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ORIGINAL ARTICLE

A metabolic model for members of the genus *Tetrasphaera* involved in enhanced biological phosphorus removal

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Members of the genus *Tetrasphaera* are considered to be putative polyphosphate accumulating organisms (PAOs) in enhanced biological phosphorus removal (EBPR) from wastewater. Although abundant in Danish full-scale wastewater EBPR plants, how similar their ecophysiology is to '*Candidatus* Accumulibacter phosphatis' is unclear, although they may occupy different ecological niches in EBPR communities. The genomes of four *Tetrasphaera* isolates (*T. australiensis*, *T. japonica*, *T. elongata* and *T. jenkinsii*) were sequenced and annotated, and the data used to construct metabolic models. These models incorporate central aspects of carbon and phosphorus metabolism critical to understanding their behavior under the alternating anaerobic/aerobic conditions encountered in EBPR systems. Key features of these metabolic pathways were investigated in pure cultures, although poor growth limited their analyses to *T. japonica* and *T. elongata*. Based on the models, we propose that under anaerobic conditions the *Tetrasphaera*-related PAOs take up glucose and ferment this to succinate and other components. They also synthesize glycogen as a storage polymer, using energy generated from the degradation of stored polyphosphate and substrate fermentation. During the aerobic phase, the stored glycogen is catabolized to provide energy for growth and to replenish the intracellular polyphosphate reserves needed for subsequent anaerobic metabolism. They are also able to denitrify. This physiology is markedly different to that displayed by '*Candidatus* Accumulibacter phosphatis', and reveals *Tetrasphaera* populations to be unusual and physiologically versatile PAOs carrying out denitrification, fermentation and polyphosphate accumulation.
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Introduction

Enhanced biological phosphorus removal (EBPR) is an environmentally friendly and cost-effective process encouraged in many wastewater treatment plants (Oehmen *et al.*, 2007). These systems exploit the ability of polyphosphate accumulating organisms (PAOs) to assimilate large amounts of phosphorus and store it intracellularly as polyphosphate granules (Fuhs and Chen, 1975). The dominance of these PAO populations in EBPR communities is achieved by cycling the biomass through alternating anaerobic (feast) and aerobic (famine) phases (Barnard, 1975).

The many models explaining PAO metabolism have been the subject of much debate. Their general features are that PAOs take up volatile fatty acids like acetate and store them as polyhydroxyalkanoates (PHAs) during the anaerobic phase. This reserve polymer is metabolized in the subsequent aerobic phase, where readily metabolizable exogenous carbon and energy sources are no longer available, to supply the energy needed for PAO to assimilate phosphate from the mixed liquor and store it as intracellular polyphosphate granules. It now appears that the energy needed for the anaerobic uptake of volatile fatty acids and PHA synthesis is

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derived from hydrolysis of the intracellular polyphosphate stores, while reducing power and additional energy is generated from glycolysis of intracellular glycogen stores (Mino *et al.*, 1987), anaerobic operation of the tricarboxylic acid (TCA) cycle (Comeau *et al.*, 1986; Louie *et al.*, 2000) or both (Pereira *et al.*, 1996; Wexler *et al.*, 2009; Zhou *et al.*, 2009).

'*Candidatus* Accumulibacter phosphatis' (subsequently referred to as Accumulibacter) is the organism thought to be largely responsible for EBPR in both laboratory- and full-scale plants (Crocetti *et al.*, 2000; and reviewed by He and McMahon, 2011). Data from laboratory-scale studies repeatedly show that this organism possesses the phenotype expected of a PAO (Pereira *et al.*, 1996; Mino *et al.*, 1998; Hesselmann *et al.*, 2000; Liu *et al.*, 2001). A metabolic model was reconstructed for the dominant Accumulibacter strain (Clade IIA str. UW-1) in a metagenome from two laboratory-scale EBPR plants enriched in it (García Martín *et al.*, 2006). The model displayed the mechanisms thought to account for phosphate accumulation, and has since provided a basis for analysis of the transcription of key genes (He *et al.*, 2010). A metagenomic study of a full-scale EBPR plant has also confirmed the presence of Accumulibacter populations although their genomes show substantial differences to that of the reference genome (Albertsen *et al.*, 2011).

Members of the Actinobacterial genus *Tetrasphaera* were suggested to be putative PAOs (Maszenan *et al.*, 2000; Kong *et al.*, 2005), but with a markedly different physiology to Accumulibacter. Both could take up phosphate aerobically and store it intracellularly as polyphosphate, and assimilate a range of substrates under anaerobic conditions. However, *in situ* staining has failed to show that *Tetrasphaera* store these in the form of intracellular PHA in EBPR systems, in contrast to the situation with Accumulibacter (Kong *et al.*, 2005; Nguyen *et al.*, 2011). Furthermore, *Tetrasphaera* appears able to ferment glucose (Kong *et al.*, 2008; Nguyen *et al.*, 2011), but like Accumulibacter, *Tetrasphaera* also assimilates phosphate into polyphosphate granules under aerobic conditions only if in a previous anaerobic phase organic substrates have been available to them. Thus, the ecophysiology of *Tetrasphaera* seems to be more versatile than that of Accumulibacter. *Tetrasphaera*-related PAOs can be detected in high relative abundances in many full-scale EBPR plants where they account for up to 30% of the total bacterial biovolume (Kong *et al.*, 2005; Nguyen *et al.*, 2011). They are also phylogenetically diverse, comprising three distinct clades, where cells within each clade exhibit a range of morphologies (Nguyen *et al.*, 2011).

Currently, the genus *Tetrasphaera* consists of six species cultured from activated sludge; *T. australiensis* (strains Ben 109 and Ben 110), *T. japonica* (strain T1-X7) (Maszenan *et al.*, 2000), *T. elongata* (strain Lp2) (Hanada *et al.*, 2002), *T. elongata* (strain ASP12) (Onda and Takii, 2002), *T. jenkinsii*, *T. veronensis* and *T. vanveenii* (McKenzie *et al.*, 2006), but no genome sequence data exist for any of these isolates. As these organisms seem to have an important role in phosphorus removal in EBPR plants (Kong *et al.*, 2005; Nguyen *et al.*, 2011), a metabolic model for *Tetrasphaera* is needed to clarify what their function in these systems is, and how their ecophysiology might differ from that of Accumulibacter. Thus, the aim of this study was to sequence the genomes of four *Tetrasphaera* isolates (*T. australiensis*, *T. elongata*, *T. japonica* and *T. jenkinsii*), and to interpret these data to develop a metabolic model for them. These models were confirmed experimentally, where possible, and a metabolic model for the *Tetrasphaera* present in EBPR configured systems is proposed.

Materials and methods

Bacterial strains

Four *Tetrasphaera* strains (*T. australiensis* str. Ben110, DSM17519; *T. elongata* str. Lp2, DSM 14184; *T. japonica* str. T1-X7, DSM13192; *T. jenkinsii* str. Ben74, DSM17519) isolated from activated sludge were used. The strains were grown in GS or R2A medium at 26 °C. Genomic DNA from each isolate was extracted using FastDNA SPIN kit for soil (MP Biomedicals, Seven Hills, NSW, Australia) according to manufacturer's instructions.

