Identification of glucose-fermenting bacteria in a full-scale enhanced biological phosphorus removal plant by stable isotope probing

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Microbiology in wastewater treatment has mainly been focused on problem-causing filamentous bacteria or bacteria directly involved in nitrogen and phosphorus removal, and to a lesser degree on flanking groups, such as hydrolysing and fermenting bacteria. However, these groups constitute important suppliers of readily degradable substrates for the overall processes in the plant. This study aimed to identify glucose-fermenting bacteria in a full-scale enhanced biological phosphorus removal (EBPR) wastewater treatment plant (WWTP), and to determine their abundance in similar WWTPs. Glucose-fermenting micro-organisms were identified by an in situ approach using RNA-based stable isotope probing. Activated sludge was incubated anaerobically with 13C6-labelled glucose, and 13C-enriched rRNA was subsequently reverse-transcribed and used to construct a 16S rRNA gene clone library. Phylogenetic analysis of the library revealed the presence of two major phylogenetic groups of Gram-positive bacteria affiliating with the genera Tetrasphaera, Propionicimonas (Actinobacteria), and Lactococcus and Streptococcus (Firmicutes). Specific oligonucleotide probes were designed for fluorescence in situ hybridization (FISH) to specifically target the glucose-fermenting bacteria identified in this study. The combination of FISH with microautoradiography confirmed that Tetrasphaera, Propionicimonas and Streptococcus were the dominant glucose fermenters. The probe-defined fermenters were quantified in 10 full-scale EBPR plants and averaged 39% of the total biovolume. Tetrasphaera and Propionicimonas were the most abundant glucose fermenters (average 33 and 4%, respectively), while Streptococcus and Lactococcus were present only in some WWTPs (average 1 and 0.4%, respectively). Thus the population of actively metabolizing glucose fermenters seems to occupy a relatively large component of the total biovolume.

INTRODUCTION

An aerobic degradation of organic matter deriving from the hydrolysis of proteins, carbohydrates and lipids is mainly carried out by fermenting micro-organisms, which ferment these molecules into short-chain fatty acids (SCFAs) and other low-molecular-mass compounds. In biological wastewater treatment, these compounds are the primary substrates for other heterotrophic organisms, especially denitrifiers and polyphosphate-accumulating micro-organisms (Vollertsen et al., 2006). The presence of an active population of fermenting bacteria that supply substrates to other functional groups is therefore of fundamental importance for efficient N and P removal carried out by the activated sludge process in wastewater treatment systems.

Fermenting micro-organisms use an internally balanced redox process in which the organic substrate becomes both oxidized and reduced. In activated sludge plants, glucose, galactose and mannose can be fermented under anaerobic conditions to a series of soluble products, such as lactic acid, acetic acid, propionic acid and formic acid (Kong...
et al., 2008). Anaerobic fermentation of amino acids generally progresses more slowly and yields similar SCFAs (Smith & Macfarlane, 1998), but very little information about the process exists from activated sludge systems (Kong et al., 2008). Access to SCFAs is often insufficient to achieve optimal removal of N and P, and many wastewater treatment plant (WWTP) operators therefore add external carbon sources (e.g. Hallin et al., 2006), which is an important economic factor in wastewater treatment. Alternatively, a pre-fermentation process with primary sludge can be implemented to ensure conversion of more complex organic compounds within the wastewater to SCFAs before the onset of P and N removal processes (e.g. Pitman et al., 1992; Vollertsen et al., 2006).

Only a little is known about the identity and ecophysiology of fermenting bacteria in activated sludge systems, and our knowledge is primarily based on culture-dependent approaches (e.g. Zhang et al., 2009). However, in the study by Kong et al. (2008), the ecophysiology of the fermenting bacteria in activated sludge was addressed by combining microautoradiography with fluorescence in situ hybridization (MAR-FISH). Their findings revealed that many monosaccharide-fermenting bacteria were members of the Gram-positive phyla Firmicutes and Actinobacteria, with some related to the genera Streptococcus and Tetrasphaera (Kong et al., 2008). They comprised up to 21% of the total population, demonstrating fermentation as a significant and important process in wastewater treatment. From the MAR analysis, Kong and co-workers also noted the presence of potential fermenters other than Streptococcus and Tetrasphaera, although these were not identified. Knowledge of the total diversity of this functional group is important for understanding their roles in the microbial ecosystem of WWTPs and how they influence the overall operation and performance of the plant.

