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Glycogen-accumulating organisms in laboratory-scale and full-scale wastewater treatment processes

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Laboratory-scale sequencing batch reactors (SBRs) as models for wastewater treatment processes were used to identify glycogen-accumulating organisms (GAOs), which are thought to be responsible for the deterioration of enhanced biological phosphorus removal (EBPR). The SBRs (called Q and T), operated under alternating anaerobic–aerobic conditions typical for EBPR, generated mixed microbial communities (sludges) demonstrating the GAO phenotype. Intracellular glycogen and poly-β-hydroxyalkanoate (PHA) transformations typical of efficient EBPR occurred but polyphosphate was not bioaccumulated and the sludges contained 1·8% P (sludge Q) and 1·5% P (sludge T). 16S rDNA clone libraries were prepared from DNA extracted from the Q and T sludges. Clone inserts were grouped into operational taxonomic units (OTUs) by restriction fragment length polymorphism banding profiles. OTU representatives were sequenced and phylogenetically analysed. The Q sludge library comprised four OTUs and all six determined sequences were 99·7% identical, forming a cluster in the *γ-Proteobacteria* radiation. The T sludge library comprised eight OTUs and the majority of clones were *Acidobacteria* subphylum 4 (49% of the library) and candidate phylum OP10 (39% of the library). One OTU (two clones, of which one was sequenced) was in the *γ-Proteobacteria* radiation with 95% sequence identity to the Q sludge clones. Oligonucleotide probes (called GAOQ431 and GAOQ989) were designed from the *γ-Proteobacteria* clone sequences for use in fluorescence *in situ* hybridization (FISH); 92% of the Q sludge bacteria and 28% of the T sludge bacteria bound these probes in FISH. FISH and post-FISH chemical staining for PHA were used to determine that bacteria from a novel *γ-Proteobacteria* cluster were phenotypically GAOs in one laboratory-scale SBR and two full-scale wastewater treatment plants. It is suggested that the GAOs from the novel cluster in the *γ-Proteobacteria* radiation be named '*Candidatus* Competibacter phosphatis'.

Keywords: GAOs, fluorescence *in situ* hybridization (FISH), wastewater treatment, EBPR

INTRODUCTION

Enhanced biological phosphorus removal (EBPR) has long been recognized for having the potential to cheaply and reliably remove soluble phosphorus (P) almost completely from wastewater. EBPR is often operated successfully in full-scale wastewater treatment plants but consistent failures resulting in release of P to the

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Abbreviations: CLSM, confocal laser scanning microscope/microscopy; COD, chemical oxygen demand; EBPR, enhanced biological phosphorus removal; FISH, fluorescence *in situ* hybridization; GAO, glycogen-accumulating organism; OTU, operational taxonomic unit; PAO, polyphosphate-accumulating organism; PHA, poly-β-hydroxyalkanoate; SBR, sequencing batch reactor; VFA, volatile fatty acid.

The GenBank accession numbers for the sequences reported in this paper are given in Methods.

environment do occur. Since the fundamental microbiology and biochemistry relevant to EBPR are poorly understood, the success of actions to remedy process failure is often erratic and unreliable (Mino *et al*., 1998).

For EBPR to occur, the sludge must be mixed with wastewater influent under anaerobic conditions. The mixture is cycled through an anaerobic zone followed by an aerobic zone and a sludge settlement zone. Polyphosphate-accumulating organisms (PAOs) release P from stored polyphosphate in the anaerobic zone and accumulate P as polyphosphate in excess of normal metabolic requirements in the aerobic zone (van Loosdrecht *et al*., 1997b). Removal of a portion of the growing biomass from the aerobic zone results in net removal of P from the wastewater. The carbon substrates utilized in EBPR are typically volatile fatty acids (VFAs) that are supplied to the sludge in the influent. PAOs take up the VFAs in the anaerobic zone and convert them to intracellular poly-β-hydroxyalkanoates (PHAs) using stored glycogen and polyphosphate for energy. In the aerobic zone, PAOs utilize their stored PHA for cellular growth, replenishment of their glycogen and uptake of P. It is thought that anaerobic uptake of influent carbon substrates like VFAs and storage as PHA and the anaerobic–aerobic polyphosphate cycling are the selective advantages for PAOs over other micro-organisms (van Loosdrecht *et al*., 1997a). One hypothesis for failure of EBPR is that non-PAOs successfully compete against PAOs for influent soluble carbon. The non-PAOs were called glycogen-accumulating organisms (GAOs) (Mino *et al*., 1995). GAOs have been reported to dominate deteriorated EBPR processes where glycogen and PHA transformations are similar to those during good EBPR but P is not transformed as in PAOs (e.g. Fukase *et al*., 1985; Satoh *et al*., 1994). Very little is known of GAOs apart from their phenotype.

The study of highly PAO-enriched laboratory-scale bioreactors allowed the understanding and identification of one confirmed PAO as '*Candidatus* Accumulibacter phosphatis', a close relative of *Rhodocyclus* in the *β-Proteobacteria* (Crocetti *et al*., 2000; Hesselmann *et al*., 1999) (henceforth called *Accumulibacter*). Studies into GAOs have been directed towards their identification (Dabert *et al*., 2001; Nielsen *et al*., 1999). However, there remain gaps in the knowledge of the identity of GAOs and there has been little attempt to directly demonstrate that putative GAOs have the GAO phenotype. In this study, we designed fluorescence *in situ* hybridization (FISH) probes from 16S rDNA clones from sludges demonstrating the GAO phenotype and evaluated them with laboratory-scale and full-scale sludges by FISH and post-FISH chemical staining for the intracellular polymers PHA and polyphosphate. Thus we have identified one GAO by linking its *in situ* identification with the GAO phenotype.

