Ecophysiology of different filamentous *Alphaproteobacteria* in industrial wastewater treatment plants

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The ecophysiology of five filamentous species affiliated to the *Alphaproteobacteria* was investigated in industrial activated sludge systems. The five species, '*Candidatus Alysiosphaera europaea*', '*Candidatus Monilibacter batavus*', '*Candidatus Alysiumicrobium bavaricum*', '*Candidatus Sphaeronema italicum*' and *Meganema perideroedes*, are very abundant in industrial wastewater treatment plants and are often involved in bulking incidents. The morphology of these filamentous bacterial species resembled Eikelboom's *Nostocoida limicola*, or Type 021N, and could only be correctly identified by using fluorescence *in situ* hybridization (FISH), applying species-specific gene probes. Two physiological groupings of the five species were found using microautoradiography combined with FISH. Group 1 ('*Ca. Monilibacter batavus*' and '*Ca. Sphaeronema italicum*') utilized many short-chained fatty acids (acetate, pyruvate and propionate), whereas Group 2 ('*Ca. Alysiosphaera europaea*', '*Ca. Alysiumicrobium bavaricum*' and *Meganema perideroedes*) could also exploit several sugars, amino acids and ethanol. All species had polyhydroxyalkanoate granules present and several of the species had a very large storage capacity. No activity was found under strict anaerobic conditions, while uptake of substrate was observed in the presence of nitrate or nitrite as potential electron acceptor. However, for all species a reduced number of substrates could be consumed under these conditions compared to aerobic conditions. Only a little exo-enzymic activity was found and nearly all species had a hydrophobic cell surface. Based on knowledge of the ecophysiological potential, control strategies are suggested.

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INTRODUCTION

Extensive growth of filamentous bacteria is a serious problem in municipal and industrial wastewater treatment plants (WWTPs). The filamentous population can cause bulking and foam formation, leading to deterioration of the settling properties of the sludge. Attempts to identify the most common filamentous bacteria have resulted in classification manuals based on morphological characteristics (Eikelboom & Geurkink, 2002; Eikelboom & van

Buijsen, 1983; Jenkins *et al.*, 1993, 2004) and on molecular methods, mainly involving development of specific oligonucleotides for identification by fluorescence *in situ* hybridization (FISH) (Amann, 1995; Kanagawa *et al.*, 2000; Wagner *et al.*, 1994).

Filamentous *Alphaproteobacteria* have been shown to be very important in industrial WWTPs where they are often associated with bulking incidents or deteriorating settling properties of the sludge (Levantesi *et al.*, 2004; van der Waarde *et al.*, 2002). The filamentous *Alphaproteobacteria* have been observed and described in several studies in the past 4 years (Dionisi *et al.*, 2002; Eikelboom & Geurkink, 2002; Levantesi *et al.*, 2004; Thomsen *et al.*, 2006; van der Waarde *et al.*, 2002) and, based on 16S rRNA gene sequences from six

Abbreviations: ELF, enzyme-labelled fluorescence; FISH, fluorescence *in situ* hybridization; MAC, microsphere adhesion to cells; MAR, microautoradiography; PHA, polyhydroxyalkanoate; SS, suspended solids; WWTP, wastewater treatment plant.

uncultured bacteria and one isolated species, they have now been described as constituting seven distantly related phylogenetic clusters: '*Candidatus* Combthrix italica', '*Candidatus* Catenimonas italica', '*Candidatus* Sphaeronema italicum', '*Candidatus* Alysiosphaera europaea', '*Candidatus* Monilibacter batavus', '*Candidatus* Alysio-microbium bavaricum' (Levantesi *et al.*, 2004) and *Meganema perideroedes* (Thomsen *et al.*, 2006). Oligo-nucleotide probes for detection of these species by FISH are also available (Levantesi *et al.*, 2004; Thomsen *et al.*, 2006).

A FISH survey applying species-specific gene probes of the filamentous *Alphaproteobacteria* has been conducted on samples originating from 86 industrial WWTPs in Denmark, the Netherlands, Germany and Italy (Levantesi *et al.*, 2004). The most common species was '*Ca. Alysiosphaera europaea*', which was seen in approximately 24 % of the WWTPs investigated, followed by '*Ca. Alysio-microbium bavaricum*' (16 %) '*Ca. Monilibacter batavus*' (10 %), '*Ca. Sphaeronema italicum*' (10 %) and *Meganema perideroedes* (2 %). '*Ca. Combthrix italica*' and '*Ca. Catenimonas italica*' were only rarely observed. In total, the *Alphaproteobacteria* were found in 66 % of all samples and the above-mentioned species were the dominant filamentous species in approximately 25 % of the samples, clearly emphasizing their importance in industrial WWTP. Unidentified filamentous *Alphaproteobacteria* in the samples were estimated to constitute 15 % based on hybridization with the group-specific alpha probe (ALF968) (Levantesi *et al.*, 2004).