Genome sequencing and assembly

From 0.5 to 1 μg of DNA, a library for Illumina paired-end sequencing was constructed using the Paired-end DNA Sample Prep Kit (PE-102-1001; Illumina, CA, USA) according to manufacturer's instructions (Part # 1005063 Rev. A), but with minor modifications. The genomic DNA was fragmented at 32 p.s.i. for 8 min, and the adaptor-modified DNA fragments were enriched by 14 PCR cycles. The purified library was sequenced using an Illumina GAII with a paired-end module. Up to 200 000 clusters were generated per tile with paired-end reads of a length of 36 bp for *T. australiensis* and *T. elongata*. For *T. japonica*, paired-end reads of both 36 bp and 72 bp were obtained, and for *T. jenkinsii*, the paired-end reads were 72 bp long.

The sequences obtained for each genome were subjected to *de novo* assembly using both CLC Genomics Workbench version 4.5.1 (CLC bio, Aarhus, Denmark) and ABySS (Simpson *et al.*, 2009) ($n = 10$, $k = 27$ for the 36-bp reads, $k = 47$ for the 72-bp reads from *T. japonica* and $k = 49$ for the reads obtained from *T. jenkinsii*). When assembling the reads with CLC Genomics Workbench, default settings were used as the assembly parameters. The minimum and maximum distances between the paired-end reads were set individually for each

assembly, depending on the approximate size of the fragment sequenced, and for the 72-bp reads, a similarity of 0.8 and length fraction of 0.5 were used. The resulting contigs from CLC Genomics Workbench version 4.5.1 and ABySS were merged into one set of contigs using Minimus from the AMOS software package (Sommer *et al.*, 2007).

Comparative genome analysis
The contigs were annotated using the web interface Magnifying Genomes (MaGe) of the MicroScope platform from GenoScope (Vallenet *et al.*, 2006, 2009). The automatic annotations provided by MaGe were curated manually to validate the presence or absence of a particular gene involved in selected pathways of interest. Based on the annotations for each genome, a statistical distribution of the protein encoding genes was determined according to their classification within the Cluster of Orthologous Groups functional categories.

The unique and conserved genes in the four *Tetrasphaera* genomes were determined by comparing the protein sequences from each *Tetrasphaera* genome against a complete *Tetrasphaera* database using blastP. The resulting genes that were $>50\%$ identical over a minimum of 50% of the length of the protein with one or more genes from the other *Tetrasphaera* genomes were considered as non-unique genes. All genes not having a hit by these criteria in the other *Tetrasphaera* genomes were considered as unique genes.

Pure culture validation experiments
Two isolates (*T. elongata* and *T. japonica*) were cultured in Erlenmeyer flasks in an orbital incubator at 22 °C in R2A broth (which includes glucose) without starch and sodium pyruvate, to generate enough biomass for biochemical studies. When an adequate amount had been produced (typically after 7 days), cells were harvested by centrifugation and resuspended in the chemically defined MSV media (Williams and Unz, 1989) without any carbon source. Biomass suspensions were incubated aerobically for 4 h to exhaust any storage polymers and residual carbon sources before experiments were initiated. All experiments were performed in duplicate at 20–22 °C.

To determine the ability of the *Tetrasphaera* strains to assimilate glucose and release phosphate under anaerobic conditions, biomass was incubated anaerobically in R2A with 1 mM ^{13}C-glucose, but without starch, sodium pyruvate and potassium dihydrogen phosphate. To ensure anaerobic conditions, cells were incubated in vials that were capped and sealed before being flushed sequentially with nitrogen gas and vacuum. During incubation, samples were removed for analyses of phosphate, ^{13}C-glucose and ^{13}C-labeled fermentation products. Cell biomass was also sampled for glycogen and PHA analyses. To determine the ability of *Tetrasphaera* strains to take up phosphate under subsequent aerobic conditions, biomass was incubated anaerobically with glucose, then harvested and washed after 3 h with MSV media under anaerobic conditions, before being incubated under aerobic conditions for a further 3 h in MSV with 0.5 mM of phosphate, but without any exogenous carbon source. Samples were taken for phosphate uptake measurements and cell contents of glycogen and PHA were measured. All experiments were performed in duplicate.

Denitrification. Inocula were obtained as above by growing cultures in shake flasks in R2A broth lacking starch and sodium pyruvate. When a sufficient inoculum was obtained, cells were harvested by centrifugation and resuspended in fresh R2A broth without starch and sodium pyruvate. To ensure that the cultures could tolerate nitrate or nitrite, low concentrations of nitrate and nitrite were added to the flasks during aerobic growth (0.25 mM of $NaNO_3$ and 0.1 mM of $NaNO_2$, respectively). To assess whether the *Tetrasphaera* isolates could denitrify, a final concentration of either 2 mM $NaNO_3$ or 0.5 mM $NaNO_2$ was added to each culture. Residual oxygen was removed as described earlier for the anaerobic incubations. Nitrate and nitrite levels were monitored. All experiments were performed in duplicate.

Anaerobic growth. Isolates were grown in R2A broth without starch and sodium pyruvate under the anaerobic conditions described above. The cell numbers were determined after 0, 1, 7, 14 and 21 days by counting DAPI-stained cells (see later).

Chemical analyses
Glycogen and PHA. Intracellular glycogen levels were determined according to the method described by Bond *et al.* (1999) with minor modifications. Ten micrograms of lyophilized biomass was resuspended in 5 ml of 0.9 M HCl and digested at 100 °C for 5 h. Glucose equivalents in the supernatant were quantified using HPLC equipped with the ICS-5000 (Dionex, Thermo Fisher Denmark A/S, Hvidovre, Denmark) column. PHA was analyzed by gas chromatography as described by Braunegg *et al.* (1978) with minor modifications. Ten micrograms of lyophilized biomass was resuspended in 2 ml chloroform, 2 ml methanol with 3% or 10% [v/v] H_2SO_4 and a hepta-decane internal standard, and digested for 6 h at 100 °C. One microliter of milliQ water was added and the sample was left to phase separate for at least 1 h. In all, 0.5 g of sodium sulfate was added to the chloroform phase to remove excess water. The samples were analyzed using an Agilent Technologies 7890A GC system (Horsholm, Denmark) equipped with a HP-5 column.

Fermentation products. In total, 10 ml aliquots of supernatant from the anaerobic cultures were lyophilized and resuspended in 600 μl of D_2O with 0.075% [w/v] of sodium 2,2,3,3-tetradeutero, 3-(trimethylsilyl) propionate, pH 7. The spectra were acquired with a Bruker AVIII-600 Spectrometer (Fällanden, Switzerland) equipped with a 5-mm TXI (H/C/N) probe operating at 600.13 MHz for ^{1}H and 150.9 MHz for ^{13}C. Fermentation products were identified by acquiring HSQC (heteronuclear single quantum coherence) and 2D-HSQC-TOCSY spectra and comparing the shifts found with the Human Metabolome Database (Wishart *et al.*, 2009) and BRUKER's BBIOREFCODE spectral database (Karlsruhe, Germany).

Orthophosphate. The samples were filtered through a 0.45-μm cellulose acetate filter, stored in 0.04 M H_2SO_4 at 4 °C and analyzed spectrophotometrically according to the ascorbic acid standard method 4500-P E (Murphy and Riley, 1962; APHA *et al*, 1995).

Biomass determination

T. elongata was grown in Erlenmeyer flasks in an orbital incubator at 22 °C in R2A broth without starch and sodium pyruvate for 7 days. Every day, 2 ml of the culture was fixed in a final concentration of 2% [w/v] formaldehyde for cell number determination. The fixed sample was homogenized and stained for 10 min with 5 μg ml^{-1} of DAPI, filtered through a 0.2-μm black polycarbonate filter and the stained cells counted using an epifluorescence microscope. On day 7, the suspended solids were also determined by filtering an aliquot through a 0.2-μm cellulose ester membrane filter that was dried overnight at 60 °C (APHA *et al.*, 1995).