METHODS

Q and T sludge operation. A 2 litre laboratory-scale sequencing batch reactor (SBR) operated under anaerobic–aerobic cycling conditions and fed with synthetic wastewater influent generated the Q and the T sludges in Brisbane, Queensland, Australia, as previously reported for the Q sludge (Bond *et al*., 1998, 1999a); and for the T sludge (Bond *et al*., 1999b). For the Q sludge, the synthetic wastewater influent contained mineral salts, 700 mg $NaCH_3CO_2.3H_2O$ l^{-1} and 15 mg PO_4-P l^{-1} to give a chemical oxygen demand (COD) : P r1atio of 22 : 1. The SBR was operated in 6 h sequences comprising 2·5 h anaerobic (first 10 min feeding 1 l influent), 2·3 h aerobic and 1·2 h settling/decanting (decant volume of 1 l supernatant). For the T sludge, the 6 h sequence comprised 2 h anaerobic (first 10 min feeding), 3·5 h aerobic and 0·5 h settling/decanting. The influent was similar to that for the Q sludge. However, during a 3 week period (corresponding to three sludge residence times) prior to collection of the T sludge, the P in the influent was progressively lowered such that for a 1 week period and at T sludge collection time, the influent P was 2 mg PO_4-P l^{-1} and the COD : P ratio was 244 : 1.

To achieve anaerobic conditions, N_2 gas was bubbled through the liquid and to achieve aerobic conditions, air was bubbled. Sludge was wasted in the aerobic zone of each cycle to achieve a sludge age of 7–8 days. The reactors were operated at a temperature of 22 ± 2 °C. Regular weekly cycle studies (samples withdrawn each 10–15 min from the reactor in the anaerobic and aerobic zones) were conducted to confirm the sludge phenotype. Samples were analysed for intracellular glycogen and PHA, and supernatant P, COD and VFA. The sludge P content (P %) was also determined on the biomass collected at the end of the aerobic period when the stored polyphosphate would be maximal. The P % is calculated by $(P_t - P_e)/MLSS \times 100$, where P_t is the total sludge phosphate in mg l^{-1}, P_e is the phosphate in the effluent in mg l^{-1}, and MLSS is the mixed liquor suspended solids in mg l^{-1}. All chemical analytical methods are reported in Bond *et al*. (1999a).

Chemical staining. Sudan black B (for lipophilic inclusions including PHAs), methylene blue (for polyanions including polyphosphate) and Gram stains (Murray *et al*., 1994) were conducted on sludges. Samples were viewed by light microscopy on either a Nikon Microphot FXA microscope or a Zeiss Axiophot microscope. Images were captured via a cooled charge-coupled device connected to a PC and prepared in Adobe PhotoShop (Adobe Systems). Additionally the Nile blue A staining method (Ostle & Holt, 1982) was used to detect intracellular PHAs. For this stain, samples were viewed by epifluorescence microscopy on a Zeiss Axiophot microscope or by confocal laser scanning microscopy (CLSM), detailed later. Sudan black, methylene blue and Nile blue staining procedures are not quantitative but simply indicate storage polymers inside specific cells.

Clone library preparation and analysis. Separate bacterial 16S rDNA clone libraries were prepared from genomic DNA extracted from frozen-stored Q and T sludge. Briefly, DNA was extracted and purified (Burrell *et al*., 1998), primers 27f and 1492r (Lane, 1991) were used for PCR amplification of near-complete 16S rDNAs and amplified genes were cloned using a TA cloning kit (Invitrogen). Inserts from individual clones in each library were amplified and grouped into operational taxonomic units (OTUs) by restriction fragment length polymorphism (RFLP) analysis using methods previously described (Burrell *et al*., 1998) and restriction enzymes *Hin*PI and *Msp*I. OTU representatives were fully sequenced (Blackall, 1994) and 16S rDNA sequences of clones were compiled using the software package SeqEd (Applied Biosystems). Each compiled sequence was compared to those in publicly available databases by use of the Basic Local

Table 1. Oligonucleotide probes used in this study

Probe name	Probe sequence (5′–3′)	rRNA target site*	Specificity	% Formamide	Reference
EUB338†	GCTGCCTCCCGTAGGAGT	16S, 338–355	Many but not all *Bacteria*	0–70	Amann *et al.* (1990)
EUB338-II†	GCAGCCACCCGTAGGTGT	16S, 338–355	*Planctomycetales*	0–50	Daims *et al.* (1999)
EUB338-III†	GCTGCCACCCGTAGGTGT	16S, 338–355	*Verrucomicrobiales*	0–50	Daims *et al.* (1999)
ALF1b	CGTTCG(C/T)TCTGAGCCAG	16S, 19–35	*α-Proteobacteria*	20	Manz *et al.* (1992)
BET42a	GCCTTCCCACTTCGTTT	23S, 1027–1043	*β-Proteobacteria*	35	Manz *et al.* (1992)
GAM42a	GCCTTCCCACATCGTTT	23S, 1027–1043	*γ-Proteobacteria*	35	Manz *et al.* (1992)
HGC69a	TATAGTTACCACCGCCGT	23S, 1901–1918	*Actinobacteria*	25	Roller *et al.* (1994)
CF319a	TGGTCCGTGTCTCAGTAC	16S, 319–336	*Cytophaga–Flavobacterium* of *Bacteroidetes*	35	Manz *et al.* (1996)
PAO651	CCCTCTGCCAAACTCCAG	16S, 651–668	'*Candidatus* Accumulibacter phosphatis'	35	Crocetti *et al.* (2000)
GAOQ431‡	TCCCCGCCTAAAGGGCTT	16S, 431–448	'*Candidatus* Competibacter phosphatis' (see Fig. 1)	35	This study
GAOQ989‡	TTCCCCGGATGTCAAGGC	16S, 989–1006	'*Candidatus* Competibacter phosphatis' (see Fig. 1)	35	This study

* rRNA, *Escherichia coli* numbering (Brosius *et al.*, 1981).
† EUB338, EUB338-II and EUB338-III were used in a mixture called EUBMIX.
‡ Other data for the designed probes are: melting temperature, 64 °C; mol% G+C, 61.