Stable-isotope probing (SIP) is an excellent approach for in situ identification of fermenting bacteria. SIP has previously been applied to identify several other important bacterial populations in activated sludge, such as denitrifying bacteria (e.g. Ginige et al., 2004), long-chain fatty acid (LCFA)-degrading anaerobic bacteria (Hatamoto et al., 2007), glycolgen-accumulating organisms (Meyer et al., 2006), and various types of environmental pollutant-degrading bacteria (e.g. Maneifeld et al., 2007). Identification of fermenting bacteria by SIP has also been successfully investigated in the human gastrointestinal tract (Egert et al., 2007; Kovatcheva-Datchary et al., 2009), in which incubation with labelled glucose or starch was carried out under fermenting conditions in an in vitro model of the human intestine.

In the present study, rRNA-based SIP was used to identify micro-organisms potentially involved in the fermentation of glucose in activated sludge from a full-scale enhanced biological phosphorus removal (EBPR) plant. The rRNA sequences obtained were used to design new oligonucleotide probes for FISH, and the combination of FISH with MAR was applied to confirm the physiology of the organisms identified. The FISH probes were then applied to study the distribution of fermenting bacteria in other WWTPs.

### METHODS

**Sampling.** Activated sludge samples for DNA extraction were obtained from Aalborg East WWTP, which is a plant with biological removal of N and P treating municipal and industrial wastewater from 235 000 population equivalent (PE). Sludge samples for SIP and clone library construction were collected from the oxic (nitrifying) tanks and transferred within 1 h to the laboratory. FISH analysis was performed on samples from Aalborg East, Aalborg West (PE 300 000), Egå (PE 160 000), Bjermarken (PE 83 000), Ejby Mølle (PE 284 000), Hjørring (PE 100 000), Skive (PE 123 000), Viby (PE 100 000), Åby (PE 93 000) and Marselisborg (PE 220 000) WWTPs. The organic matter content (volatile suspended solids) was determined from loss of ignition (105 °C for 24 h followed by 550 °C for 3 h).

**SIP.** Replicate activated sludge samples diluted with effluent water to 2 g MLSS (mixed liquor suspended solids) 1−1 were prepared, and 2 ml was transferred to 9 ml serum bottles closed with thick butyl rubber stoppers, evacuated with oxygen-free nitrogen, and left for 30 min to ensure anaerobic conditions. Two samples were incubated for 6 and 24 h with unlabelled glucose, and two were incubated with labelled [13C]glucose to a final concentration of 200 mg l−1. Removal of glucose and formation of SCFA were measured by HPLC, as described previously (Kong et al., 2008). Subsequently, RNA was extracted using the FastRNA Pro Blue kit in combination with the FastPrep Instrument according to the protocol recommended by the manufacturer (Qbiogene) and stored at −80 °C. The RNA concentration was measured on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

**Isolation of 13C-labelled RNA.** Isopycnic separation of ‘heavy’ and ‘light’ RNA was performed on each RNA extract as described elsewhere (Meyer et al., 2006). Briefly, 500 ng RNA from each sample was mixed with buffer (0.1 M Tris/HCl, pH 8, 0.1 M KCl, 1 mM EDTA) to obtain a total volume of 0.9 ml. Deionized formamide (0.175 ml) and 4.1 ml caesium trifluoroacetate (CsTFA; GE Healthcare) were then added, resulting in a mean density of 1.794 g ml−1. Solutions were sealed in 5.1 ml polyallomer tubes (Beckman Coulter) and ultracentrifuged in a Vti 65.2 vertical rotor (Beckman Coulter) at 5 °C at 130 000 g for 60 h. Immediately after centrifugation, the density gradients were fractionated into 10 volumes of approximately 400 μl. The density of each fraction was estimated by weighing 50 μl of the sample, and RNA from the remaining 350 μl was isolated by 2-propanol precipitation (Sambrook & Russell, 2001).