Nucleotide sequence accession numbers

These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under the accession numbers CAIZ01000001-CAIZ01000173, CAJA 01000001-CAJA01000528, CAJB01000001-CAJB010 00435 and CAJC01000001-CAJC01000215 for *T. elongata, T. australiensis, T. japonica* and *T. jenkinsii*, respectively.

Results and discussion

Tetrasphaera genome characteristics

Of the phylogenetic clades proposed for the genus *Tetrasphaera* (Nguyen *et al.,* 2011), *T. elongata* belongs to clade 1, *T. australiensis* and *T. jenkinsii* to clade 2, while *T. japonica* does not belong to any of the described clades (Figure 1). The morphology of these isolates varies, with *T. australiensis* and *T. japonica* always growing as cocci in tetrads or clusters (Maszenan *et al.*, 2000), *T. elongata* as rod or oval unicells (Hanada *et al.*, 2002), while the morphology of *T. jenkinsii* depends on the medium on which it is cultured; being filamentous on GS media and growing as irregularly sized cocci in clusters on R2A media (McKenzie *et al.*, 2006).

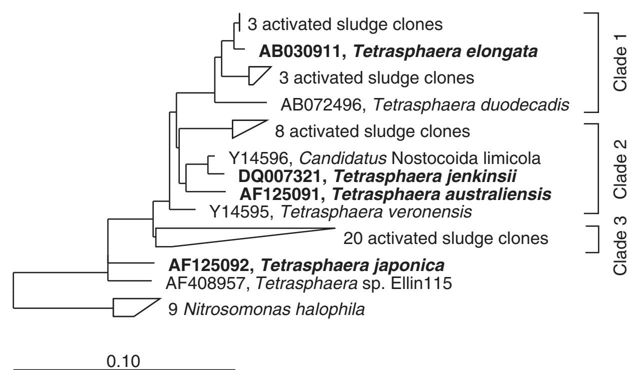


Figure 1 Phylogenetic tree of the *Tetrasphaera* genus showing the grouping of *Tetrasphaera* isolates in three clades described by Nguyen *et al.* (2011). Names of isolates used to construct the *Tetrasphaera* genomes are indicated with boldface font. The outgroup used to construct this tree was nine strains of *Nitrosomonas halophila*.

To reconstruct the genomes of these four *Tetrasphaera* species, the reads were assembled *de novo* and annotated using the MicroScope platform. The assembly resulted in genomes comprising 173–526 contigs (Table 1). The genome size varied from 3.15 to 5.16 Mbp with an average genome coverage ranging from 316 × to 1327 ×. According to Farrer *et al.* (2009), a sequence depth above 30 × should be sufficient to assemble >96% of the genome *de novo* with the remaining unassembled part of the genome likely to be mainly composed of non-coding RNA and mobile genetic elements. Based on the presence of 106 essential genes, the completeness of the genomes is above 95%. These four *Tetrasphaera* genomes consist of 3258–5003 coding sequences, constituting up to 92.37% of their total genomes. An analysis of the Clusters of Orthologous Groups classification distribution of the coding sequences was conducted, and in general, none of the genomes is distinctly different from the others (Supplementary Material; Supplementary Figure S1). For all isolates, the coverage of the 16S rRNA gene was 1.3–1.5 times higher than the average coverage of the genome, which indicates that each isolate contains 1 or 2 copies of the rRNA operon.

Conserved and unique genes were identified in the four *Tetrasphaera* isolates. Recognizing whether an individual gene is unique or conserved depends heavily on the criteria used. As no definitions exist for determining which represent core genes, it is difficult to elucidate the core genome (Bentley, 2009). In this study, we have recognized the genes involved in central metabolic pathways as being conserved genes, and those involved in a pathway unique to a single isolate as unique genes. With these criteria, the conserved genes from all four genomes constitute 1283 sequences (Figure 2). They

Table 1 Genome characteristics of four sequenced *Tetrasphaera* isolates

Organism	*Sequence length (bp)*	*Genome completeness (%)*[a]	*mol% GC*	*Coverage*	*Number of contigs*	*N50*	*Number of CDS*	*Protein coding density (%)*
T. australiensis	4 282 171	96.2	69.01	366	526	13 130	4748	89.40
T. elongata	3 157 491	95.3	68.99	316	173	35 110	3258	92.37
T. japonica	5 162 321	98.1	71.51	737	435	19 833	5003	87.83
T. jenkinsii	3 809 986	100	68.24	1327	215	29 678	3983	91.78

Abbreviation: CDS, coding sequences.
[a]Based on percentage essential genes present.

Figure 2 Venn diagram of unique and conserved genes for four *Tetrasphaera* isolates (*T. australiensis*, *T. elongata*, *T. japonica* and *T. jenkinsii*). *T. japonica* has 2924 unique genes and *T. elongata* has 1183 unique genes. *T. australiensis* and *T. jenkinsii*, both belonging to clade 2, have 1974 and 1469 unique genes, respectively, and share 582 genes. The conserved genes constitute 1283 genes of each genome.

include those involved in the central metabolic pathways possessed by all four isolates, and include polyphosphate metabolism, glycolysis and the TCA cycle (see below). The unique genes constitute from 1183 to 2924 sequences, and for example are genes involved in pathways for PHA synthesis, and assimilatory nitrate reduction to ammonia (see below).

Metabolic reconstruction

The annotation of the four *Tetrasphaera* genomes showed that many of the genes involved in the central metabolic pathways in Accumulibacter are also found in *Tetrasphaera*. These pathways include the TCA cycle, glycolysis, gluconeogenesis and polyphosphate metabolism. However, as detailed below, they differ fundamentally in several important respects, especially in the ability of these *Tetrasphaera* species to ferment.

Polyphosphate metabolism. Polyphosphate metabolism has been detailed in many bacteria including *Escherichia coli*. Orthologous genes involved in polyphosphate metabolism are also possessed by each of these *Tetrasphaera* species (Table 2; Supplementary Table S1). These include the genes important in both polyphosphate degradation and synthesis, and in transporting inorganic phosphate across the membrane using a low-affinity inorganic phosphate transporter (Pit) system and a high-affinity phosphate ABC transporter (Pst) system (Willsky and Malamy, 1980). *E. coli* is thought to synthesize polyphosphate using a reversible PPK1 (polyphosphate kinase 1), which transfers high-energy phosphate groups from ATP to a growing polyphosphate chain (Akiyama *et al.*, 1992). Intracellularly stored polyphosphate can be degraded to yield energy in reactions catalyzed by a polyphosphate kinase 2 (Ishige *et al.*, 2002), exophosphatase (PPX) (Akiyama *et al.*, 1993), PAP (polyphosphate AMP phosphotransferase) (Bonting *et al.*, 1991), or the combined enzymatic activity of ADK (adenylate kinase) and PPK1 (Shiba *et al.*, 2000). The PAP/ADK reaction may also serve as an additional mechanism for polyphosphate synthesis (García Martín *et al.*, 2006).

These observations suggest that *Tetrasphaera* may share much of their polyphosphate metabolic machinery with Accumulibacter (García Martín *et al.*, 2006). Thus, intracellular polyphosphate in *Tetrasphaera* may be degraded to Pi during the EBPR anaerobic phase, generating energy, with the Pi transported out of the cell, and the energy generated supporting substrate uptake and conversion into storage reserve material. In the subsequent aerobic phase, polyphosphate storage levels are replenished by cells assimilating Pi from the bulk liquid.