Alignment Search Tool (BLAST, Altschul *et al.*, 1997) to determine approximate phylogenetic affiliations and detect sequences with high identity. The compiled sequences were aligned using the ARB software package (http://www.arb-home.de) and alignments were refined manually. Phylogenetic analysis of the sequences was by methods previously reported (Hugenholtz *et al.*, 2001b). Distance and parsimony methods were carried out in PAUP* version 4.0b2a, with and without corrections for rate variation and GC bias. The robustness of the tree topology was tested by bootstrap analysis with a range of outgroups (Dalevi *et al.*, 2001).

GenBank accession numbers. The EMBL accession numbers for the 15 sequences reported in this paper are as follows. Novel *γ-Proteobacteria* cluster: SBRQ171, AF361089; SBRQ185, AF361090; SBRQ191, AF361091; SBRQ157, AF361092; SBRQ196, AF361093; SBRQ152, AF361094; SBRH10, AF361095; SBRT185, AF361096. Candidate phylum OP10: SBRT152, AF368186; SBRT161, AF368187; SBRT197, AF368188; SBRT162, AF368189. *Acidobacteria*: SBRT166, AF368181. *α-Proteobacteria*: SBRT155, AF368183. *Bacteroidetes*: SBRT303, AF368190.

FISH and post-FISH chemical staining. Published probes and two designed probes (Table 1) were used in FISH (Amann, 1995; Manz *et al.*, 1992). Probes were commercially synthesized and 5′ labelled with either the fluorochrome FITC or with one of the sulfoindocyanine dyes Cy3 and Cy5 (ThermohybaidInteractiva, Ulm, Germany). The probe design tool of the ARB software package was used to design two probes from the clone library sequences (GAOQ probes in Table 1). The design parameters used were as described by Hugenholtz *et al.* (2001a, b). Probe sequences were subsequently confirmed for specificity using BLAST, commercially synthesized and evaluated and optimized for FISH with paraformaldehyde- and ethanol-fixed Q sludge and the pure cultures *Rhodocyclus purpureus* DSM168 [from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany] and *Desulfobulbus propionicus* DSM2032 (from DSMZ) using methods previously described (Crocetti *et al.*, 2000).

The confocal laser scanning microscope (CLSM) used for image capture was a Bio-Rad MRC-1024. The illumination source was a 15 mW argon/krypton laser (American Laser Corporation) with excitation peaks at 488 nm (blue), 568 nm (green) and 647 nm (red). The images were captured in three different photomultiplier tubes. A 560 nm long pass emission filter separated the green signal (FITC) from the red signal and a 640 nm short pass emission filter separated the far-red signal (from Cy5) from the near-red signal (from Cy3). The CLSM was controlled by an OS/2 PC running the Bio-Rad Laser-Sharp Software package. Images were collected and final image evaluation was done in Adobe PhotoShop. The green emission was presented in the green channel of the colour image, the red emission as the red channel and, by convention, far-red emission (Cy5) as the blue channel.

In some preparations, pot-FISH chemical staining was done. FISH was carried out and representative images captured, then the coverslip was removed from the slide, the mountant washed off and the chemical staining carried out. Fields photographed in FISH were relocated and rephotographed with the chemical stain. This procedure was first reported by Crocetti *et al.* (2000) for FISH and methylene blue staining to identify a PAO.

One laboratory-scale anaerobic–aerobic cycling SBR and four full-scale activated sludge mixed liquors (from Noosa, Loganholme, Gibson Island and Luggage Point Sewage Treatment Plants, South-East Queensland, Australia) were collected from the end of the anaerobic zone and the aerobic zone and fixed. They were FISH probed with EUBMIX and GAOQ431 (Table 1). The laboratory-scale SBR and two full-scale sludges (Noosa and Loganholme Sewage Treatment Plants) were post-FISH chemically stained for PHA and polyphosphate.

Microbial quantification. Methods used were essentially those reported previously (Bouchez *et al.*, 2000). Samples from both Q and T sludges were pipetted into thick smears on slides to achieve reasonable homogeneity of sample. FISH preparations on these samples were viewed on the CLSM. Twenty different fields of view were selected randomly in the *X*, *Y* and *Z* planes for each reported population measurement. Area measurements with all probes (labelled with Cy3 or Cy5; Table 1) were reported as a proportion of the area of all *Bacteria* in each field according to the probe set EUBMIX (labelled with FITC). Area measurements were performed on CLSM images using Image-Pro Plus (Version 4.0 for Windows; Media Cybernetics). The area of all pixels above a manually determined minimum pixel intensity was measured from the greyscale image of each probe in a field. The upper greyscale pixel intensity value remained constant at the maximum greyscale value of 255, thus allowing for the varying fluorescent signals from different populations of cells to be measured above the highest possible background and non-specific levels of fluorescence. The proportion of Cy5-labelled EUBMIX-binding cells to SYBR Green I (Molecular Probes)-binding cells was also determined (Schmid *et al.*, 2000).

RESULTS

Q and T sludge operation and chemical staining

Both the Q and the T sludges operated at deteriorated EBPR in that they did not release P anaerobically (Table 2) nor take it up aerobically. However, both sludges carried out carbon transformations typical of EBPR (Table 2) and therefore demonstrated the GAO phenotype.

