+	+
Ethanol	[1-^{3}H]Ethanol	−	−	−	−
Glucose	D-[2-^{3}H]Glucose	+/−	+/−	+/−	+/−
Mannose	D-[2-^{3}H]Mannose	−	−	−	−
Oleic acid	[9,10(n)-^{3}H]Oleic acid	−	−	−	−
Aspartic acid	D-[2,3-^{3}H]Aspartic acid	−	−	−	−
Glutamic acid	L-[G-^{3}H]Glutamic acid	−	−	−	−
Leucine	[4,5-^{3}H]Leucine	−	−	−	−
Glycine	[2-^{3}H]Glycine	−	−	−	−
Thymidine	[*methyl*-^{3}H]Thymidine	−	−	−	−
Amino acids	^{3}H-labelled amino acids§	ND	ND	+	+

*The final concentration of all unlabelled substrates used was 1.5 mM, except for oleic acid (1.0 mM) and amino acid mixture (1.0 mM for each amino acid).

†The amount of radiolabelled substrates used was 10 μCi ml^{-1} (1.85×10^{7} Bq ml^{-1}).

‡+, >90 % of cells MAR positive; +/−, 25–90 % of cells MAR positive; −, 0 % of cells MAR positive; ND, not determined.

§Details of radiolabelled amino acids have been previously described (Kong *et al.*, 2005).

Fate of substrate assimilated by Cluster 2 *Defluviicoccus* spp.

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* spp. in all four sludges

Table 3. Simultaneous uptake of two carbon substrates under anaerobic incubation conditions (i.e. no external electron acceptor) by Cluster 2 *Defluviicoccus*, as demonstrated by MAR

Unlabelled substrates	Radiolabelled substrate	Uptake*
Acetate (1.5 mM) + propionate (1.5 mM)	[^{3}H]Acetate (Na)	+
Acetate (3.0 mM) + propionate (0.5 mM)	[1-^{14}C]Propionate (Na)	+
Acetate (1.5 mM) + butyrate (1.5 mM)	[2,3-^{3}H]Butyric acid	−
Acetate (1.5 mM) + oleic acid (1.0 mM)	[9,10(n)-^{3}H]Oleic acid	−
Acetate (1.5 mM) + leucine (1.5 mM)	[4,5-^{3}H]Leucine	+
Acetate (1.5 mM) + thymidine (1.5 mM)	[*methyl*-^{3}H]Thymidine	+

*+, >90 % of cells MAR positive; −, 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

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

*Anaerobic, agitation with nitrogen gassing (no external electron acceptor provided); aerobic, agitation with air gassing (oxygen is external electron acceptor); anoxic, agitation with nitrogen gassing (either 0.3 mM nitrate or 0.3 mM nitrite provided as external electron acceptor at $t=0$, $t=1.5$, $t=3$ and $t=4.5$ h for pre-incubation and 0.3 mM nitrate or 0.3 mM nitrite provided as external electron acceptor at $t=0$ h for incubation).

†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.

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 fluoroacetate (at 0.5, 1.0 or 2.0 mM), an inhibitor of the TCA cycle enzyme aconitase.

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

1 *Defluviicoccus*. Micro-organisms related to *Sphingomonas* spp. were not observed in any of the 10 plants, suggesting that they are unimportant. 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.*, 2006). 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.

REFERENCES

Amann, R. I. (1995). In situ identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes. In *Molecular Microbial Ecology Manual*, pp. 1–15. Edited by A. D. L. Akkermans, J. D. van Elsas & F. J. de Bruijn. London: Kluwer.

Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R. & Stahl, D. A. (1990). Combination of 16S ribosomal RNA-targeted oligonucleotide probes with flow-cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56**, 1919–1925.

Beer, M., Kong, Y. H. & Seviour, R. J. (2004). Are some putative glycogen accumulating organisms (GAO) in anaerobic : aerobic activated sludge systems members of the *α-Proteobacteria*? *Microbiology* **150**, 2267–2275.

Bickis, I. J. & Quastel, J. H. (1965). Effects of metabolic inhibitors on energy metabolism of Ehrlich ascites carcinoma cells. *Nature* **205**, 44–46.