Substrate uptake and storage polymers. *Tetrasphaera* appears able to assimilate a range of substrates, including acetate, propionate, glucose, glutamate and aspartate. Previous *in situ* ecophysiological investigations have shown that they utilize glucose and amino acids, but controversy exists regarding their ability to utilize acetate (Kong *et al.*, 2004; Nguyen *et al.*, 2011). All four *Tetrasphaera* genomes contain an acetate transporter gene (*actP*) and genes involved in acetate activation (*acs*, *ackA* and *pta*). Thus, they clearly have the potential to take up and utilize acetate. Furthermore, the genes encoding a general sugar transporter and glucokinase are present in all four *Tetrasphaera* genomes, as are the genes encoding for a glutamate/aspartate transporter. Thus, it seems

Table 2 Key metabolic pathways as predicted from the genomes from four *Tetrasphaera* isolates

Pathway	*Accumulibacter*	*T. australiensis*	*T. elongata*	*T. jenkinsii*	*T. japonica*
Glycolysis	+	+	+	+	+
Gluconeogenesis	+	+	+	+	+
Glycogen synthesis	+	+	+	+	+
Glycogenolysis	+	+	+	+	+
TCA cycle	+	+	+	+	+
Glyoxylate shunt	+	−	−	−	−
PHA synthesis[a]	+	−	−	−	+
Polyphosphate metabolism	+	+	+	+	+
Acetate uptake	+	+	+	+	+
Glucose uptake	−	+	+	+	+
Glutamate/aspartate uptake	−	+	+	+	+

Abbreviations: PHA, polyhydroxyalkanoate; TCA, tricarboxylic acid.
+, Candidate genes coding for all enzymes involved in the pathway have been located in the genome; −, Not all candidate genes for the enzymes involved in the pathway have been found in the genome.
[a]Based on the presence of *phaC* gene.

that *Tetrasphaera* is versatile in the substrates it can potentially utilize. The genome for Accumulibacter lacks genes encoding for assimilation of glucose, agreeing with *in situ* observations that unlike *Tetrasphaera* which can utilize glucose, Accumulibacter cannot (Kong *et al*., 2004; Burow *et al*., 2008; Nguyen *et al*., 2011). This major metabolic difference between *Tetrasphaera* and Accumulibacter might have a key role in deciding which of these PAO populations outcompete the other in EBPR plants.

All genes encoding for enzymes involved in the forward TCA cycle, glycolysis, gluconeogenesis, glycogen synthesis and glycogenolysis were present in all four *Tetrasphaera* genomes. However, those encoding for enzymes involved in the glyoxylate shunt (isocitrate lyase (*icl*) and malate synthase (*mas*)) indicating that *Tetrasphaera* does not seem to bypass the steps in the TCA cycle where carbon is lost as CO_2 as Accumulibacter has been proposed to do (García Martín *et al*., 2006). The absence of genes for the glyoxylate shunt may explain the poor growth when acetate is provided as the sole carbon source, despite the presence of the genes for acetate transport and activation.

Both PHA and glycogen are important bacterial storage polymers. Candidate genes for both acetyl-CoA acetyltransferase (*phaA*) and acetoacetyl-CoA reductase (*phaB*) were identified in all four *Tetrasphaera* genomes, but PHA synthase (*phaC*) was only found in the *T. japonica* genome (Table 2). Thus, only *T. japonica* appears to have the potential to synthesize PHA as a storage compound. This partly disagrees with previous published data. Of the four isolates studied here, *T. jenkinsii* was the only isolate shown previously by histochemical staining to contain ‘PHA’, although the positive staining reaction was reported to be inconsistent for this organism (Blackall *et al.,* 2000; McKenzie *et al.,* 2006) and other lipidic material may also have given a positive signal with the applied Nile blue A staining method (Serafim *et al*., 2002). On the other hand, all four isolates have the potential to transform substrate into glycogen, another common carbon and energy storage polymer produced by bacteria. In Accumulibacter, glycogen is synthesized in the aerobic EBPR phase, and hydrolyzed in the subsequent anaerobic phase to provide energy and reducing power for synthesis of PHA from assimilated volatile fatty acids. However, glycogen and glycogen-like inclusions can also be synthesized under anaerobic conditions, as in *Methanothrix* str. FE (Pellerin *et al*., 1987). Thus, it is possible that glycogen is synthesized as the anaerobic storage polymer in *Tetrasphaera*, and used under subsequent aerobic conditions as an energy source for Pi assimilation and polyP synthesis. No ecophysiological studies have investigated this possibility because of the lack of a staining method for the specific detection of glycogen *in situ* (Serafim *et al*., 2002).

Any discussion on which source of reducing power Accumulibacter uses to synthesize PHA anaerobically from acetate (glycolysis, TCA, split TCA or a combination, reviewed by He and McMahon, 2011) is not relevant for *Tetrasphaera* clade 1 and 2 members because no PHA is formed, although their ability to ferment (see below) would provide an alternative source of energy.

Fermentation. *In situ* studies of the *Tetrasphaera* populations have shown that they may possess a fermentative metabolism (Kong *et al*., 2008; Nguyen *et al*., 2011). The genes important in glucose fermentation in *E. coli* (Clark, 1989) and *Bacillus subtilis* (Nakano *et al*., 1997) are indeed present in these four *Tetrasphaera* genomes (Table 3). This includes the candidate gene encoding an alanine dehydrogenase (*ald*), which suggests that all isolates may produce alanine as an end product of glucose fermentation, whereas the genes coding for ethanol production (acetaldehyde dehydrogenase *adhE* and alcohol dehydrogenase (adh)) have only been observed in *T. australiensis*. The gene responsible for lactate formation (lactate dehydrogenase (*ldh*)) was detected only in *T. elongata* and *T. japonica*.

Table 3 Genes coding for enzymes involved in fermentation

Gene	*Protein*	*EC no.*	*Accumulibacter*	*T. australiensis*	*T. elongata*	*T. jenkinsii*	*T. japonica*
pdc	Pyruvate decarboxylase	4.1.1.1	−	−	−	−	−
mhpF	Acetaldehyde dehydrogenase	1.2.1.10	−	+	−	−	−
aldA	Aldehyde dehydrogenase	1.2.1.3	−	+	+	+	+
adh	Alcohol dehydrogenase	1.1.1.1	−	+	+	+	+
ldh	Lactate dehydrogenase	1.1.1.27	−	−	+	−	+
pfl	Pyruvate formate lyase	2.3.1.54	−	−	+	−	−
gcvP	Glycine dehydrogenase	1.4.4.2	+	+	+	+	+
gcvT	Aminomethyltransferase	2.1.2.10	+	+	+	+	+
lpd	Dihydrolipoyl dehydrogenase	1.8.1.4	+	+	+	+	+
gcvH	Gcv-system lipoylprotein		+	+	+	+	+
ltaE	Threonine aldolase	4.1.2.5	−	+	+	+	+
tdh	Threonine 3-dehydrogenase	1.1.1.103	−	+	+	+	−
kbl	Glycine acetyl transferase	2.3.1.29	−	+	+	+	−

+, Candidate gene present in the genome; −, a candidate gene has not been identified in the genome.