For complete details of the Q sludge operation, Bond *et al.* (1998, 1999a) should be consulted. Briefly, the SBR was being operated to achieve EBPR and had been functioning for a period of 67 days with initially 30 days of erratic P removal, followed by consistent, meagre P removal. In weekly cycle studies during the meagre P removal, the SBR demonstrated anaerobic carbon transformations (rapid uptake of acetate, production of cellular PHA and degradation of glycogen) and aerobic carbon transformations (degradation of cellular PHA and production of glycogen) characteristic of EBPR, but not P transformations. The sludge P% was only 1·8%. After 31 days of consistent, meagre P removal (day 61), the Q sludge sample was taken from the end of the aerobic period, a portion was fixed and an aliquot frozen. The Q sludge was dominated by large clusters of Gram-negative coccobacilli approximately 2 μm × 2–3 μm in size. These cell types in this aerobic period sample did not contain polyphosphate or PHA, supporting an earlier characterization of the Q sludge (Bond *et al.*, 1999a). In addition, it had been previously reported that the abundant coccobacilli stored PHA anaerobically but they did not accumulate polyphosphate (Bond *et al.*, 1999a).

Table 2. Stoichiometry of transformations observed during the anaerobic period of the SBR cycle studies compared with theoretical values

Sludge type	Acetate uptake (mol)	Cell glycogen consumed (mol)	PHA* units produced (mol)	Phosphate released (mol)
EBPR†	6	1	4	6
Q sludge‡	6	2·6	4·4	0·3
T sludge§	6	2·3	5·0	0·1
Non-EBPR‖	6	2·5	5·5	0

* PHA is typically a mixture of poly-β-hydroxybutyrate and poly-β-hydroxyvalerate.

† Theoretical ratios for EBPR model (Smolders, 1995).

‡ From Bond *et al*. (1999a).

§ From Bond *et al*. (1999b).

‖ Theoretical ratios for non-EBPR model (Satoh *et al*., 1994).

The SBR from which the T sludge was obtained had been operating for 198 days, with the latter 88 days of operation showing good to excellent EBPR (Bond *et al*., 1999b). Beginning on day 198 and spanning a 3 week period, the P in the influent was progressively lowered from 30 mg PO_4-P l^{-1} to 14 mg l^{-1}, to 4 mg l^{-1} and then on day 219 to 2 mg PO_4-P l^{-1}. During this reduction, the P% in the sludge dropped from 12% to 1·5%, but carbon transformations (PHA and glycogen) remained essentially the same as during good EBPR. After approximately a week of operation at 2 mg PO_4-P l^{-1} in the influent (day 225), the T sludge sample was taken from the end of the aerobic period, a portion was fixed and an aliquot frozen. We observed a high proportion of both Gram-negative and Gram-positive cells in the T sludge. Two abundant morphotypes of Gram-negative cells were large cocci arranged in tetrads and clusters of coccobacilli (approx. 1·5 μm diameter). Cells in this aerobic period sample did not contain polyphosphate or PHA. It had been previously reported that many cell types but not the cocci in tetrads stored PHA anaerobically (Bond *et al*., 1999b).

Data for the stoichiometry of the anaerobic carbon and P transformations in both the Q and T sludges presented in Table 2 confirm the dominant GAO phenotype in these reactors due to their high similarity with model data for non-EBPR. Data for EBPR are presented for comparison.

Clone library analysis

By RFLP analysis, 50 clones from the Q sludge fell into four OTUs, and 53 T sludge clones produced eight OTUs.

Consistent with the morphological uniformness in the Q sludge micro-organisms, the clones in the library fell into only four OTUs, which all had very similar RFLP profiles. When representatives of these OTUs were sequenced (six near-complete inserts), the sequences were 99·7% identical and according to BLAST, their closest organismal match (88% identity) in GenBank was '*Nitrosococcus halophilus*' from the *γ-Proteobacteria* subphylum. Also highlighted by BLAST were sequences from three previously reported studies of wastewater treatment reactors (Dabert *et al*., 2001; Liu *et al*., 2000; Nielsen *et al*., 1999). Sequences reported in two studies (Liu *et al*., 2000; Nielsen *et al*., 1999) were very short due to the method of obtaining them, and although of limited value from a phylogenetic standpoint, were useful for discussion due to their relatively high percentage identity with our Q sludge sequences (approx. 93–95%). One sequence (PHOS-HE54, GenBank accession no. AF314424) from Dabert *et al*. (2001) was 1433 nt long and 94·4% identical to the Q sludge sequences, and was used in phylogenetic analysis. One further sequence from an unpublished sludge clone study (SBRH10) was 94·5% identical to the Q sludge clone sequences and was fully sequenced (GenBank accession no. AF361095) so that it could be included in the analysis. Fig. 1 shows the phylogenetic tree from the analysis of the Q sludge clone sequences. These sequences, along with SBRH10, PHOS-HE54, and a clone from the T sludge clone library (SBRT185) (detailed later) form one cluster with a mean of 96·7% identity (Fig. 1). In the phylogenetic analysis, we used many different outgroups (Dalevi *et al*., 2001) but could never confidently affiliate the Q sludge clone cluster with either the *β-Proteobacteria* or the well-known *γ-Proteobacteria* and this cluster always fell as an outlier of the *γ-Proteobacteria*. Thus, we concluded the Q sludge cluster was a monophyletic group well supported by bootstrap analysis and in the '*γ-Proteobacteria* radiation' (Fig. 1). The short sequences highlighted by BLAST were added to the tree using the parsimony insertion tool of ARB and are indicated with dashed lines.