**cDNA synthesis, PCR and cloning.** A PCR using the primers GM3F and GM4R (Muyzer et al., 1995) at an annealing temperature of 42 °C and 25 cycles was performed on RNA precipitated from each gradient fraction to check that DNA was absent. RNA from each fraction was then reverse-transcribed using the Superscript III kit (Invitrogen), and the subsequent PCR used the primers 27F (Lane, 1991) and 1390R (Zheng et al., 1996) at 57 °C annealing temperature and 27 cycles. A clone library was prepared from the PCR product obtained from the gradient fraction with the highest density, using the pGEM-T cloning vector (Promega).

**Phylogenetic analysis.** 16S rRNA gene sequences were aligned and phylogenetic trees constructed in the ARB software package (Ludwig et al., 2004) with the SILVA reference database (release 106; Pruesse et al., 2007). The unambiguously aligned sequences were checked for
chimeric artefacts using the CHECK_CHIMERA tool (Maidak et al., 2001) and Bellerophon (Huber et al., 2004), before being compared in GenBank using the BLAST program (Altschul et al., 1997). Phylogenetic trees were calculated and built based on the neighbor-joining method with a Poisson correction model and a 10-replicate bootstrap analysis. The branching pattern was compared with trees generated using the neighbor-joining algorithm.

**FISH and probe design.** Oligonucleotide probes were designed using the function provided in the ARB software. The specificity of these probes was further confirmed by the use of the Check Probe program in the Ribosomal Database Project (Maidak et al., 2001). The specific formamide concentration used for a probe was determined by FISH probing of biomass from Aalborg East WWTP at a series of formamide concentrations from 0 to 60 % (v/v) at 5 % increments. Moreover, all new probes were applied in a hierarchical approach with either an LGC354C (Firmicutes; Meier et al., 1999) or an HGC69a (Actinobacteria; Roller et al., 1994) probe to further confirm their specificity. In all FISH probing, probe NONEUB was used as a negative control.

Sludge fixation and FISH were carried out as described previously (Nielsen, 2009). The oligonucleotide probes used were: EUBmix (equimolar concentrations of EUB338 (Amann et al., 1990), EUB338II and EUB338III (Daims et al., 1999)), NONEUB (Wallner et al., 1993), and LGCmix (equimolar concentrations of LGC354A, LGC354B and LGC354C (Meier et al., 1999)). Details and specificities of the specific oligonucleotide probes used in this study are shown in Table 1. All the probes were labelled with Cy3, except EUBmix, which was labelled with FLUOS.

FISH for quantitative biovolume analysis was carried out on six-well gelatin-coated slides by measuring the percentage of the area fluorescing with a probe (Cy3-labelled) with respect to the area fluorescing with EUBmix probe (FLUOS-labelled) in the same microscope field. For each enumeration, at least 24 images were taken from three different hybridizations. The mean values from each microscope field. For each enumeration, at least 24 images were taken from three different hybridizations. The mean values from each microscope field.

**RESULTS**

The investigated WWTPs had all been running for years with stable N and P removal at the time of sampling. SIP experiments were carried out on sludge from Aalborg East WWTP, in which the fermentation rates were previously found to follow first-order kinetics at 0.122 ± 0.027 mmol glucose (g volatile suspended solids$^{-1}$ h$^{-1}$), as reported previously (Kong et al., 2008). Removal of glucose was measured in samples incubated with unlabelled glucose in parallel with SIP and MAR experiments. Glucose was transformed into a number of different SCFAs (formic acid, acetic acid, propionic acid, butyric acid and lactic acid), similarly to the previous report from the same system by Kong et al. (2008).

RNA extraction from the SIP incubations yielded more than 100 μg RNA (g volatile suspended solids$^{-1}$), and after microscopy. An epifluorescence microscope (Axioskop 2, Carl Zeiss) was used in all FISH and MAR-FISH analyses.

**MAR-FISH.** MAR-FISH was carried out following the procedures described previously (Nielsen & Nielsen, 2005). Briefly, activated sludge samples were diluted to 1 g MLSS l$^{-1}$ with nitrate-free effluent water and incubated with labelled glucose (3.7 × 10$^5$ Bq) (δ-[$^{2}$H]$_{2}$) and unlabelled glucose to a final concentration of 1.5 mM under anaerobic conditions for 6 h (labelled and unlabelled glucose were added at time 0, and again after 3 h). Addition of 1 mM molybdate and 10 mM BES (bromoethane sulfonate) was done 20 min prior to addition of tracer to inhibit sulfate reduction (Oremland & Capone, 1988) and methanogenic activity (Nollet et al., 1997). The incubations were terminated by addition of 50 % (v/v) ethanol (final concentration). The samples were immobilized and hybridized on cover glasses as described previously (Nielsen & Nielsen, 2005). The samples were coated with Kodak NTB liquid film emulsion, and exposed in the dark for 3–6 days, after which the films were developed and examined by microscopy.