Table 4 Genes coding for enzymes involved in denitrification and dissimilatory nitrate reduction to ammonia

Gene	*Protein*	*EC no.*	*Accumulibacter*	*T. australiensis*	*T. elongata*	*T. jenkinsii*	*T. japonica*
narI	Nitrate reductase (γ subunit)	1.7.99.4	−	+	+	+	+
narJ	Nitrate reductase (δ subunit)	1.7.99.4	−	+	+	+	+
narH	Nitrate reductase (β subunit)	1.7.99.4	−	+	+	+	+
narG	Nitrate reductase (α subunit)	1.7.99.4	−	+	+	+	+
napA	Nitrate reductase	1.9.6.1	+	−	−	−	+
nirB	Nitrite reductase, large subunit	1.7.1.4	+	−	−	−	+
nirD	Nitrite reductase, small subunit	1.7.1.4	+	−	−	−	+
nirK	Copper-containing nitrite reductase	1.7.2.1	−	+	+	+	+
norB	Nitric oxide reductase, large subunit	1.7.99.7	−	+[a]	−	−	+[a]
norC	Nitric oxide reductase, small subunit	1.7.99.7	−	−	−	−	−
norZ	Nitric oxide reductase	1.7.99.7	+	+[a]	−	−	+[a]
nosZ	Nitrous oxide reductase	1.7.99.6	+	−	−	−	−

+, Candidate gene present in the genome; −, a candidate gene has not been identified in the genome.
[a]*norB* and *norZ* have amino-acid identities of 90%. Therefore, this sequence may be either of these sequences.

However, the lactate dehydrogenase in *T. japonica* (1.1.2.3 and 1.1.2.4) is theoretically metabolically irreversible, catalyzing the conversion of lactate into pyruvate, while that present in the *T. elongata* genome (1.1.1.27) is theoretically reversible (Stansen *et al.*, 2005). From this observation, we hypothesize that only *T. elongata* has the potential to produce lactate as an end product of glucose fermentation. Acetate and succinate may also be produced as fermentation end products from glucose in all four isolates. If the annotated succinate dehydrogenase is capable of operating in reverse, then the observed anaerobically produced succinate (see later) may be a by-product of fermentation via the forward and/or reverse operation of the TCA cycle. The ability of these *Tetrasphaera* to generate energy from fermentation is an additional key metabolic feature distinguishing them from Accumulibacter.

Denitrification. All four *Tetrasphaera* isolates possess the potential to utilize nitrate and/or nitrite as terminal electron acceptors in anaerobic respiration or denitrification. Genes involved in the reduction of nitrate to nitrite were detected in each species. In all four genomes *nirK*, the defining gene for denitrification was similar phylogenetically to *nirK* sequences in other Gram-positive bacteria (data not shown). However, sets of genes allowing complete denitrification were not found (Table 4). Interestingly, those encoding the nitrate reductases responsible for reducing nitrate to ammonia (*nirB* and *nirD*) were detected in the genome of *T. japonica* (Table 4), suggesting that this organism can reduce nitrate by dissimilatory nitrate reduction to ammonia. Thus, *Tetrasphaera* may be able to couple nitrate and/or nitrite reduction to phosphorus uptake, which is similar to the behavior described previously for Accumulibacter clade IA members (Flowers *et al.*, 2009).

Experimental metabolic investigations

The genomic investigations of the four *Tetrasphaera* isolates provided insight into the metabolic potential of *Tetrasphaera* isolates and how they may survive and compete successfully in EBPR plants. The hypotheses developed and discussed above were validated through experimental investigations

of their ability to consume glucose and acetate, to ferment, to synthesize different storage compounds, to denitrify and to actively take up and release P. However, only *T. elongata* and *T. japonica* were studied as the other isolates grew too slowly in all the media tested. *T. elongata* represents the most abundant clade (clade 1) observed in full-scale plants in Denmark (based on FISH (fluorescent *in situ* hybridization)), while *T. japonica* has not been found so far in any Danish full-scale plants (Nguyen *et al*., 2011).

All the potential pathways revealed by the genome sequencing were shown to operate in these two species. Both consumed acetate and glucose, but as they grew very poorly on acetate, all subsequent experiments were carried out with glucose in R2A media. *T. elongata* grew at 20 °C with a doubling time of ∼4 and 19 h under aerobic and anaerobic conditions, respectively. During an anaerobic-aerobic cycle, *T. elongata* demonstrated a typical PAO phenotype (Figure 3). Thus, it consumed glucose and released Pi during the anaerobic phase, and then assimilated Pi during the subsequent aerobic conditions. In the absence of glucose in the anaerobic phase, no Pi was assimilated aerobically (data not shown). A fraction of the glucose was stored as glycogen (Figure 3) and a fraction was fermented to succinate, lactate, alanine, and acetate as determined by ^{13}C-labeling (data not shown). Glycogen was only produced as stored Pi was released, which is similar to the pattern observed in Accumulibacter for the anaerobic formation of PHA from acetate (Oehmen *et al*., 2007), suggesting that the energy required is sourced from the hydrolysis and release of intracellular polyphosphate. In the aerobic phase, the glycogen was slowly consumed with time (Figure 3) and no fermentation products were observed (data not shown).

The amount of phosphate released after 3 h incubation was ∼19 mgP g^{-1} dry biomass and the amount taken up was ∼9 mgP g^{-1} dry biomass. A sample was also taken after 24 h of aerobic incubation which showed that the isolate had taken up ∼16 mgP g^{-1} dry biomass (corresponding to ∼1.4×10^{-16} mol per cell). It is difficult to extrapolate results from pure cultures to the complex conditions of full-scale plants where the P content may be even higher when considering the experiments only covered a single cycle, and P-uptake capacity may increase over time. The experimental design was intended to demonstrate the ability of *Tetrasphaera* to release/uptake P and not to determine the maximum uptake capacity.

T. japonica could ferment glucose, generating acetate and alanine as end products. *T. japonica* also produced intracellular PHA anaerobically from glucose. Following uptake, the glucose is likely activated and degraded to pyruvate through the glycolysis pathway. The produced pyruvate may be further converted into PHA as polyhydroxybutyrate via acetyl-CoA and polyhydroxyvalerate through propionyl-CoA.

Both *T. japonica* and *T. elongata* can potentially denitrify, using both nitrate and nitrite as electron acceptors (Supplementary Figure S2). Both isolates reduced the nitrate supplied completely within 48 h. During nitrate reduction, nitrite accumulated in the cultures and was not reduced until the nitrate was completely removed. Whether nitrite reduction proceeded completely to N_2 was not pursued.