The T sludge clone library was more complex than the Q library, with 53 clones from eight OTUs. A representative clone from each OTU was near-completely sequenced and comparative analysis enabled the clones to be placed into recognized phyla or recently described candidate phyla (Hugenholtz *et al*., 1998). One OTU containing two clones (SBRT185 was fully sequenced) was affiliated with the cluster of Q sludge clones in the '*γ-Proteobacteria* radiation' (Fig. 1), being 95%

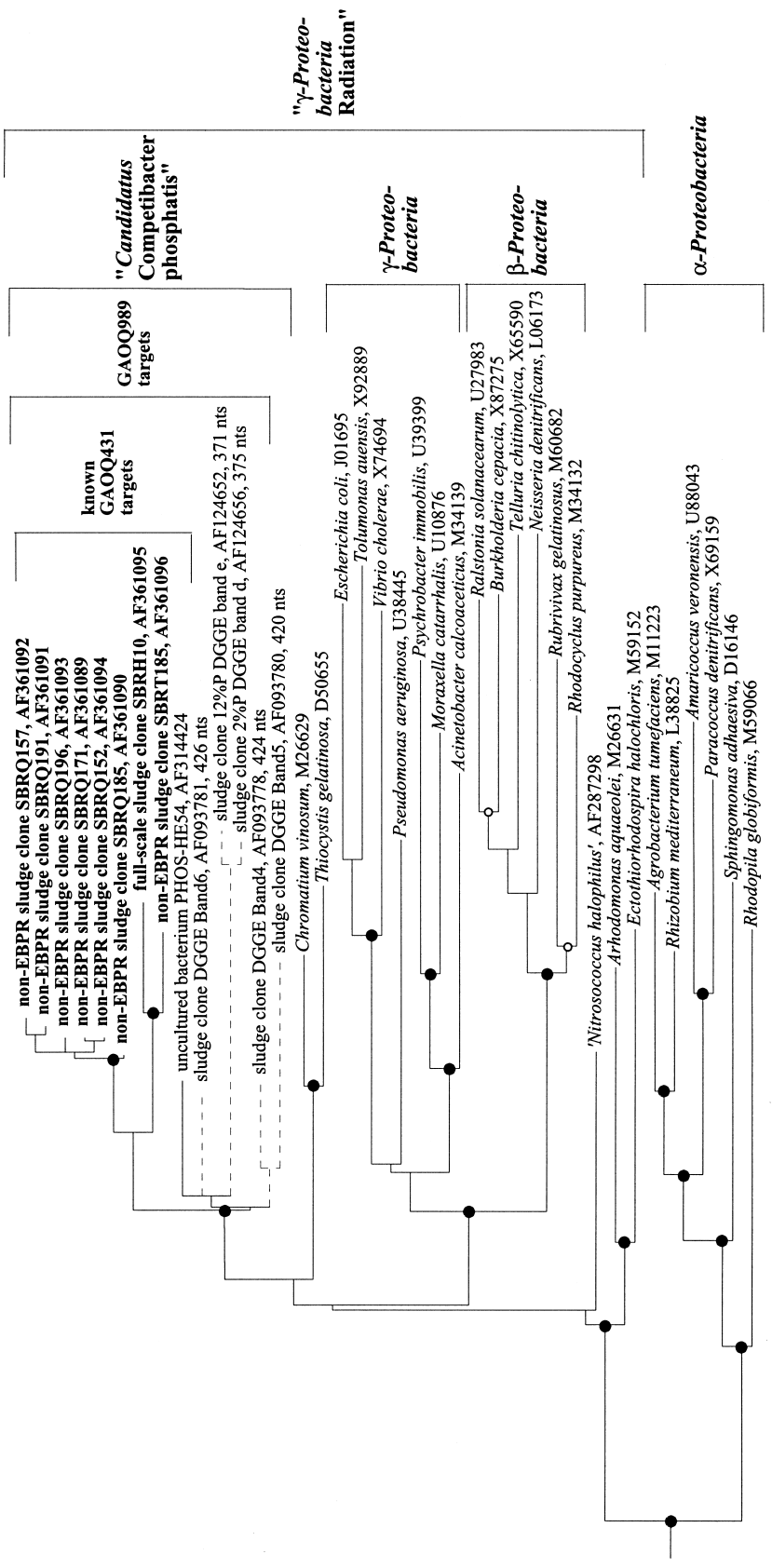

Fig. 1. For legend see facing page.

Table 3. Data for different bacterial groups in the Q and T sludges according to FISH

Bacterial phylum, subphylum or group	Probe (see Table 1)	FISH (% of EUBMIX binding to probe)	
		Q sludge*	T sludge†
α-Proteobacteria	ALF1b	2	28
β-Proteobacteria	BET42a	83	18
'*Candidatus* Accumulibacter phosphatis'	PAO651	2	3
γ-Proteobacteria	GAM42a	10	16
'*Candidatus* Competibacter phosphatis' (Fig. 1)	GAOQ431	92	28
Actinobacteria	HGC69a	6	34
Cytophaga–Flavobacterium of *Bacteroidetes*	CF319a	ND	4

ND, Not done.

* A total of 91% of SYBR Green-positive cells bound EUBMIX in Q sludge.

† A total of 84% of SYBR Green-positive cells bound EUBMIX in T sludge.

identical to them. One OTU was affiliated with the *Acidobacteria* subphylum 4 (26 clones; 49% of library; SBRT166, GenBank AF368181), four OTUs were affiliated with candidate phylum OP10 (21 clones; 39% of library; 1 representative from each OTU was sequenced), one OTU was in the *α-Proteobacteria* subphylum (1 clone; SBRT155, GenBank AF368183), and one OTU was in the *Bacteroidetes* phylum (3 clones; SBRT303, GenBank AF368190).

Only the sequences from Q and T sludge in the '*γ-Proteobacteria* radiation' were considered further in this paper.