**Table 1. Oligonucleotide probes used in this study**

<table>
<thead>
<tr>
<th>Probe</th>
<th>Abbreviation</th>
<th>Target group</th>
<th>Sequence (5’–3’)</th>
<th>Formamide (%)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-S-Str-56-a-A-18</td>
<td>Str56</td>
<td>Streptococcus spp.</td>
<td>ATCCGCGTTCTCTTGC</td>
<td>20</td>
<td>This study</td>
</tr>
<tr>
<td>S-S-Lac-93-a-A-18</td>
<td>Lac93</td>
<td>Lactococcus spp.</td>
<td>GCTACTCAAAAGACGG</td>
<td>10</td>
<td>This study</td>
</tr>
<tr>
<td>S-S-Pro-60-a-A-18</td>
<td>Pro60</td>
<td>Propionimonomas spp.</td>
<td>CTCCCTCAGGCGTGC</td>
<td>0</td>
<td>This study</td>
</tr>
<tr>
<td>S-S-LGC354C-a-A-18</td>
<td>LGC354C</td>
<td>Firmicutes (Lactobacilales)</td>
<td>CCGAGATTCCCTACTGC</td>
<td>35</td>
<td>Meier et al. (1999)</td>
</tr>
<tr>
<td>S-S-Tet1-266-a-A-18</td>
<td>Tet1-266</td>
<td>Uncultured Tetrathera (clade 1)</td>
<td>CCGTGCCTGCGGCTGTAGC</td>
<td>25</td>
<td>Nguyen et al. (2011)</td>
</tr>
<tr>
<td>S-S-Tet2-892-a-A-18</td>
<td>Tet2-892</td>
<td>Uncultured Tetrathera (clade 2)</td>
<td>TAGTTAGCCTTGGCGCCG</td>
<td>5</td>
<td>Nguyen et al. (2011)</td>
</tr>
<tr>
<td>S-S-Tet2-174-a-A-18</td>
<td>Tet2-174</td>
<td>Cultured Tetrathera (clade 2)</td>
<td>GCTCGGTCTGATCCGG</td>
<td>20</td>
<td>Nguyen et al. (2011)</td>
</tr>
<tr>
<td>S-S-Tet3-19-a-A-18</td>
<td>Tet3-19</td>
<td>Uncultured Tetrathera (clade 3)</td>
<td>CAGCGTTGCGTCTACAC</td>
<td>0</td>
<td>Nguyen et al. (2011)</td>
</tr>
</tbody>
</table>
the isopycnic separation, no contamination by DNA could be visualized by formation of PCR products after 27 cycles of amplification. Separation of 'heavy' and 'light' RNA resulted in a linear isopycnic gradient from 1.77 g ml\(^{-1}\) (fraction 1) to 1.85 g ml\(^{-1}\) (fraction 10) in which RNA could be amplified by RT-PCR in the fractions from 1.77 to 1.805 g ml\(^{-1}\) (fractions 1–5) for the unlabelled incubations, while amplificates were seen in the fractions from 1.77 to 1.825 g ml\(^{-1}\) (fractions 1–7) for the \(^{13}\text{C}_6\)glucose incubations (data not shown). Similar results were seen after 6 and 24 h incubations, and it was therefore decided that the fraction with a density of 1.825 g ml\(^{-1}\) (fraction 7), from the 3 h incubation, would be used to create a clone library.

Phylogenetic analysis of the clone library sequenced from the \(^{13}\text{C}\)-enriched rRNA revealed a presence of three major phyla: Firmicutes, Actinobacteria and Proteobacteria. The most abundant operational taxonomic units (OTUs) are shown in Table S1, and phylogenetic trees from the clones affiliated with the Firmicutes and Actinobacteria are shown in Figs 1 and 2. Quantitative FISH analysis with the applied FISH probes did not reveal major changes in the population composition due to isotope incubation (data not shown).