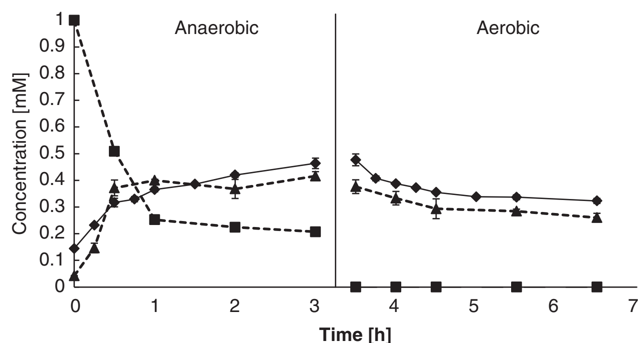


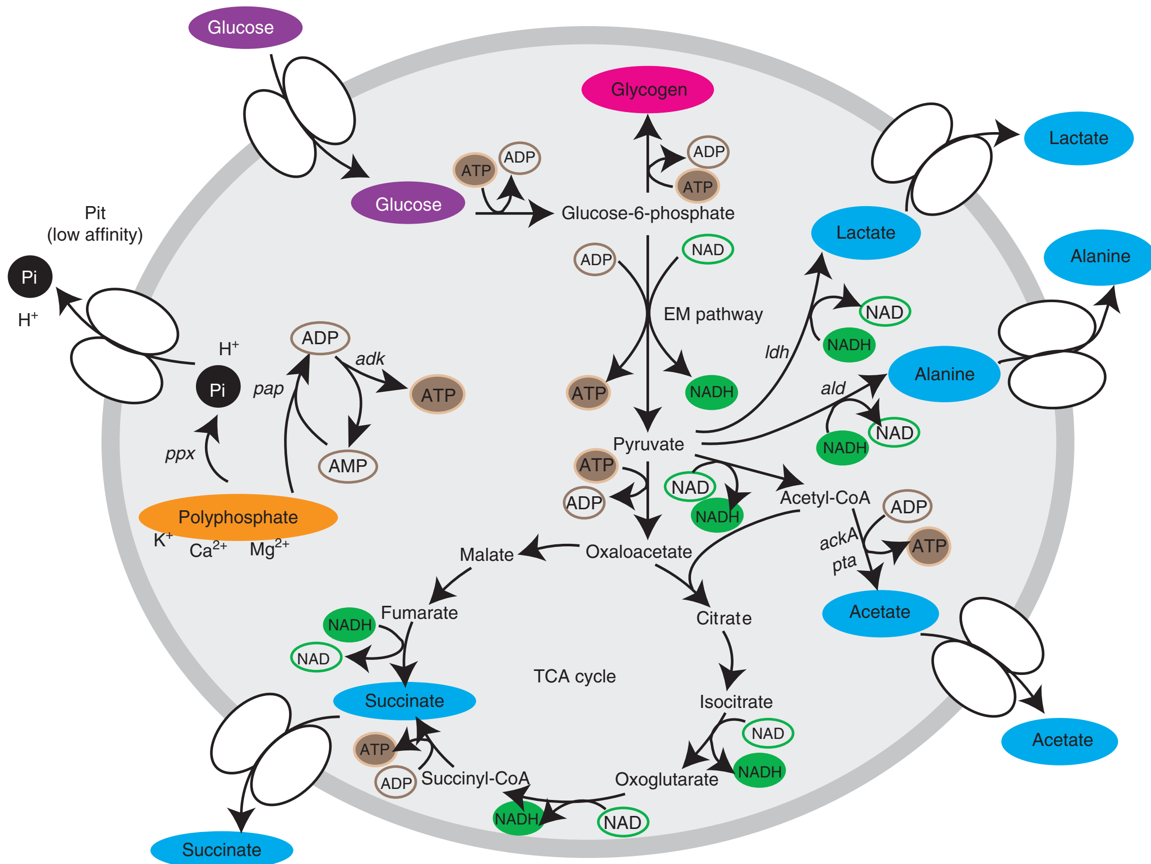
Figure 3 The ability of *T. elongata* to release Pi and consume glucose in the anaerobic phase (0–3 h) and take up Pi in the aerobic phase (3.5–6.5 h), and to produce and consume glycogen in the anaerobic and aerobic phases, respectively. The average concentration and standard deviation from duplicate experiments are shown for the Pi concentration of the media (diamonds) and glycogen content of the biomass (triangles). Media glucose concentration is also shown (squares).

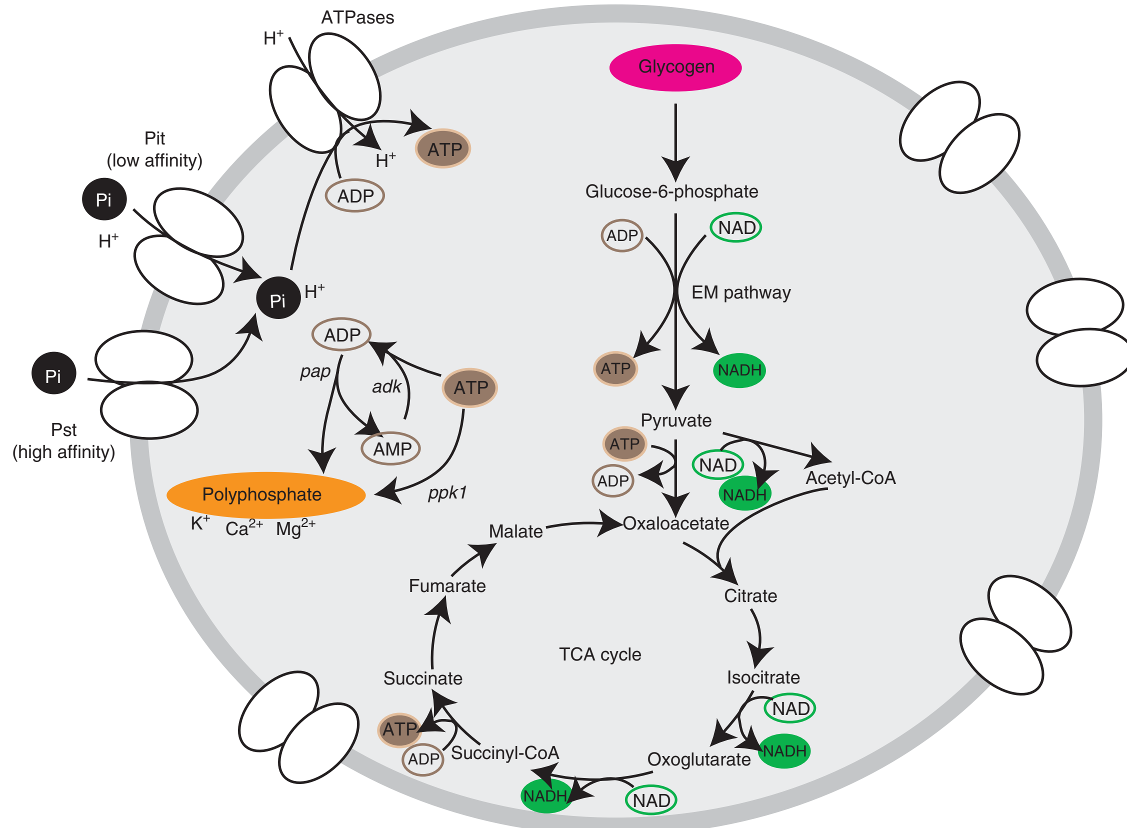
Developing a metabolic model for Tetrasphaera

Based on the genomic sequence data from these four *Tetrasphaera* isolates and experimental validation of pathways of special interest with *T. elongata* and *T. japonica*, we propose the following model for their metabolism in EBPR systems.

The genome sequence of *T. japonica* suggests it has the potential to synthesize PHA, glycogen and polyphosphate, and to reduce nitrate and nitrite, thus sharing key features of its metabolism with Accumulibacter (García Martín *et al*., 2006; He and McMahon, 2011). Furthermore, *T. japonica* has a fermentative ability, and thus can grow anaerobically. However, this species has not been detected by FISH in full-scale plants in Denmark (Nguyen *et al*., 2011), and so more data are needed from plants in other countries before its importance in EBPR can be properly assessed.