Several attempts to isolate and grow these filamentous *Alphaproteobacteria* have largely failed and currently little information on their physiology is available. Such knowledge is of major importance for developing efficient control strategies. At present only isolates of *Meganema perideroedes* exist in pure culture (Levantesi *et al.*, 2004; Thomsen *et al.*, 2006), but isolates of '*Ca. Alysiosphaera europaea*' coexisting with a yeast (*Debaryomyces hansenii*) are available (Snaird *et al.*, 2002). The physiological properties of *Meganema perideroedes* have been examined in pure culture (Levantesi *et al.*, 2004; Thomsen *et al.*, 2006) and data on the ecophysiology of *Meganema perideroedes* have been obtained (Kragelund *et al.*, 2005). This filamentous bacterium is a very versatile consumer of a variety of organic substrates under aerobic conditions and it has a very large storage capacity, measured as polyhydroxyalkanoates (PHAs) formed from different substrates in the presence of different electron acceptors (e-acceptors). Denitrification was not observed in pure culture, although nitrate could be reduced to nitrite by some isolates. However, studies on ecophysiology showed that different substrates were taken up and stored under different redox conditions (Kragelund *et al.*, 2005), demonstrating the use of nitrate and nitrite as e-acceptors. These data indicate that *Meganema perideroedes* is most probably able to perform denitrification (Kragelund *et al.*, 2005).

The purpose of this study was to carry out a comprehensive ecophysiological investigation of the five most important

filamentous alphaproteobacterial species known to date. Ecophysiological data on *Meganema perideroedes* have been included (Kragelund *et al.*, 2005) and the different alphaproteobacterial species have been compared to find common physiological traits within this group with the aim of developing potential control strategies.

METHODS

Activated sludge sampling. The experiments were carried out with sludge from seven different industrial WWTPs in Denmark, Italy and the Netherlands. For wastewater treatment plant details, see Table 1. In general, most of the industrial sites only carried out organic carbon removal, nitrification and chemical phosphorus removal. The temperature range in the plants was from 20 to 40 °C and the sludge age was between 12 and 37 days.

The activated sludge was collected the day before the experiments, kept on ice and sent to Aalborg, Denmark, by express mail. The sludge was diluted with filtered nitrate- and nitrite-free sludge water to a final concentration of 1 g suspended solids (SS) l⁻¹ before performing experiments.

Morphological description. The filamentous bacteria present in the sludge were morphologically identified using the Eikelboom classification system for municipal and industrial WWTPs (Eikelboom, 2002; Eikelboom & Geurkink, 2002; Eikelboom & van Buijsen, 1983). Morphological identification involved the use of phase-contrast microscopy, and Gram-, Neisser- and PHA-staining properties (Eikelboom & van Buijsen, 1983; Ostle & Holt, 1982).

FISH and gene probe design. FISH was applied using group- and species-specific 16S rRNA-targeted nucleic acid probes; their details are described in probeBase (Loy *et al.*, 2003) and are depicted in Table 2. The probes were labelled with 5(6)-carboxyfluorescein-N-hydroxy-succinimide ester (FLUOS) or with the sulfoindocyanine dyes (Cy3 and Cy5) (ThermoHybaid Interactive). The FISH procedure was conducted as described elsewhere (Amann, 1995). A confocal laser scanning microscope (LSM 510; Carl Zeiss) equipped with a UV laser (351 and 364 nm), an Ar ion laser (458 and 488 nm) and two HeNe lasers (543 and 633 nm) was used to record fluorescent signals from the gene probes. Light microscopy was used to assess silver grain density from the microautoradiography (MAR) procedure. FISH was combined with other *in situ* methods as listed below and modification of procedures can be found elsewhere (Kragelund *et al.*, 2005). An overview of theoretical perfect-match organisms targeted by the different probes is shown in Fig. 1. New more specific probes were constructed for '*Ca. Alysio-microbium bavaricum*' and for '*Ca. Sphaeronema italicum*' as the ecophysiological investigation suggested that the existing probes for these two species were unspecific and hybridized with more than one species. Therefore, the specificity of the two probes was investigated using Probe Match (<http://rdp.cme.msu.edu/index.jsp>) and this confirmed that more sequences were targeted than just '*Ca. Alysio-microbium bavaricum*' and '*Ca. Sphaeronema italicum*' sequences. Probe PPx3-1428 was also found to target sequences within other phyla, e.g. *Deltaproteobacteria* (1), *Firmicutes* (15) and *Actinobacteria* (3). Probe Sita-649 targeted unclassified sequences within the *Proteobacteria* (1) and *Firmicutes* (2). A hierarchical approach was therefore used, where the group-specific probe (ALF968) was applied together with more specific probes, which ensured that only filamentous *Alphaproteobacteria* were investigated. These theoretical probe match searches, however, indicated that it is very likely that two probes, Sita-649 and PPx3-1428, could hybridize with more than one species of filamentous *Alphaproteobacteria* and therefore additional probes were designed.

Table 1. Overview of wastewater treatment plants

(+) , Only during summer months.

WWTP	Type of industry	Nitrification	Denitrification	Phosphorous removal (biological/chemical)	Temperature of process tank (°C)	Sludge age (days)	Selector present
TNO43	Brewery	+	+	—	20	?	—
CNR1	Tannery	+	+	Ch	18–25	15	—
TNO18	Chemical	+	+	—	20–30	30	+*
AAU12	Paper	+	—†	Ch	41	25	Anox. nitrate
AAU23	Yeast and ethanol	+	+	B	0–25	37	—
BIO6	Potato	—	—	—	?	?	—
AAU20	Pharmaceutical	(+)	(+)	Ch	10–35	7–10	—

*Incorrectly designed aerobic selector with no aeration.

†Only in anoxic selector.