Blackall, L. L., Crocetti, G. R., Saunders, A. M. & Bond, P. L. (2002). A review and update of the microbiology of enhanced biological phosphorus removal in wastewater treatment plants. *Antonie Van Leeuwenhoek* **81**, 681–691.

Bond, P. L., Erhart, R., Wagner, M., Keller, J. & Blackall, L. L. (1999). Identification of some of the major groups of bacteria in efficient and nonefficient biological phosphorus removal activated sludge systems. *Appl Environ Microbiol* **65**, 4077–4084.

Crocetti, G. R., Hugenholtz, P., Bond, P. L., Schuler, A., Keller, J., Jenkins, D. & Blackall, L. L. (2000). Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. *Appl Environ Microbiol* **66**, 1175–1182.

Crocetti, G. R., Banfield, J. F., Keller, J., Bond, P. L. & Blackall, L. L. (2002). Glycogen-accumulating organisms in laboratory-scale and full-scale wastewater treatment processes. *Microbiology* **148**, 3353–3364.

Daims, H., Bruhl, A., Amann, R., Schleifer, K. H. & Wagner, M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* **22**, 434–444.

Filipe, C. D. M., Daigger, G. T. & Grady, C. P. L. (2001). A metabolic model for acetate uptake under anaerobic conditions by glycogen accumulating organisms: stoichiometry, kinetics, and the effect of pH. *Biotechnol Bioeng* **76**, 17–31.

Kong, Y. H., Ong, S. L., Ng, W. J. & Liu, W. T. (2002). Diversity and distribution of a deeply branched novel proteobacterial group found in anaerobic-aerobic activated sludge processes. *Environ Microbiol* **4**, 753–757.

Kong, Y. H., Nielsen, J. L. & Nielsen, P. H. (2004). Microautoradiographic study of Rhodocyclus-related polyphosphate accumulating bacteria in full-scale enhanced biological phosphorus removal plants. *Appl Environ Microbiol* **70**, 5383–5390.

Kong, Y. H., Nielsen, J. L. & Nielsen, P. H. (2005). Identity and ecophysiology of uncultured actinobacterial polyphosphate-accumulating organisms in full-scale enhanced biological phosphorus removal plants. *Appl Environ Microbiol* **71**, 4076–4085.

Kong, Y. H., Xia, Y., Nielsen, J. L. & Nielsen, P. H. (2006). Ecophysiology of a group of uncultured gammaproteobacterial glycogen-accumulating organisms in full-scale enhanced biological phosphorus removal wastewater treatment plants. *Environ Microbiol* **8**, 479–489.

Lee, N., Nielsen, P. H., Andreasen, K. H., Juretschko, S., Nielsen, J. L., Schleifer, K. H. & Wagner, M. (1999). Combination of fluorescent in situ hybridization and microautoradiography – a new tool for structure–function analyses in microbial ecology. *Appl Environ Microbiol* **65**, 1289–1297.

Lindrea, K. C., Seviour, E. M., Seviour, R. J., Blackall, L. L. & Soddell, J. A. (1999). Practical methods for the examination and characterization of activated sludge. In *The Microbiology of Activated Sludge*, pp. 257–300. Edited by R. J. Seviour & L. L. Blackall. Dordrecht: Kluwer.

Liu, W. T., Mino, T., Nakamura, K. & Matsuo, T. (1996). Glycogen accumulating population and its anaerobic substrate uptake in anaerobic-aerobic activated sludge without biological phosphorus removal. *Water Res* **30**, 75–82.

Lotspeich, W. D., Peters, R. A. & Wilson, T. H. (1952). The inhibition of aconitase by inhibitor fractions isolated from tissues poisoned with fluoroacetate. *Biochem J* **51**, 20–25.

Maszenan, A. M., Seviour, R. J., Patel, B. K. C., Janssen, P. H. & Wanner, J. (2005). *Defluvicoccus vanus* gen. nov., sp. nov., a novel Gram-negative coccus/coccobacillus in the '*Alphaproteobacteria*' from activated sludge. *Int J Syst Evol Microbiol* **55**, 2105–2111.

Meyer, R. L., Saunders, A. M. & Blackall, L. L. (2006). Putative glycogen-accumulating organisms belonging to the *Alphaproteobacteria* identified through rRNA-based stable isotope probing. *Microbiology* **152**, 419–429.