Figure 4 Metabolic model for *T. elongata*. The key metabolic pathways enabling *Tetrasphaera* to compete in full-scale EBPR plants are shown. (**a**) In the anaerobic phase, glucose is taken up and either stored as glycogen or fermented to acetate, lactate, succinate and alanine (highlighted in blue circles). The energy required for glycogen synthesis is supplied by fermentation and polyphosphate degradation to orthophosphate (Pi). (**b**) In the aerobic phase, the stored glycogen is degraded, supplying energy for growth and replenishing the polyphosphate stores.


a
ANAEROBIC PHASE
Glucose
Glucose
Glycogen
ADP
ATP
ATP
ADP
Glucose-6-phosphate
Lactate
Lactate
Alanine
Alanine
Pit
(low affinity)
Pi
H+
H+
Pi
pap
ADP
adk
ATP
AMP
ppx
Polyphosphate
K+ Ca2+ Mg2+
ADP
NAD
EM pathway
ATP
NADH
ldh
NAD
NADH
ald
NAD
NADH
Pyruvate
ATP
ADP
NAD
NADH
Acetyl-CoA
ADP
ATP
ackA
pta
Acetate
Acetate
Malate
Oxaloacetate
Fumarate
Citrate
NADH
NAD
Succinate
TCA cycle
Isocitrate
NAD
NADH
ATP
ADP
Succinyl-CoA
Oxoglutarate
NADH
NAD
Succinate


b
AEROBIC PHASE
ATPases
H+
H+
ATP
ADP
Glycogen
Glucose-6-phosphate
Pit
(low affinity)
Pi
H+
Pi
H+
Pi
Pst
(high affinity)
ADP
pap
adk
ATP
AMP
Polyphosphate
K+ Ca2+ Mg2+
ppk1
ADP
NAD
EM pathway
ATP
NADH
Pyruvate
ATP
ADP
NAD
NADH
Acetyl-CoA
Malate
Oxaloacetate
Fumarate
Citrate
TCA cycle
Succinate
Isocitrate
NAD
NADH
ATP
ADP
Succinyl-CoA
Oxoglutarate
NADH
NAD

The dominant *Tetrasphaera* in Danish full-scale EBPR plants belonging to clades 1 and 2 (*T. australiensis*, *T. elongata* and *T. jenkinsii*) seem unable to synthesize PHA anaerobically, but instead produce glycogen and polyphosphate *in situ*. They can also denitrify and ferment. These attributes agree with the analysis presented in this study and imply that these *Tetrasphaera* species are metabolically highly versatile. Consequently, they may be active during all stages of EBPR, being involved in key processes that contribute to efficient nutrient removal (Nielsen *et al.*, 2012). We propose in the model that these *Tetrasphaera* can grow and ferment glucose and produce glycogen under anaerobic conditions, and that the energy required for these anabolic reactions is obtained from fermentation and polyphosphate degradation (Figure 4). During the aerobic phase, where exogenous energy and carbon sources are considered to be in short supply, the stored glycogen is degraded to provide carbon and energy for growth, phosphate assimilation and polyphosphate formation. The fermentation capability that allows *Tetrasphaera* to grow under anaerobic conditions and the ability, like Accumulibacter, to store substrate under the carbon-rich anaerobic conditions, for later use in the carbon-deficient anoxic/aerobic periods, are the key physiological traits that potentially allow them to be so successful in EBPR plants.

The metabolic model proposed in this study should also provide a strong basis for future investigations into regulation of gene expression and the niche ecology of *Tetrasphaera* PAOs in EBPR systems. More detailed kinetic studies, expanding on the findings of this study, will be important in understanding the competition between the *Tetrasphaera* and other key organisms in activated sludge; such as the Accumulibacter PAO and the glycogen accumulating organisms. Although, the demonstrated ability of the *Tetrasphaera* PAO to ferment glucose and utilize amino acids indicates that they occupy a slightly different niche. Incorporation of such information into more comprehensive process models will also markedly advance our understanding of the structure and population dynamics of the EBPR community, and its functional stability, which is important if these biotechnological processes are to be operated more efficiently.

Conflict of Interest

The authors declare no conflict of interest.

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References

Akiyama M, Crooke E, Kornberg A. (1992). The polyphosphate kinase gene of *Escherichia coli*. Isolation and sequence of the ppk gene and membrane location of the protein. *J Biol Chem* **267**: 22556–22561.

Akiyama M, Crooke E, Kornberg A. (1993). An exopolyphosphatase of *Escherichia coli*. The enzyme and its ppx gene in a polyphosphate operon. *J Biol Chem* **268**: 633–639.

Albertsen M, Hansen LBS, Saunders AM, Nielsen PH, Nielsen KL. (2011). A metagenome of a full-scale microbial community carrying out enhanced biological phosphorus removal. *ISME J* **6**: 1094–1106.

APHA, AWWA, WPCF (1995). *Standard Methods for the Examination of Water and Wastewater*. Port City Press: Baltimore.

Barnard JL. (1975). Biological nutrient removal without the addition of chemicals. *Water Res* **9**: 485–490.

Bentley S. (2009). Sequencing the species pan-genome. *Nature Rev Microbiol* **7**: 258–259.

Blackall LL, Seviour EM, Bradford D, Rossetti S, Tandoi V, Seviour RJ. (2000). 'Candidatus Nostocoida limicola', a filamentous bacterium from activated sludge. *Int J Syst Evol Microbiol* **50**: 703–709.

Bond PL, Erhart R, Wagner M, Keller J, Blackall LL. (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.

Bonting CF, Kortstee GJ, Zehnder AJ. (1991). Properties of polyphosphate: AMP phosphotransferase of *Acinetobacter strain* 210A. *J Bacteriol* **173**: 6484–6488.

Braunegg G, Sonnleitner B, Lafferty RM. (1978). A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. *Eur J Appl Microbiol Biotechnol* **6**: 29–37.

Burow LC, Mabbett AN, Blackall LL. (2008). Anaerobic glyoxylate cycle activity during simultaneous utilization of glycogen and acetate in uncultured *Accumulibacter* enriched in enhanced biological phosphorus removal communities. *ISME J* **2**: 1040–1051.

Clark DP. (1989). The fermentation pathways of *Escherichia coli*. *FEMS Microbiol Rev* **5**: 223–234.

Comeau Y, Hall KJ, Hancock REW, Oldham WK. (1986). Biochemical model for enhanced biological phosphorus removal. *Water Res* **20**: 1511–1521.

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

Farrer RA, Kemen E, Jones JDG, Studholme DJ. (2009). De novo assembly of the *Pseudomonas syringae* pv.syringae B728a genome using Illumina/Solexa short sequence reads. *FEMS Microbiol Lett* **291**: 103–111.

Flowers JJ, He S, Yilmaz S, Noguera DR, McMahon KD. (2009). Denitrification capabilities of two biological phosphorus removal sludges dominated by different *'Candidatus Accumulibacter'* clades. *Environ Microbiol Rep* **1**: 583–588.

Fuhs GW, Chen M. (1975). Microbiological basis of phosphate removal in the activated sludge process for the treatment of wastewater. *Microb Ecol* **2**: 119–138.