The new probes enabled a distinction between at least two species in the two populations in the different WWTP samples investigated. The probes were designed using the probe design software in ARB (Ludwig *et al.*, 2004). To evaluate the formamide concentration for optimum stringency, the designed probes were analysed on formamide-fixed activated sludge from the plant where the filaments were originally observed, by applying hybridization buffer containing 0–60 % formamide (in 5 % increments) using image analysis software with a designed macro (ImageJ 1.33s, Rasband W; National Institutes of Health, USA; <http://rsb.info.nih.gov/ij/>).

MAR. MAR experiments were performed with ³H- or ¹⁴C-labelled tracers. The procedure is described briefly here, but further details are available in Lee *et al.* (1999) and Kragelund *et al.* (2005). MAR-positive and -negative filaments were assessed by comparing silver grains on top of the filaments to the background. A minimum of 30 gene-probe-defined filaments were investigated for each incubation. Aerobic uptake of short- and long-chained fatty acids, sugars, alcohol, amino acids and bicarbonate with thiosulfate for autotrophic/mixotrophic behaviour was tested for the five *Alphaproteobacteria*

species. Diluted sludge (1 g SS l⁻¹) was added to a glass serum vial and mixed with tracer (10 µCi = 3.7 × 10⁵ Bq) and the unlabelled organic substrate to a final concentration of 2 mM (except oleic acid, final concn 0.5 mM), and the vial was closed with an airtight rubber stopper. The use of other potential e-acceptors apart from oxygen was tested with selected substrates, including nitrate (2 mM) or nitrite (0.5 mM) as potential e-acceptors, or under anaerobic conditions (without oxygen, nitrate or nitrite present). Nitrate and nitrite concentrations were measured in the sludge water using sticks. If either nitrate or nitrite was present, incubation of sludge was performed to remove these prior to MAR experiments. This was done by adding acetate and degassing the samples, thus allowing the consumption of nitrate or nitrite using these as e-acceptors. Moreover, a preincubation step of 2 h with unlabelled organic substrate (with nitrate or nitrite present as e-acceptor) was included before labelled substrate was added. This was done to ensure that only bacteria able to take up large amounts of substrates (for growth or storage) would be MAR-positive (Andreassen & Nielsen, 2000). Pasteurized sludge was used for chemography. A 3 h incubation period was used in all experiments except 5 h for bicarbonate

Table 2. Overview of specificity, sequences and hybridization conditions of oligonucleotide probes used

Probe name	Specificity	Probe sequence (5'–3')	FA (%)*	Reference
EUB338	Most bacteria	GCTGCCTCCCGTAGGAGT	0–60	Amann <i>et al.</i> (1990)
EUB338-II	<i>Planctomycetales</i>	GCAGCCACCCGTAGGTGT	0–35	Daims <i>et al.</i> (1999)
EUB338-III	<i>Verrucomicrobiales</i>	GCTGCCACCCGTAGGTGT	0–60	Daims <i>et al.</i> (1999)
ALF968	Most <i>Alphaproteobacteria</i>	GGTAAGGTTCTGCGCGTT	35	Neef (1997)
PPx3-1428	' <i>Ca. Alysiumicrobium bavaricum</i> '	TGGCCACCCGCTTCGGG	50	Levantesi <i>et al.</i> (2004)
PPx1002	' <i>Ca. Alysiumicrobium bavaricum</i> '	GGTCTCCCCGGGCCGCGG	10	This study
Noli-644	' <i>Ca. Alysiosphaera europaea</i> '	TCCGCTCTCCAGCCACA	35	Levantesi <i>et al.</i> (2004)
Sita-649	' <i>Ca. Sphaeronema italicum</i> '	CCWCTCCCGGACCCAG	50	Levantesi <i>et al.</i> (2004)
Nost993	' <i>Ca. Sphaeronema italicum</i> '	CAGCCGAAACGGGCATGT	20	This study
Helper1010	' <i>Ca. Sphaeronema italicum</i> '	GAAAGCCGCGTCTCCGG	20	This study
MC2-649	' <i>Ca. Monilibacter batavus</i> '	CTCTCCCGGACTCGAGCC	35	Levantesi <i>et al.</i> (2004)
Meg983 + Meg1028	<i>Meganema perideroedes</i>	CGGGATGTCAAAGGTGG + CTGTCACCGAGTCCCTTGC	35	Thomsen <i>et al.</i> (2006)

*Formamide concentration in hybridization buffer (v/v).

Fig. 1. A phylogenetic tree based on the 16S rRNA gene showing the affiliation of filamentous *Alphaproteobacteria*. The tree was calculated by the neighbour-joining method using ARB default settings. The scale bar corresponds to 10% estimated sequence divergence. Perfect-match organisms targeted by gene probes are illustrated with grey boxes. *Thiothrix* sp. was chosen as outgroup.

and thiosulfate. After the incubation period, the sludge was prepared and processed as described by Lee *et al.* (1999). The exposure time was 4–6 days. Details on the radiochemicals used are listed elsewhere (Kragelund *et al.*, 2005).