Specific oligonucleotide probes for FISH targeting Lactococcus, Propionicimonas and Streptococcus were designed to target most members of each of the three genera. Their hybridization stringency was optimized on biomass from the 10 Danish full-scale WWTPs. The sequence target regions and specificities of all probes applied are listed in Table 1. The hierarchical applications of specific probes and phyllum-specific probes (LGC354C and HGC69a) revealed complete congruence.

The probe-defined ability of organisms to take up glucose under anaerobic conditions was tested in situ by MAR-FISH. Indeed, all the FISH-positive bacteria identified with specific probes for Lactococcus, Propionicimonas, Streptococcus and Tetrassphaera clades 1, 2 and 3 were also MAR-positive (Fig. 3), and therefore most likely glucose fermenters. It is always a concern that the incubation conditions during a SIP experiment alter the microbial community composition by enriching the organisms capable of taking up the specific substrate provided. Using the newly designed FISH probes, we therefore quantified the fermenting genera immediately before and after a 3 h anaerobic incubation with glucose, and confirmed that the incubation did not induce changes in the abundance of these genera (data not shown). A few MAR-positive cells were identified which hybridized with the FISH probe targeting the classes Betaproteobacteria and Gammaproteobacteria (BET42a and GAM42a, respectively). These groups cover some of the other sequences identified by the SIP approach, but as the total numbers of these MAR-FISH-positive Proteobacteria were estimated to be below 2 % of the total EUBmix cell numbers, it was decided not to pursue these groups further. Based on combinations of DAPI staining with MAR and FISH, it was estimated that approximately 85 % of all glucose fermenters were identified by the probes applied in this study (data not shown).

![Phylogenetic neighbor-joining tree representing the Firmicutes 16S rRNA gene sequences recovered from the \(^{13}\text{C}\)-enriched RNA after incubation with \(^{13}\text{C}_6\)glucose under fermenting conditions. Scale bar, 1 % sequence difference.](http://mic.sgmjournals.org)
The oligonucleotide probes were applied to survey the abundance of Gram-positive fermenting bacteria in 10 Danish full-scale WWTPs (Table 2). With a quantification limit of 0.25%, estimated by the use of NONEUB, most probes showed the low abundance or completely absence of bacteria affiliating with *Lactococcus* and *Streptococcus*, while *Propionicimonas*, detected by probe Pro60, was present in all EBPR plants investigated and constituted 3.5–5% of the bacterial biovolume (Table 2). The morphology of the cells targeted by Pro60 (small rods and cocci) was identical in all plants analysed, while the few cells affiliating with *Lactococcus* and *Streptococcus* appeared as rod-shaped and as cocci, respectively (Fig. 3).

*Streptococcus* was targeted by the newly designed Str56 probe, but FISH-positive cells with this probe were only found in three WWTPs and in less than 0.9% of the total biovolume (Table 2). However, the broader Strept probe, which targets most members of the genus *Streptococcus*, revealed that members of this genus were present in all 10 WWTPs tested, and that their abundance was similar to the previously published findings from similar WWTP configurations (Kong *et al.*, 2008). MAR-FISH analysis of these bacteria revealed that they were all capable of fermenting glucose.

The probes targeting the three clades of *Tetrasphaera* were applied to 10 WWTPs, and the abundance of cells targeted by these probes ranged from 22 to 44% with an average of 33% of the total EUBmix cell biovolume, which is in accordance with the numbers that we recently published from the same WWTPs (Nguyen *et al.*, 2011). The highest numbers were seen in Ejby Mølle, which has a BioDeniphos configuration, and the lowest in Bjergmarken, which has a sequencing batch reactor configuration. The *Tetrasphaera* clade 3 probe (Tet3-19) targeting the single *Tetrasphaera* sequence obtained in the SIP experiment revealed that this clone represented an average of 3.9% of the total biovolume. Microautoradiographic studies confirmed that all predefined groups of *Testrasphaera* were anaerobically metabolizing glucose.

**Fig. 2.** Phylogenetic neighbor-joining tree representing the actinobacterial 16S rRNA gene sequences recovered from the $^{13}$C-enriched RNA after incubation with $[^{13}$C$_6$]glucose under fermenting conditions. Scale bar, 1% sequence difference.