Probe design and use with Q and T sludge

Two probes called GAOQ431 and GAOQ989 were designed to target the eight sequences from Q and T sludges, and SBRH10 and PHOS-HE54 in the *γ-Proteobacteria* cluster (Table 1, Fig. 1). Conditions for their use were optimized with Q sludge as a positive control. For GAOQ431, *Rhodocyclus purpureus* (2-base mismatch) was used as a negative control, while for GAOQ989, *Desulfobulbus propionicus* (2-base mismatch) was the negative control. For GAOQ431, there are strains with a 1-base mismatch and therefore the specificity of this probe might not be perfect. Results with Q sludge for both probes were identical and quantification was only done with GAOQ431. GAOQ431 specifically bound 92% of the EUBMIX-positive cells in the Q sludge (Table 3) and they were large coccobacillus-shaped bacteria. Some 88% of these GAOQ431-binding cells also bound the BET42a probe (for *β-Proteobacteria*) and 11% bound the GAM42a probe (for *γ-Proteobacteria*) (Fig. 2a). Other results for FISH with the Q sludge are presented in Table 3.

GAOQ431 was used with the T sludge, where 28% of EUBMIX-positive cells bound this probe (Table 3) and their morphology was similar to the abundant GAOQ431-binding cells in the Q sludge (Fig. 2b). About half of the GAOQ431-binding cells in the T sludge also bound BET42a and the other half bound GAM42a. Other results for FISH with the T sludge are shown in Table 3.

FISH with GAOQ431 and post-FISH chemical staining for PHA (Sudan black B) and polyphosphate (methylene blue) demonstrated that GAOQ431-binding coccobacilli in Q and T aerobic sludges did not contain PHA or polyphosphate.

Probing of laboratory-scale and full-scale activated sludges

The sludge from a laboratory-scale SBR operating in consistent deteriorated EBPR mode contained numerous GAOQ431-binding cells with intracellular PHA at the end of the anaerobic zone (Fig. 2c, d) but lacking PHA at

Fig. 1. Evolutionary distance dendrogram of *α-Proteobacteria*, *β-Proteobacteria* and *γ-Proteobacteria* based on phylogenetic analyses of 16S rDNA data where 1335 nt are compared. The '*γ-Proteobacteria* radiation' is marked and includes the novel cluster (labelled '*Candidatus* Competibacter phosphatis') of non-EBPR sludge clones including PHOS-HE54 from Dabert *et al.* (2001). Short sequences from denaturing gradient gel electrophoresis (DGGE) bands 4, 5 and 6 (420–426 nt), and d and e (375 and 371 nt, respectively) from previous studies were added to the tree by the parsimony insertion tool of ARB and are indicated by dashed lines. Subphylum designations are bracketed on the right and specificities of GAOQ431 and GAOQ989 probes are indicated. Branch points supported (bootstrap values, >74%) by all inference methods used are indicated by solid circles, and those supported by one inference method are indicated by open circles. Branch points without circles were not resolved (bootstrap values from all analyses <75%). Clones sequenced in the present study are in boldface. Outgroups (not shown) were *Synergistes jonesii* and *Deferribacter thermophilus*. The bar represents 10% estimated sequence divergence.