Storage capabilities. The PHA storage capabilities of the gene-probe-defined filamentous *Alphaproteobacteria* species were investigated using MAR incubations with different carbon substrates with different e-acceptors. Samples were spread out on gelatin-coated slides and stained according to the protocol for Nile blue (Ostle & Holt, 1982); details are described by Kragelund *et al.* (2005). The intensity was measured using image analysis software (ImageJ 1.33s, Rasband W). For each filament, a segment of at least 15 µm was analysed by recording the maximum intensity in each horizontal line perpendicular to the filament image. The mean value of these measurements (min. 100) was calculated for each analysed filament in a given incubation. At least 22 filaments were examined for each substrate incubation and mean fluorescence intensity $\pm$ SD was calculated. Student's *t*-test was used as a statistical tool to evaluate significant PHA formation compared to the corresponding control. To determine the background level of PHA in the different species, both pasteurized samples and formate incubations were used. No PHA accumulation was ever observed after the addition of formate.

Enzyme-labelled fluorescence (ELF) FISH. The presence of exoenzyme activity was determined using ELF (ELF-97; Molecular Probes), where substrates form a fluorescent precipitate on the surfaces of bacteria upon enzymic cleavage (Kragelund *et al.*, 2005). The following enzymes were evaluated: ELF-97 esterase substrate (ELF-97 acetate), ELF-97 lipase substrate (ELF-97 palmitate), ELF-97 β -D-galactosidase substrate (ELF-97 β -D-galactopyranoside), ELF-97 β -D-glucuronidase substrate (ELF-97 β -D-glucuronide), ELF-97 chitinase/*N*-acetylglucosaminidase substrate (ELF-97 *N*-acetylglucosaminide; ELF-97 NAG) and the ELF-97 Endogenous Phosphatase detection kit. Sludge (100 µl) was mixed with 0.2 mM of either substrate and was placed in the dark for 2–3 h. Five microlitres of this mix was spread out on a gelatin-coated glass slide and images were taken. The slide was washed with dH₂O prior to FISH. The enzyme reaction was

subsequently fixed in paraformaldehyde for 1 h and washed in tap water prior to the normal FISH procedure. The remaining enzyme reaction was resuspended in PBS and stored at 4 °C.

Microsphere adhesion to cells (MAC) FISH. To determine the surface properties of the filamentous bacteria, sulfate-modified microspheres (Molecular Probes) were applied (Nielsen *et al.*, 2001; Kragelund *et al.*, 2005). A 0.002% solution of microspheres with a diameter of 0.02 µm was sonicated for 100 min at 60 W. Activated sludge (10 µl, 4–5 g SS l⁻¹), 100 µl sterile, deionized water and 0.5 µl microsphere solution were mixed. Subsamples were spread out on gelatin-coated glass slides, images of filamentous bacteria with and without microspheres were recorded using a confocal laser scanning microscope and their positions were recorded. The identity of the filamentous bacteria was subsequently determined by FISH.

RESULTS

Identification and description

The six different industrial WWTPs investigated in this study were all dominated by a single-probe-defined species of filamentous *Alphaproteobacteria* and all had a filament index between 3 and 4, indicating bulking problems. In the sludge samples studied, all five species had a morphology resembling that of *Nostocoida limicola*, or Type 021N: coiled/twisted filaments, consisting of disc-shaped, round or short rod-shaped cells (Table 3). The diameter varied between 1 and 2 µm, without a visible sheath. Epiphytic growth was rarely observed. Other granules apart from sulfur were frequently seen. All species contained PHA (see below). They were usually Gram-negative, but occasionally filaments or parts of filaments were Gram-positive. No uniform Neisser staining was found. As evident in Table 3, any clear distinction between these species was impossible using only morphological methods, confirming

Table 3. Morphological description of the different filamentous *Alphaproteobacteria* species

Data from Levantesi *et al.* (2004) for all *Candidatus* and from Kragelund *et al.* (2005) and Thomsen *et al.* (2006) for *Meganema perideroedes*. $\pm$, Parts of filaments stain Gram-positive.

Character	' <i>Ca. Monilibacter batavus</i> '	' <i>Ca. Sphaeronema italicum</i> '	' <i>Ca. Alysiomicrobium bavaricum</i> '	' <i>Ca. Alysiosphera europaea</i> '	<i>Meganema perideroedes</i>
Shape of filament	Robust curled	Robust curled	Curled/coiled	Curled	Robust curled
Cell shape	Disc-shaped	Disc-/rod-shaped	Disc- to draught-shaped	Disc-shaped/rounded	Disc-shaped
Diameter (μm)	Approx. 1.3–2.0	0.8–1.4	Approx. 1.5	1.1–1.6	1.5–2.2
Length (μm)	<200	200	200	200	<500
Gram-staining	$\pm$	–	–	$\pm$	$\pm$
Neisser-staining	+	+	–	+	–
PHA granules	+	+	+	+	+

the requirements of species-specific gene probes for their identification. In one WWTP (AAU12), an additional very thin morphotype that showed a positive reaction with oligonucleotide probe PPx3-1428 was observed. However, it was present in small amounts only in this treatment plant and was not targeted by the newly developed probe PPx1002 (see below); therefore, it was not investigated further.