**Fig. 3.** FISH and MAR images showing (a) *Propionicimonas* (probe Pro60) and (b) *Lactococcus* (probe Lac93). Red/yellow microcolonies and cells are an overlay of red and green fluorescence images (specific probes in red and the bacterial probes EUBmixture in green). MAR image (c) and FISH image (d) show bacteria targeted by probe Pro60 (red/yellow) taking up $[^3$H]glucose under fermenting conditions. Bars, 10 $\mu$m.
Glucose fermenters in activated sludge

**Table 2. Abundance of glucose fermenters in 10 full-scale EBPR plants as determined by quantitative FISH**

Numbers are percentages of specific probes of the total biovolumes (EUBmix). P, Strong positive FISH signals, but abundance not significant above detection limit (0.25%, see Methods). ND, Not detectable.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Target group</th>
<th>WWTP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ejby Mølle</td>
</tr>
<tr>
<td>Str56</td>
<td>Streptococcus spp.</td>
<td>0.31</td>
</tr>
<tr>
<td>Lac93</td>
<td>Lactococcus spp.</td>
<td>P</td>
</tr>
<tr>
<td>Pro60</td>
<td>Propionicimonas spp.</td>
<td>3.56</td>
</tr>
<tr>
<td>LGC354C</td>
<td>Firmicutes (Lactobacillales)</td>
<td>0.85</td>
</tr>
<tr>
<td>Strept</td>
<td>Streptococcaceae</td>
<td>1.75</td>
</tr>
<tr>
<td>Tet1-266</td>
<td>Uncultured Tetrasphaera</td>
<td>11.68</td>
</tr>
<tr>
<td></td>
<td>(clade 1)</td>
<td></td>
</tr>
<tr>
<td>Tet2-892</td>
<td>Uncultured Tetrasphaera</td>
<td>2.75</td>
</tr>
<tr>
<td></td>
<td>(clade 2)</td>
<td></td>
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<tr>
<td>Tet2-174</td>
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<tr>
<td></td>
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<tr>
<td>Tet3-654</td>
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<td>Tet3-19</td>
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</table>

**DISCUSSION**

A substantial part of the readily biodegradable substrates in wastewater may be transformed into biomass before the wastewater arrives at the WWTP. Hydrolysis and fermentation are therefore important processes that supply soluble compounds for biological N and P removal. Only two genera have hitherto been proposed as the main fermentation are therefore important processes that supply soluble compounds for biological N and P removal. Only two genera have hitherto been proposed as the main fermenters in activated sludge systems from EBPR plants: *Tetrasphaera* and *Streptococcus* (Kong et al., 2008). In this study we investigated the bacterial population capable of fermentation and by employing specific inhibitors of sulphate reducers and estimated their abundance by MAR-FISH, using genus-specific oligonucleotide probes. The risk of cross feeding by other species taking up labelled fermentation products in the applied SIP and MAR approach was minimized by the relatively short incubation time (3 h), and by employing specific inhibitors of sulphate reducers and methanogens in the MAR approach. The anaerobic physiology of the SIP-identified organisms supports the suitability of this methodological approach.

The most abundant glucose-fermenting bacteria in activated sludge from the EBPR plants were *Tetrasphaera*, *Propionicimonas* and *Streptococcus*. *Tetrasphaera* and *Streptococcus* belong to the phyla Actinobacteria and Firmicutes, respectively, which harbour a number of well-known glucose fermenters from many other environments, e.g. in the human gut (Kovatcheva-Datchary et al., 2009). These genera have previously been identified as fermenters in full-scale EBPR plants (Kong et al., 2008). *Tetrasphaera* is a large and diverse group of glucose-fermenting organisms, which form three distinct phylogenetic clades (Nguyen et al., 2011). All three clades were represented in the 10 WWTPs investigated, and constituted an average of 33 ± 6% of the total biovolume, which supports previous studies showing that they are very abundant in activated sludge (Nguyen et al., 2011; Nielsen et al., 2010). Only one clone from the present study affiliated with *Tetrasphaera* (clade 3), but quantification of this organism by FISH revealed that it represents a surprisingly large fraction (3.6–4.9%) of the total biovolume in the 10 WWTPs examined. The morphology of these cells was coccoid in clusters of tetrads, as described previously (Nguyen et al., 2011). While a large fraction of *Tetrasphaera* have been shown to be putative polyphosphate-accumulating organisms, the Tet3-19-positive cells, representing *Tetrasphaera* clade 3, did not show any ability to accumulate polyphosphate and were not able to utilize acetic acid and amino acids like most other *Tetrasphaera* (Nguyen et al., 2011). MAR studies of this group of Tet3-19-positive cells revealed, however, that they were capable of taking up glucose and glutamic acid under both anaerobic and aerobic conditions, which supports their ability to ferment glucose.