García Martín H, Ivanova N, Kunin V, Warnecke F, Barry KW, McHardy AC *et al.* (2006). Metagenomic analysis

of two enhanced biological phosphorus removal (EBPR) sludge communities. *Nat Biotechnol* **24**: 1263–1269.
Hanada S, Liu WT, Shintani T, Kamagata Y, Nakamura K. (2002). Tetrasphaera elongata sp. nov., a polyphosphate-accumulating bacterium isolated from activated sludge. *Int J Syst Evol Microbiol* **52**: 883.
He S, Kunin V, Haynes M, Martin HG, Ivanova N, Rohwer F *et al.* (2010). Metatranscriptomic array analysis of *'Candidatus Accumulibacter phosphatis'*-enriched enhanced biological phosphorus removal sludge. *Environ Microbiol* **12**: 1205–1217.
He S, McMahon K. (2011). *'Candidatus Accumulibacter'* gene expression in response to dynamic EBPR conditions. *ISME J* **5**: 329–340.
Hesselmann RPX, Von Rummell R, Resnick SM, Hany R, Zehnder AJB. (2000). Anaerobic metabolism of bacteria performing enhanced biological phosphate removal. *Water Res* **34**: 3487–3494.
Ishige K, Zhang H, Kornberg A. (2002). A polyphosphate kinase (PPK2) widely conserved in bacteria. *Proc Natl Acad Sci USA* **99**: 16678–16683.
Kong Y, Nielsen JL, Nielsen PH. (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, Nielsen JL, Nielsen PH. (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, Xia Y, Nielsen PH. (2008). Activity and identity of fermenting microorganisms in full-scale biological nutrient removing wastewater treatment plants. *Environ Microbiol* **10**: 2008–2019.
Liu JR, McKenzie CA, Seviour EM, Webb RI, Blackall LL, Saint CP *et al.* (2001). Phylogeny of the filamentous bacterium *'Nostocoida limicola'* III from activated sludge. *Int J Syst Evol Microbiol* **51**: 195–202.
Louie TM, Mah TJ, Oldham W, Ramey WD. (2000). Use of metabolic inhibitors and gas chromatography/mass spectrometry to study poly-[beta]-hydroxyalkanoates metabolism involving cryptic nutrients in enhanced biological phosphorus removal systems. *Water Res* **34**: 1507–1514.
Maszenan AM, Seviour RJ, Patel BKC, Schumann P, Burghardt J, Tokiwa Y *et al.* (2000). Three isolates of novel polyphosphate-accumulating Gram-positive cocci, obtained from activated sludge, belong to a new genus, Tetrasphaera gen. nov., and description of two new species, Tetrasphaera japonica sp. nov. and Tetrasphaera australiensis sp. nov. *Int J Syst Evol Microbiol* **50**: 593–603.
McKenzie CM, Seviour EM, Schumann P, Maszenan AM, Liu JR, Webb RI *et al.* (2006). Isolates of *'Candidatus Nostocoida limicola'* Blackall *et al.* (2000) should be described as three novel species of the genus Tetrasphaera, as *Tetrasphaera* jenkinsii sp. nov. *Tetrasphaera vanveenii* sp. nov. and *Tetrasphaera veronensis* sp. nov. *Int J Syst Evol Microbiol* **56**: 2279–2290.
Mino T, van Loosdrecht MCM, Heijnen JJ. (1998). Microbiology and biochemistry of the enhanced biological phosphate removal process. *Water Res* **32**: 3193–3207.
Mino T, Tsuzuki Y, Matsuo T. (1987). Effect of phosphorus accumulation on acetate metabolism in the biological phosphorus removal process. *Adv Water Pollut Control* **27**: 27–38.
Murphy J, Riley JP. (1962). A modified single solution method for the determination of phosphate in natural waters. *Anal Chim Acta* **27**: 31–36.
Nakano MM, Dailly YP, Zuber P, Clark DP. (1997). Characterization of anaerobic fermentative growth of *Bacillus subtilis*: identification of fermentation end products and genes required for growth. *J Bacteriol* **179**: 6749–6755.
Nguyen HT, Le VQ, Hansen AA, Nielsen JL, Nielsen PH. (2011). High diversity and abundance of putative polyphosphate-accumulating Tetrasphaera-related bacteria in activated sludge systems. *FEMS Microbiol Ecol* **76**: 256–267.
Nielsen PH, Saunders AM, Hansen AA, Larsen P, Nielsen JL. (2012). Microbial communities involved in enhanced biological phosphorus removal from wastewater—a model system in environmental biotechnology. *Curr Opin Biotechnol* **23**: 452–459.
Oehmen A, Lemos PC, Carvalho G, Yuan Z, Keller J, Blackall LL *et al.* (2007). Advances in enhanced biological phosphorus removal: from micro to macro scale. *Water Res* **41**: 2271–2300.
Onda S, Takii S. (2002). Isolation and characterization of a Gram-positive polyphosphate-accumulating bacterium. *J Gen Appl Microbiol* **48**: 125–133.
Pellerin P, Gruson B, Prensier G, Albagnac G, Debeire P. (1987). Glycogen in Methanothrix. *Arch Microbiol* **146**: 377–381.
Pereira H, Lemos PC, Reis MA, Crespo JPS, Carrondo MJ, Santos H. (1996). Model for carbon metabolism in biological phosphorus removal processes based on *in vivo* 13C-NMR labelling experiments. *Water Res* **30**: 2128–2138.
Serafim LS, Lemos PC, Levantesi C, Tandoi V, Santos H, Reis MA. (2002). Methods for detection and visualization of intracellular polymers stored by polyphosphate-accumulating microorganisms. *J Microbiol Methods* **51**: 1–18.
Shiba T, Tsutsumi K, Ishige K, Noguchi T. (2000). Inorganic polyphosphate and polyphosphate kinase: their novel biological functions and applications. *Biochemistry (Mosc)* **65**: 315–323.
Simpson JT, Wong K, Jackman SD, Schein JE, Jones SJM, Birol I. (2009). ABySS: A parallel assembler for short read sequence data. *Genome Res* **19**: 1117–1123.
Sommer DD, Delcher AL, Salzberg SL, Pop M. (2007). Minimus: a fast, lightweight genome assembler. *BMC Bioinformatics* **8**: 64.
Stansen C, Uy D, Delaunay S, Eggeling L, Goergen JL, Wendisch VF. (2005). Characterization of a *Corynebacterium glutamicum* lactate utilization operon induced during temperature-triggered glutamate production. *Appl Environ Microbiol* **71**: 5920–5928.
Vallenet D, Labarre L, Rouy Z, Barbe V, Bocs S, Cruveiller S *et al.* (2006). MaGe: a microbial genome annotation system supported by synteny results. *Nucleic Acids Res* **34**: 53–65.
Vallenet D, Engelen S, Mornico D, Cruveiller S, Fleury L, Lajus A *et al.* (2009). MicroScope: a platform for microbial genome annotation and comparative

genomics. *Database (Oxford)* 2009: doi:10.1093/database/bap021.
Wexler M, Richardson DJ, Bond PL. (2009). Radiolabelled proteomics to determine differential functioning of Accumulibacter during the anaerobic and aerobic phases of a bioreactor operating for enhanced biological phosphorus removal. *Environ Microbiol* **11**: 3029–3044.
Williams TM, Unz RF. (1989). The nutrition of Thiothrix, Type 021N, Beggiatoa and Leucothrix strains. *Water Res* **23**: 15–22.
Willsky GR, Malamy MH. (1980). Characterization of two genetically separable inorganic phosphate transport systems in *Escherichia coli*. *J Bacteriol* **144**: 356–365.
Wishart DS, Knox C, Guo AC, Young N, Gautam B, Hau DD *et al.* (2009). HMDB: a knowledgebase for the human metabolome. *Nucleic Acids Res* **37**: D603–D610.
Zhou Y, Pijuan M, Zeng RJ, Yuan Z. (2009). Involvement of the TCA cycle in the anaerobic metabolism of polyphosphate accumulating organisms (PAOs). *Water Res* **43**: 1330–1340.

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