FISH and gene probe design

Additional oligonucleotide probes were constructed to target '*Ca. Alysiomicrobium bavaricum*' and '*Ca. Sphaeronema italicum*' as some variation in ecophysiological data was observed, indicating the presence of several genotypes. It was possible to design an additional probe (PPx1002) targeting only '*Ca. Alysiomicrobium bavaricum*' present in AAU 12 and not AAU23, strongly indicating the presence of two different species in the two plants. Probe PPx1002 gave the strongest signal at 10% formamide concentration as quantified by image analysis. Also, probe Nost993 was designed to reveal whether two different genotypes of '*Ca. Sphaeronema italicum*' were present in CNR1 and TNO18. Although a perfect match to the publicly available '*Ca. Sphaeronema italicum*' sequences, no hybridization signal was observed even at 0% formamide concentration. To overcome this, a helper probe (Helper1010) was constructed to enhance accessibility of the target site (Behrens *et al.*, 2003). When the probes Nost993 and Helper1010 were applied at an optimal formamide concentration (20%), filamentous '*Ca. Sphaeronema italicum*' gave a clear fluorescent signal in CNR1 but not in TNO18, also suggesting the presence of two different genotypes in these plants.

Ecophysiology

Uptake of organic substrates under aerobic conditions. Substrate uptake of the different filamentous species under aerobic conditions was determined in various treatment plants by MAR-FISH and the results are listed in Table 4. Data obtained from the four different alphaproteobacterial species were compared to that of *Meganema perideroedes* reported previously (Kragelund *et al.*, 2005). All the probe-defined species consumed acetate and most

also consumed other short-chain fatty acids (propionate and pyruvate), but not formate and butyrate. The results also show that the five species could be divided into two major physiological groups: Group 1 that primarily consumed short-chain acids ('*Ca. Monilibacter batavus*' and '*Ca. Sphaeronema italicum*') and Group 2 that, besides the short-chain acids, were able to consume various carbohydrates, amino acids and ethanol ('*Ca. Alysiomicrobium bavaricum*', '*Ca. Alysiosphera europaea*' and *Meganema perideroedes*).

'*Ca. Sphaeronema italicum*' (defined by probe Sita-649) was investigated in two different plants (CNR1 and TNO18). They showed minor differences in substrate uptake, but both clearly belonged to Group 1. As the new probe Nost993 only hybridized with filaments in CNR1, it is likely that the population showing a different substrate uptake profile in TNO18 was a different genotype. Also '*Ca. Alysiomicrobium bavaricum*' filaments were present in two plants (AAU12 and AAU23) and despite minor differences in substrate uptake they belonged to Group 2. Probe PPx1002 targeted only filaments present in AAU12, again indicating two different genotypes with slight differences in substrate uptake.

Substrate uptake under denitrifying conditions. The ability of the filamentous *Alphaproteobacteria* to take up substrates under anaerobic conditions or denitrifying conditions (with nitrate or nitrite added) is shown in Table 5.

When nitrate served as potential e-acceptor, all species were able to take up acetate and a few other substrates. However, it was clear that a narrower range of substrates was consumed by the individual species here compared to under aerobic conditions. Two of the species from Group 2 still consumed glucose, but '*Ca. Alysiomicrobium bavaricum*' did not. In general, compared to aerobic conditions, less substrate was taken up by the individual filaments as assessed by the number of silver grains on top of the filaments.

When nitrite acted as potential e-acceptor, all Group 1 species and one Group 2 species ('*Ca. Alysiomicrobium*

Table 4. Uptake of substrates by the different filamentous *Alphaproteobacteria* species under aerobic conditions, as investigated by MAR

ND, Not determined; –, no substrate uptake (no silver grains); (+), some filaments slightly positive (some silver grains); +, a little uptake, but clearly active (few silver grains); ++, active uptake (many silver grains). No uptake of formate or bicarbonate + thiosulfate was observed in any species. Data for *Meganema perideroedes* are from Kragelund *et al.* (2005).

Substrate	'Ca. Monilibacter batavus'		'Ca. Sphaeronema italicum'		'Ca. Alysiomicrobium bavaricum'		'Ca. Alysiosphera europaea'		<i>Meganema perideroedes</i>	
	WWTP: TNO43		CNR1		AAU12		BIO6			
	Probe:	MC2-649	Nost993	Sita-649	PPx1002	PPx3-1428	Noli-644		Meg983 +	Meg1028
Acetate		++	+	++	++	++	++		++	
Propionate		++	–	+	++	–	ND		++	
Butyrate		–	–	+	(+)	–	(+)		+	
Pyruvate		++	++	++	++	+	++		–	
Oleic acid		–	++	–	–	–	–		++	
Glucose		–	–	–	++	++	++		++	
Mannose		–	–	–	++	++	+		+	
Galactose		–	–	–	(+)	+	(+)		+	
Leucine		–	–	–	++	+	+		+	
Glycine		–	–	–	–	–	–		(+)	
Ethanol		–	–	–	++	–	–		–	

bavaricum') took up the same substrates as when nitrate was e-acceptor (Table 5). No substrate uptake was observed for 'Ca. Alysiosphera europaea', while *Meganema perideroedes* utilized only glucose and acetate.

Under anaerobic conditions, none of the alphaproteobacterial species investigated showed any uptake of substrates, indicating a lack of anaerobic growth or storage capabilities (data not shown).

Storage capacity

In fresh activated sludge samples, all gene-probe-defined filamentous *Alphaproteobacteria* species had small PHA granules as visualized by Nile blue staining. A number of substrates were tested for uptake and potential conversion into PHA under different e-acceptor conditions by measuring the increase in fluorescence intensity during a 3 h incubation with different substrates (Table 6 and Table 7). The

Table 5. Uptake of substrates by the different filamentous *Alphaproteobacteria* species under anaerobic conditions with nitrate or nitrite as e-acceptor, as investigated by MAR

ND, Not determined; –, no substrate uptake (no silver grains); (+), some filaments slightly positive (some silver grains); +, a little uptake, but clearly active (few silver grains); ++, active uptake (many silver grains). Data for *Meganema perideroedes* are from Kragelund *et al.* (2005).