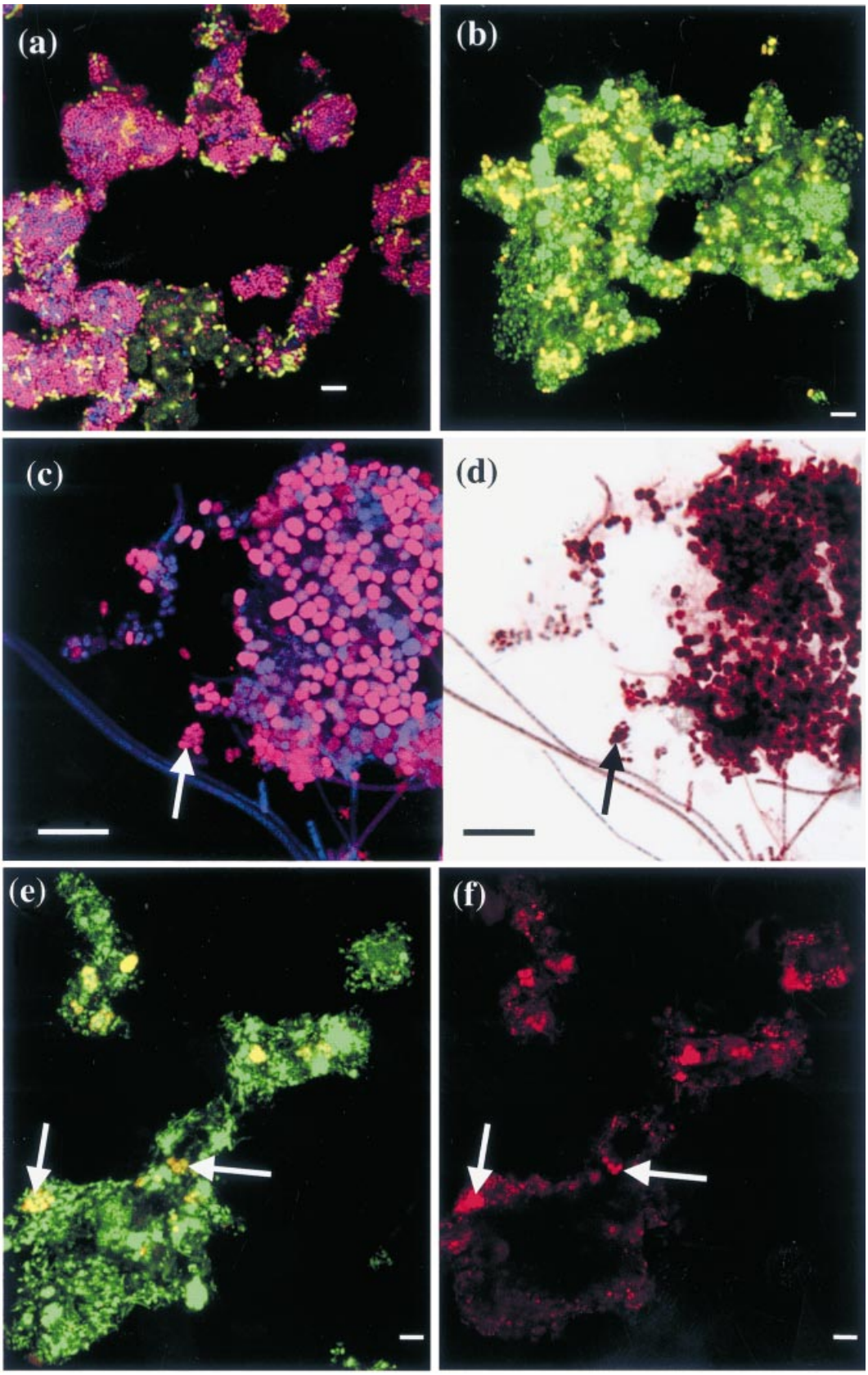

Fig. 2. For legend see facing page.

the end of the aerobic zone (data not shown). Of the four full-scale plants, Loganholme and Noosa Sewage Treatment Plants were operating for EBPR. All four plants contained GAOQ431-binding cells, and in both EBPR plants, post-FISH chemical staining for PHA demonstrated the EBPR anaerobic–aerobic cycling of this polymer in GAOQ431-binding cells. FISH (Fig. 2e) and post-FISH (Fig. 2f) Nile blue A staining results for anaerobic Noosa sludge demonstrate this.

DISCUSSION

Reactors demonstrate the GAO phenotype

The GAO phenotype occurs when mixed microbial communities have the capacity to take up soluble carbon substrates anaerobically and store them as PHAs, while concomitantly using stored glycogen. In the aerobic phase, GAOs utilize intracellular PHAs and produce intracellular glycogen. GAOs do not cycle P according to EBPR, and therefore they cannot perform the anaerobic P release and aerobic P uptake characteristic of PAOs. It is hypothesized that GAOs in EBPR processes can compete with PAOs for soluble carbon, thus adversely affecting the P removal process (Mino *et al*., 1995; Satoh *et al*., 1994). The identification of GAOs is important, so that diagnostic procedures for them can be devised; and if pure cultures of them could be obtained, their phenotype could be better studied. The Q sludge reactor clearly demonstrated the GAO phenotype (see Table 2) over a sustained time period and was probably enriched for GAOs. The T sludge reactor also displayed a GAO phenotype (Table 2), but its microbial community was probably in transition from one dominated by PAOs to one dominated by GAOs due to the fact that only 2 weeks of operation at very low influent P had elapsed.

GAOs are identified in laboratory-scale and full-scale sludges

The Q and T sludges were chosen to generate 16S rDNA sequences because they demonstrated the GAO phenotype and were therefore a good source of GAO sequences from which specific FISH oligonucleotides could be designed. Bond *et al*. (1999a) previously described the highly abundant clusters of Gram-negative coccobacilli anaerobically storing PHA granules but not polyphosphate in the Q sludge. The abundance of GAOQ431-binding cells in the Q sludge (92%) was combined with the conspicuous GAO phenotype of this reactor (Table 2 and Bond *et al*., 1999a) to draw a correlation between the GAOQ431-binding cells and the GAO phenotype. GAO phenotype was demonstrated in the T sludge (Table 2 and Bond *et al*., 1999b), where the biodiversity by microbial morphology and FISH (Table 3) was greater than that in the Q sludge. GAOQ431-binding cells were present at 28% in the T sludge; their morphology was similar to the GAOQ431-binding cells in the Q sludge and they demonstrated some of the GAO phenotype (no PHA or polyphosphate at the end of the aerobic phase). Therefore, we suggest they were probably one of the GAOs in this sludge, but others could have been GAOs.

A laboratory-scale SBR operating in deteriorated EBPR and two full-scale EBPR processes (Loganholme and Noosa) were confirmed to contain GAOQ431-binding cells which stored PHA anaerobically and utilized it aerobically. However, in these sludges, GAOQ431-binding cells did not accumulate polyphosphate aerobically. Thus GAOQ431-binding cells were strongly suggested to be GAOs. Other organisms in the sludges also contained PHA (Fig. 2c–f) and possibly transformed it in accordance with EBPR (anaerobic production and aerobic utilization) but we only demonstrated this feature in GAOQ431-binding cells.

Relationship between our results and those of other GAO studies

One near-complete (Dabert *et al*., 2001) and five partial (Liu *et al*., 2000; Nielsen *et al*., 1999) 16S rDNA sequences from other sludge studies were found to be highly identical to sequences in our *γ-Proteobacteria* cluster shown in Fig. 1. Two of the studies (Dabert *et al*., 2001; Nielsen *et al*., 1999) aligned the organisms with poor EBPR and in all three studies (Dabert *et al*.,