Mino, T., Liu, W. T., Kurisu, F. & Matsuo, T. (1995). Modeling glycogen-storage and denitrification capability of microorganisms in enhanced biological phosphate removal processes. *Water Sci Technol* **31**, 25–34.

Mino, T., Van Loosdrecht, M. C. M. & Heijnen, J. J. (1998). Microbiology and biochemistry of the enhanced biological phosphate removal process. *Water Res* **32**, 3193–3207.

Nielsen, J. L., Christensen, D., Kloppenberg, M. & Nielsen, P. H. (2003). Quantification of cell-specific substrate uptake by probe-defined bacteria under in situ conditions by microautoradiography and fluorescence in situ hybridization. *Environ Microbiol* **5**, 202–211.

Oehmen, A., Yuan, Z. G., Blackall, L. L. & Keller, J. (2005). Comparison of acetate and propionate uptake by polyphosphate accumulating organisms and glycogen accumulating organisms. *Biotechnol Bioeng* **91**, 162–168.

Ostle, A. & Holt, J. G. (1982). Nile blue-A as a fluorescent stain for poly-beta-hydroxybutyrate. *Appl Environ Microbiol* **44**, 238–241.

Satoh, H., Mino, T. & Matsuo, T. (1992). Uptake of organic substrates and accumulation of polyhydroxyalkanoates linked with glycolysis of intracellular carbohydrates under anaerobic conditions in the biological excess phosphate removal processes. *Water Sci Technol* **26**, 933–942.

Saunders, A. M., Oehmen, A., Blackall, L. L., Yuan, Z. & Keller, J. (2003). The effect of GAOs (glycogen accumulating organisms) on anaerobic carbon requirements in full-scale Australian EBPR (enhanced biological phosphorus removal) plants. *Water Sci Technol* **47**, 37–43.

Seviour, R. J., Maszenan, A. M., Soddell, J. A., Tandoi, V., Patel, B. K. C., Kong, Y. H. & Schumann, P. (2000). Microbiology of the 'G-bacteria' in activated sludge. *Environ Microbiol* **2**, 581–593.

Seviour, R. J., Mino, T. & Onuki, M. (2003). The microbiology of biological phosphorus removal in activated sludge systems. *FEMS Microbiol Rev* **27**, 99–127.

van Loosdrecht, M. C. M., Smolders, G. J., Kuba, T. & Heijnen, J. J. (1997). Metabolism of micro-organisms responsible for enhanced biological phosphorus removal from wastewater – use of dynamic enrichment cultures. *Antonie Van Leeuwenhoek* **71**, 109–116.

Wallner, G., Amann, R. & Biesker, W. (1993). Optimizing fluorescent in situ hybridisation with rRNA-targeted oligonucleotide probes for low cytometric identification of microorganisms. *Cytometry* **14**, 136–143.

Wong, M.-T., Tan, F. M., Ng, W. J. & Liu, W. T. (2004). Identification and occurence of tetrad-forming *Alphaproteobacteria* in anaerobic-aerobic activated sludge processes. *Microbiology* **150**, 3741–3748.

Wong, M. T., Mino, T., Seviour, R. J., Onuki, M. & Liu, W. T. (2005). In situ identification and characterization of the microbial community structure of full-scale enhanced biological phosphorous removal plants in Japan. *Water Res* **39**, 2901–2914.

Zeng, R. J., Saunders, A. M., Yuan, Z. G., Blackall, L. L. & Keller, J. (2003a). Identification and comparison of aerobic and denitrifying polyphosphate-accumulating organisms. *Biotechnol Bioeng* **83**, 140–148.

Zeng, R. J., van Loosdrecht, M. C. M., Yuan, Z. G. & Keller, J. (2003b). Metabolic model for glycogen-accumulating organisms in anaerobic/aerobic activated sludge systems. *Biotechnol Bioeng* **81**, 92–105.

Zeng, R. J., Yuan, Z. G. & Keller, J. (2003c). Enrichment of denitrifying glycogen-accumulating organisms in anaerobic/anoxic activated sludge system. *Biotechnol Bioeng* **81**, 397–404.

Edited by: W. Liesack