Substrate	'Ca. Monilibacter batavus' TNO43		'Ca. Sphaeronema italicum' TNO18		'Ca. Alysiomicrobium bavaricum' AAU12		'Ca. Alysiosphera europaea' BIO6		<i>Meganema perideroedes</i>	
	Probe:	MC2-649	Sita-649		PPx1002		Noli-644		Meg983 +	Meg1028
e-acceptor		NO ₃ [–]	NO ₂ [–]	NO ₃ [–]	NO ₂ [–]	NO ₃ [–]	NO ₂ [–]	NO ₃ [–]	NO ₂ [–]	
Acetate		(+)	(+)	+	(+)	++	++	(+)	++	+
Propionate		–	–	–	–	++	+	ND	++	–
Butyrate		ND	ND	–	–	–	–	–	–	–
Pyruvate		++	++	+	+	+	+	–	ND	ND
Oleic acid		ND	ND	ND	ND	ND	ND	ND	–	–
Glucose		ND	ND	ND	ND	–	–	++	+	+
Leucine		ND	ND	ND	ND	–	–	–	–	–
Glycine		ND	ND	ND	ND	ND	ND	ND	–	–
Ethanol		ND	ND	ND	ND	–	–	–	ND	ND

Table 6. Storage capabilities of the different filamentous *Alphaproteobacteria* species under aerobic conditions, as investigated by fluorescence intensity measurements of total PHA content

ND, Not determined because no uptake was observed in MAR incubation; +, PHA formation after incubation with substrate for 3 h; –, no detectable PHA formation after 3 h incubation. Data for *Meganema perideroedes* are from Kragelund *et al.* (2005).

Substrate	' <i>Ca. Monilibacter batavus</i> '		' <i>Ca. Sphaeronema italicum</i> '		' <i>Ca. Alysiumicrobium bavaricum</i> '		' <i>Ca. Alysiosphaera europaea</i> '		<i>Meganema perideroedes</i>	
	WWTP:		CNR1		AAU12		BIO6			
	Probe:		Nost993		PPx1002		Noli-644		Meg983 + Meg1028	
		TNO43		TNO18		AAU23				
		MC2-649		Sita-649		PPx3-1428				
Acetate		+		–		+		+		+
Propionate		+		+		+		ND		+
Butyrate		ND		–		–		+		–
Pyruvate		+		+		–		+		ND
Oleic acid		ND		ND		ND		ND		+
Glucose		ND		ND		+		+		–
Mannose		ND		ND		–		(+)		+
Galactose		ND		ND		+		+		+
Leucine		ND		ND		+		+		–
Glycine		ND		ND		ND		ND		–
Ethanol		ND		ND		–		ND		ND

substrates tested for PHA accumulation were those resulting in a positive signal using MAR for the different species (Tables 4 and 5). For some substrates, substantial uptake and subsequent storage was observed, where the individual cells in the filaments were almost completely filled with PHA granules. There was some variation between filaments, but with similar PHA content in all cells within each filament.

Under aerobic conditions, members of Group 1 were able to convert at least some of the short-chain fatty acids into PHA (Table 6). Group 2 species also converted almost all substrates tested into PHA, e.g. short-chain fatty acids, sugars and amino acids.

When nitrate was present as e-acceptor, PHA formation was observed for all substrates taken up, as determined by MAR (Table 7). PHA formation with nitrite as e-acceptor showed

the same pattern as with nitrate for all species except '*Ca. Alysiosphaera europaea*'.

Exoenzyme activity and surface properties

The investigated species present in the bulk liquid showed no surface-associated exoenzymic activity with any of the ELF enzymes tested, with the exception of some '*Ca. Monilibacter batavus*' filaments which exhibited lipase activity. Also *Meganema perideroedes* showed lipase activity on one occasion after a factory shutdown (Kragelund *et al.*, 2005). Filamentous bacteria in the different samples exhibited some enzyme activity, observed as fluorescent precipitates along the filament, but these bacteria were not identified. Enzyme activity at the surface of microcolonies and filamentous organisms in the flocs was always present. In general, very high glucuronidase and esterase activity was

Table 7. Storage capabilities of the different filamentous *Alphaproteobacteria* species investigated by fluorescence intensity measurements of total PHA content under anaerobic conditions with nitrate and nitrite as e-acceptor

ND, Not determined because no uptake was observed in MAR incubation; +, PHA formation after incubation with substrate for 3 h; –, no detectable PHA formation after 3 h incubation. Data for *Meganema perideroedes* are from Kragelund *et al.* (2005).