One probe (Str56) was designed to specifically target the sequences affiliating with *Streptococcus*. The probe-targeted cells were found in most of the WWTPs but only in very low amounts (<0.9% of the EUBmix biovolume). The specificity of the Str56 probe was in accordance with the hierarchical overlap with FISH signals targeted by the LGC354C probe (Firmicutes). Application of the Strept probe revealed overlap with the Str56 probe, and probe-defined cells were present in all WWTPs with abundances of up to 1.8% of the total biovolume. The ecophysiology
tested by MAR-FISH with these probes confirmed that the cells took up glucose under anaerobic conditions. Furthermore, all probes designed in this study were made to specifically target members of the genera identified, and application of the FISH probes revealed that all probe-defined populations were also targeted using a hierarchical probe set and that all or nearly all of these had a fermenting physiology.

*Propionicimonas* has not previously been associated with glucose fermentation in activated sludge but was found in all 10 WWTPs in significant numbers (4.3 ± 0.5 % of the total biovolume). *Propionicimonas* is described as a fermenter capable of fermenting glucose and has been isolated from plant residues in rice-field soil environments (Akasaka *et al.*, 2003), and pure culture studies of this organism have described it as being facultatively anaerobic with production of propionic acid, acetic acid and lactic acid during anaerobic fermentation of glucose.

*Lactococcus* was also identified by SIP, and a probe was designed to target these sequences (Lac93). This probe revealed its presence in most WWTPs, but only insignificantly in significant numbers (0.8 ± 0.3 % of the total biovolume) in the Marselisborg WWTP. The Lac93-targeted population was also covered by the broader LGC354C probe, and the ability of these probe-defined organisms to take up glucose under anaerobic conditions was also confirmed by MAR-FISH. *Tolumonas* and *Burkholderia* were also identified in the 13C-enriched RNA fraction, but no attempt was made to design probes to target these groups, and their ability to ferment glucose was therefore not verified by MAR-FISH. Both genera are affiliated with the Betaproteobacteria, and MAR-FISH examination with a probe targeting this class revealed the presence of glucose-fermenting bacteria. These genera of potentially glucose-fermenting bacteria have been associated with fermentation in other systems. For example, *Tolumonas* has been isolated from eutrophic lake sediments, and acetic acid, ethanol and formic acid were the major fermentation products when it was grown on glucose (Fischer-Romero *et al.*, 1996). Members of the genus *Burkholderia* have to our knowledge not previously been found to possess the ability to ferment. The remaining sequences obtained from the 13C-enriched rRNA affiliated with the phyla Bacteroidetes and TM7, but their ability to perform glucose fermentation was not further investigated.

Collectively, the fermenters quantified by FISH in the 10 EBPR plants investigated in this study constituted approximately 38.9 ± 7 % of the entire bacterial biovolume. Although this number seems to be surprisingly large, most seem to be facultative fermenters and may therefore also be active under other conditions prevailing in the WWTP. Some *Tetrasphaera* have for example also been characterized as polyphosphate-accumulating organisms (Kong *et al.*, 2005; Nguyen *et al.*, 2011), and similarly the *Propionicimonas* identified in this study as a widespread and important fermenter in activated sludge are closely related to *Microlunatus phosphovorus*, another putative polyphosphate-accumulating organism (Nakamura *et al.*, 1995). The percentage of MAR-positive cells with respect to the total DAPI count and the FISH/DAPI ratio indicates that most glucose fermenters in activated sludge from the investigated systems have been identified by the probes designed in this study.

The identification of up to 38.9 % of the total biovolume with *Tetrasphaera, Propionicimonas* and *Streptococcus* as the most abundant genera shows that fermentation is a common and important process in activated sludge that is carried out by a relatively large group of predominantly Gram-positive bacteria. This diversity probably ensures a broad mixture of fermentation products that can be used to sustain other groups of micro-organisms in the plants.

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