Fig. 2. CLSM micrographs of FISH and Nile blue A staining (a–c; e and f) and a bright-field micrograph of a Sudan black B stain (d) of mixed liquors from laboratory-scale sequencing batch reactors and from a full-scale EBPR process. Probes indicated are described in Table 1. Bars (in all images), 10 μm. (a) FISH of aerobic zone Q sludge with GAOQ431 (Cy3), GAM42a (FITC) and BET42a (Cy5). Yellow cells are *γ-Proteobacteria* (11% GAOQ431-binding cells) *Competibacter* and magenta cells are *β-Proteobacteria* (88% of GAOQ431-binding cells) *Competibacter* in the Q sludge. (b) FISH of aerobic zone T sludge with GAOQ431 (Cy3) and EUBMIX (FITC). Yellow cells (28% of bacteria) have bound both probes in the T sludge. (c) FISH of anaerobic zone laboratory-scale, non-EBPR SBR sludge with GAOQ431 (Cy3) and EUBMIX (FITC – but coloured blue in this image). Magenta cells have bound both probes. (d) Anaerobic-zone laboratory-scale, non-EBPR SBR sludge stained with Sudan black B for PHAs, which appear as black inclusions in red cells. Cells lacking PHAs are red. The images in (c) and (d) are of the same field and were obtained by capturing a CLSM image from FISH, then a bright-field microscopy image of the same field from post-FISH Sudan black B staining (see Methods). Examples of GAOQ431-binding cells (magenta in c) containing PHAs (black in d) are shown by the arrows. (e) FISH of anaerobic zone Noosa Sewage Treatment Plant sludge with GAOQ431 (Cy3) and EUBMIX (FITC). Yellow cells have bound both probes. (f) Anaerobic zone Noosa Sewage Treatment Plant sludge stained with Nile blue A for PHAs, which are red in this image. The images in (e) and (f) are of the same field and were obtained by capturing an image from FISH, then an image of the same field from post-FISH Nile blue A staining (see Methods). Examples of GAOQ431-binding cells (yellow in e) containing PHAs (red in f) are shown by the arrows.

2001; Liu *et al*., 2000; Nielsen *et al*., 1999), sequences with high identity to our *γ-Proteobacteria* sequences were found in deteriorated EBPR reactors. Although there is corroboration between all these studies, ours markedly extends the knowledge by generating eight near-complete 16S rDNA sequences, adding to the one currently available, near-complete sequence (Dabert *et al*., 2001), thus allowing precise phylogenetic analysis (Fig. 1) and extensive probe design capacity.

Nielsen *et al*. (1999) designed FISH probes (GAM1019 and GAM1278) to be specific for the novel *γ-Proteobacteria* cluster from sequences only 420 nt in length. These probes have one or more mismatches to most of the near-complete sequences in this cluster shown in Fig. 1. Probe GAOQ989 targets with no mismatches all the near-complete sequences and all but one (AF093780) of the partial sequences in the novel *γ-Proteobacteria* cluster (Fig. 1). Probe GAOQ431 targets with no mismatches all the near-complete sequences but none of the partial sequences, due to the fact that none of them extend to this part of the 16S rDNA. However, GAM1019 and GAM1278 are likely to be of use to target different populations within the novel *γ-Proteobacteria* cluster and their abundance would also be relevant in the study of GAOs.

In our study, we have directly linked cells with aspects of the GAO phenotype (PHA cycling and not polyphosphate cycling) with their phylotype, and have therefore been able to determine the *in situ* morphology of GAOs. Previous studies also attempted this but they were less convincing due to the use of quite narrow-specificity probes for the *γ-Proteobacteria* cluster (Liu *et al*., 2001; Nielsen *et al*., 1999). Additionally, we used FISH and post-FISH chemical staining to demonstrate that GAOQ431-binding cells were GAOs in one laboratory-scale deteriorated EBPR SBR and two full-scale EBPR plants, establishing the utility of GAOQ431 probe in understanding deterioration of EBPR. The determination of GAO competition for VFAs with PAOs should now be determined in full-scale processes.

The phylogeny of the *γ-Proteobacteria* GAOs (Fig. 1) and the FISH results with GAOQ431 warrant further comment. The GAOQ431 and GAOQ989 probes were designed from sequences forming a highly supported monophyletic lineage (Fig. 1), but in the Q sludge 88 % of the GAOQ431-binding cells bound BET42a (for *β-Proteobacteria*) and 11 % bound GAM42a (for *γ-Proteobacteria*) (Fig. 2a). The BET42a and GAM42a probe targets are in the 23S rRNA and only differ from each other by one central nucleotide (Table 1). There are no pure cultures of the *γ-Proteobacteria* GAOs and there is no information on their 23S rDNA sequences. Other researchers (Liu *et al*., 2001; Nielsen *et al*., 1999) also reported probing inconsistencies where only a portion of the *γ-Proteobacteria* GAOs also bound the GAM42a probe. However, BET42a was not simultaneously used in these studies. Thus, elucidation of the reason why the Q sludge contains mostly *β-Proteobacteria* GAOs and fewer *γ-Proteobacteria* GAOs awaits information on the BET42a–GAM42a target region of the 23S rDNA for these organisms.

Factors favouring GAOs and PAOs

The complex interactions that lead to the selection of different micro-organisms in a wastewater treatment system are not well understood. When we used conditions that should select for PAOs (Q sludge operation), GAOs predominated. However, *Accumulibacter* was present at very low levels (2 % in Q sludge; Table 3) and although this organism has the ability to cycle P and carbon according to EBPR, it was out-competed in this experiment by the novel *γ-Proteobacteria* functioning as GAOs. It could be that competition for carbon was the deciding factor in which organism would predominate, as proposed previously (Mino *et al*., 1998). We found the novel *γ-Proteobacteria* in all four full-scale activated sludges examined, and in two EBPR plants demonstrated they had aspects of the GAO phenotype. We have also found that *Accumulibacter* are common in full-scale activated sludges and in EBPR processes they are PAOs (data not shown). We hypothesize that most activated sludges will contain both the novel *γ-Proteobacteria* and *Accumulibacter* but the reason why one or the other predominates in EBPR conditions (anaerobic–aerobic cycling) must now be determined.

Proposal for '*Candidatus* Competibacter phosphatis'

On several occasions we have attempted isolation to pure culture of the novel *γ-Proteobacteria* reported in this paper but have not been successful (results not shown). Thus, we propose that the organisms from which the novel *γ-Proteobacteria* cluster sequences originated be named '*Candidatus* Competibacter phosphatis' (see Fig. 1 for sequences encompassed), due to their ability to compete with polyphosphate-accumulating organisms in EBPR processes.

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