Substrate	' <i>Ca. Monilibacter batavus</i> ' TNO43		' <i>Ca. Sphaeronema italicum</i> ' TNO18		' <i>Ca. Alysiumicrobium bavaricum</i> ' AAU12		' <i>Ca. Alysiosphaera europaea</i> ' BIO6		<i>Meganema perideroedes</i>	
	Probe:		Sita-649		PPx1002		Noli-644		Meg983 + Meg1028	
e-acceptor	NO ₃ [–]	NO ₂ [–]	NO ₃ [–]	NO ₂ [–]	NO ₃ [–]	NO ₂ [–]	NO ₃ [–]	NO ₂ [–]	NO ₃ [–]	NO ₂ [–]
Acetate	+	+	+	+	+	+	+	–	+	+
Propionate	ND	ND	ND	ND	+	+	ND	ND	+	ND
Pyruvate	+	+	+	+	+	+	ND	ND	ND	ND
Glucose	ND	ND	ND	ND	ND	ND	+	ND	+	+

observed in the flocs. No attempts were made to study possible exoenzyme activity inside sludge flocs.

The surface properties of the filamentous alphaproteobacterial species were investigated using MAC-FISH. The adherence of surface-modified microspheres was investigated for the filaments and for the flocs. Both hydrophobic and more hydrophilic areas were observed in the flocs.

The three species belonging to Group 2 (see above) were all completely covered with microspheres and had relatively more microspheres than most other organisms in the sludges. This indicates the presence of a hydrophobic sheath. Group 1 '*Ca. Monilibacter batavus*' filaments appeared much less hydrophobic as only very few microspheres attached to the surfaces. '*Ca. Sphaeronema italicum*' filaments did not show the same surface properties in the two different plants investigated, reflecting the two different genotypes. The gene-probe-defined population in CNR1 targeted by Nost993 had no microspheres attached to the surface, whereas the filaments in TNO18 targeted by Sita-649 were completely covered.

DISCUSSION

Filamentous *Alphaproteobacteria* have recently been shown to be very common in industrial WWTPs and they often cause serious bulking problems (Eikelboom & Geurkink, 2002; Levantesi *et al.*, 2004; van der Waarde *et al.*, 2002). Of the five species hitherto described, only the ecophysiology of *Meganema perideroedes* has been investigated (Kragelund *et al.*, 2005), so the present study provides a more comprehensive study of almost all filamentous *Alphaproteobacteria* encountered in industrial WWTPs. All species had a morphology resembling that of Type 021N, or *Nostocoida limicola*, and could only be correctly identified to species level when FISH was applied. Hardly anything is known about the physiology of the different species, so specific control strategies have not yet been developed to combat this group of filamentous organisms.

The substrate uptake data suggest that the five species can be divided into two groups with a very similar physiology. Group 1 consists of '*Ca. Monilibacter batavus*' and '*Ca. Sphaeronema italicum*' and they took up mainly short-chain fatty acids. Group 2 ('*Ca. Alysiumicrobium bavaricum*', '*Ca. Alysiosphaera europaea*' and *Meganema perideroedes*) was more versatile and able to take up carbohydrates, amino acids and ethanol in addition to the short-chain fatty acids. Furthermore, the capability to form the storage product PHA was generally large for all species in Group 1 and Group 2 (except for '*Ca. Alysiosphaera europaea*'). Interestingly, when we compare this grouping with the phylogenetic analysis of the species, it appears that the species in Group 1 cluster together, as do two of the Group 2 species. *Meganema perideroedes* does not cluster with any of the other four species. Thus, it seems that the physiological properties to some extent follow the phylogenetic affiliation of the species.

It is not clear whether the five species were able to perform full denitrification to gaseous nitrogen, but under conditions where nitrate served as potential e-acceptor they were definitely physiologically very active, and able to take up substrates and form PHA from all substrates. When nitrite served as potential e-acceptor all but '*Ca. Alysiosphaera europaea*' were active and took up largely the same substrates, although none of the species took up substrates under strict anaerobic conditions. This suggests that denitrification to gaseous nitrogen probably took place with some substrates and reduction of nitrate to nitrite occurred only in the presence of a few substrates (e.g. propionate for *Meganema perideroedes*). It was interesting to note that for all species the number of substrates that could be taken up with nitrate or nitrite as e-acceptor was almost identical, but clearly reduced compared to uptake under aerobic conditions. A similar observation has also been reported for growth of isolates of the denitrifying genus *Thauera* (Foss & Harder, 1998) and it reinforces the idea that it is virtually impossible to predict the substrate uptake pattern of these bacteria under denitrifying conditions on the basis of only aerobic investigations.

PHA granules were present in all five species. Extensive PHA formation has been reported for *Meganema perideroedes* (Kragelund *et al.*, 2005; Levantesi *et al.*, 2004) and for an unidentified alphaproteobacterial *Nostocoida limicola*-like organism (Dionisi *et al.*, 2002). With the results presented here, this seems to be a common trait in filamentous *Alphaproteobacteria* investigated so far. The ability to form PHA is an obvious advantage, since the storage material can be utilized as energy and/or as a reserve of carbon during periods of unbalanced growth due to unfavourable conditions (Dawes, 1991). '*Ca. Alysiumicrobium bavaricum*' (AAU12) had a very large storage capacity, as described for *Meganema perideroedes* (Kragelund *et al.*, 2005), and the variation in storage of PHA in each species may reflect physiological differences or differences in actual physiological status.

Comparing MAR results and PHA quantification, information regarding consumption and transformation of various substrates was obtained. With oxygen as e-acceptor, most short-chain fatty acids were taken up by the species; the Group 2 species ('*Ca. Alysiumicrobium bavaricum*', '*Ca. Alysiosphaera europaea*' and *Meganema perideroedes*) in particular transformed most substrates into PHA. In two cases PHA formation was observed after incubation with glucose, which is in agreement with studies on other bacteria (Dawes, 1991; Haywood *et al.*, 1990, 1991). However, this was not observed for *Meganema perideroedes* under aerobic conditions (Kragelund *et al.*, 2005). This could potentially illustrate differences in the metabolic pathways amongst these species, since *Meganema perideroedes* only formed PHA from glucose when nitrate or nitrite served as e-acceptor. At present, it is not possible to study glycogen formation at the single-cell level, so differentiation between the storage of PHA and glycogen remains to be investigated.

The ecophysiology of '*Ca. Sphaeronema italicum*' (CNR1) and '*Ca. Alysiumicrobium bavaricum*' was not identical

in the two plants investigated for each species. This is different from most other ecophysiological investigations we have conducted on other filamentous bacteria (Nielsen *et al.*, 2002), glycogen-accumulating organisms (Kong *et al.*, 2006) and polyphosphate-accumulating bacteria (Kong *et al.*, 2004), where the physiology for a certain gene-probe-defined species appears very similar in different plants. Therefore, additional gene probes were designed and revealed that different populations were probably present in the different WWTPs, explaining the observed differences in ecophysiology. This seems plausible as there are a number of differences in growth conditions in the plants in terms of temperature and wastewater type. The existence of several closely related species belonging to the two candidate species is not unexpected and a more comprehensive phylogenetic overview must await the determination of more sequences of these filamentous *Alphaproteobacteria*.

Surface-associated exoenzymic activity was barely observed in these species. '*Ca. Monilibacter batavus*' showed some lipase activity, but was not able to consume the only offered long-chain fatty acid (oleic acid). However, other long-chain fatty acids that were not tested could potentially be taken up, explaining the observed lipase activity. Also, *Meganema perideroedes* showed lipase activity on one occasion after a factory shutdown, indicating an ability to turn on enzyme activity under periods of stress and/or starvation (Kragelund *et al.*, 2005). The ecophysiology results suggest that all five species utilize mainly low-molecular-mass dissolved organic compounds present in the wastewater rather than particulate organic matter requiring an enzymic step before uptake.

The surface of most of the filamentous *Alphaproteobacteria* was very hydrophobic, particularly amongst the Group 2 members. It is not known how they benefit from this as they mainly consume hydrophilic soluble substrates and not hydrophobic substrate as does *Microthrix parvicella*, for example (Andreasen & Nielsen, 2000; Nielsen *et al.*, 2002).

Another factor is the ability of these species to form flocs. It is essential for the filaments to stay attached to the floc material in order to settle in the final clarifier and not to be washed out with the effluent. Previous studies have revealed that most single cells in bulk water in WWTPs are hydrophilic, while many filamentous bacteria protruding from flocs are often hydrophobic (Zita & Hermansson, 1997a, b). Most of the *Alphaproteobacteria* were observed to be incorporated into the floc material as long as they did not dominate the population. Thus, the primary function of the relatively hydrophobic surface of these bacteria may be attachment to the floc. In several cases we have observed filamentous *Alphaproteobacteria* in foam in various WWTPs, also reflecting their hydrophobic nature (data not shown).

Possible control measures

The filamentous *Alphaproteobacteria* are responsible for many bulking incidents, especially in industrial WWTPs.

Evidence from this and others studies emphasizes that molecular identification is necessary to distinguish between the different filamentous *Alphaproteobacteria*. From a physiological point of view, all five of the filamentous *Alphaproteobacteria* species discussed in this study can be divided into two groups. However, from the point of view of control, they can be considered as a single group as they are all very well adapted to industrial plants with a high content of soluble, readily consumable organic compounds. The survey conducted by Levantesi *et al.* (2004), and unpublished data (C. Kragelund & P.H. Nielsen), show that the filamentous *Alphaproteobacteria* in the versatile Group 2 are twice as abundant as Group 1 species in WWTPs.

Currently, the most efficient control strategy for most filamentous bacteria is the introduction of a selector in the plant configuration (Eikelboom, 2002; Wanner, 1994). The advantage of small compartmentalized selectors with a short residence time is the creation of a substrate gradient when raw influent is mixed with sludge. Here, easily degradable substrates are taken up by bacteria with high substrate uptake rates and storage capacities. These properties are generally associated with floc-forming bacteria rather than filamentous bacteria and should thus select for floc-forming bacteria (Shao & Jenkins, 1989; Wanner, 1994). However, both groups of the filamentous *Alphaproteobacteria* seem to have high substrate uptake rates and storage capacities, probably comparable to floc-formers, so a selector may only work under conditions where the substrate uptake is more restricted. An anaerobic selector would probably work very well, but this is usually difficult to install except in plants with biological phosphate removal. This study showed that nitrate, and in particular nitrite, significantly reduced the number of substrates that can be consumed by these filamentous *Alphaproteobacteria*, so anoxic selectors using nitrate, or even better nitrite, should be tested. Although the costs of adding nitrate, and particularly nitrite, can be high, it may in some cases be worth consideration. The selector must be designed properly to optimize the amount of soluble substrate to be removed (Martins *et al.*, 2004; Wanner, 1994). For Group 1 members it may also be possible to look at the wastewater stream and see whether it is possible to reduce the amount of short-chain fatty acids.

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