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Micromanipulation and further identification of FISH-labelled microcolonies of a dominant denitrifying bacterium in activated sludge

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Summary

The activated sludge process relies on the formation of strong microbial flocs. The knowledge about dominant floc-forming bacteria is at present very limited, especially from a phylogenetic perspective. In this study, numerous microcolonies in the activated sludge flocs were found to be targeted by a *Betaproteobacteria*-group-specific oligonucleotide probe using fluorescence *in situ* hybridization (FISH). Some of these were micromanipulated and further identified by reverse transcriptase polymerase chain reaction (RT-PCR) and sequencing to belong to the *Aquaspirillum* genus in the *Neisseriaceae* family. A specific oligonucleotide probe, Aqs997, was designed to target the identified bacteria. A survey in nine different wastewater treatment plants with nutrient removal (WWTP) showed a high abundance of bacteria hybridizing to the oligonucleotide probe developed. Microautoradiography (MAR) combined with FISH on activated sludge incubated with radiolabelled substrate showed uptake of substrate with oxygen, nitrate and nitrite as electron acceptor demonstrating a denitrifying potential of the bacteria investigated. The *Aquaspirillum*-related bacteria seemed to be abundant denitrifiers in WWTPs with nitrogen removal and they were particularly numerous in plants mainly receiving domestic wastewater, where they constituted up to 30% of all bacteria.

Introduction

In activated sludge wastewater treatment plants (WWTP), formation of strong microbial flocs, is of key importance for an efficient separation of activated sludge and treated wastewater (Eikelboom and van Buijsen, 1983; Bidault *et al*., 1997). Therefore, dominant floc-forming bacteria are important for the floc properties and thus the quality of the solid-liquid separation in wastewater treatment plants.

The microbial population in activated sludge has often been described from a functional point of view as aerobic heterotrophs, nitrifiers, denitrifiers, sulphate reducers, iron reducers and phosphate-accumulating organisms (PAOs), e.g. (Nielsen and Nielsen, 2002a). However, a substantial amount of these bacteria are still phylogenetically undescribed, especially the aerobic heterotrophs and the denitrifiers from WWTPs with nitrogen removal. These two functional groups of bacteria represent 70–80% of all bacteria in activated sludge (Nielsen and Nielsen, 2002a) and they can thus be anticipated to be of key importance to the floc formation. Most bacteria in activated sludge plants belong to the *Betaproteobacteria,* e.g. (Juretschko *et al*., 2002; Wagner *et al*., 2002; Nielsen *et al*., 2004; Schmid *et al*., 2003), and recently it was shown that microcolonies belonging to this group form very strong microcolonies that can be very resistant to shear stress in contrast to other phylogenetic groups (Nielsen *et al*., 2004). Among known genera in this group present in activated sludge are the *Nitrosomonas* (Wagner *et al*., 2002), *Zoogloea* (Lajoie *et al*., 2000), *Azoarcus* (Wagner and Loy, 2002), and bacteria belonging to the *Rhodocyclus* genus, e.g. *Candidatus* 'Accumulibacter phosphatis' (Hesselmann *et al*., 1999; Crocetti *et al*., 2000). However, there are still many unknown bacterial species hidden in this group and very little is understood about their function.

In several WWTPs with nitrogen removal we have observed unidentified large microcolony-forming bacteria belonging to the *Betaproteobacteria.* They appeared to be abundant in some of the WWTPs, so the aim of this study was to identify these bacteria capable of forming large microcolonies. Instead of performing an extensive clone library, we used micromanipulation (Skerman, 1968; Blackall, 1991; Hornsby and Horan, 1994; Bradford *et al*., 1996) and the full cycle rRNA approach (Amann *et al*., 1995; Snaidr *et al*., 2002) to identify the bacteria and confirm their abundance by fluorescence *in situ* hybridiza-

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tion (FISH). The great advantage of this technique is that a highly enriched sample can be obtained for further phylogenetic investigations. Here we present an additional improvement of the method where the micromanipulation was performed on probe-labelled samples, that enabled enrichment and identification of specific bacteria of interest, which could not be otherwise distinguished by morphological means. The method was tested on some filamentous bacteria and subsequently improved for identifying microcolony-forming bacteria. A new specific oligonucleotide probe was developed, confirming the presence of an abundant, denitrifying, and microcolony-forming species, closely related to bacteria in the *Aquaspirillum* genus in the *Neisseriaceae* family.

Results

Micromanipulation and phylogenetic analysis

The new combination that uses micromanipulation on samples hybridized with specific oligonucleotide probes was initially tested on filamentous bacteria from an industrial WWTP, as filamentous bacteria are easier to micromanipulate than microcolonies. One *Thiothrix* filament was micromanipulated on the basis of morphological characteristics (Eikelboom and van Buijsen, 1983), and four filamentous bacteria, all *Meganema perideroedes*, were micromanipulated after hybridization to a species-specific oligonucleotide probe. They all gave a suitable PCR product for cloning. Two PCR products were cloned, one of each type. The *Thiothrix* was identified as *Thiothrix ramosa* (98.0% similarity), whereas the other filament was confirmed to be *Meganema perideroedes* (100.0% similarity). These results demonstrated the applicability of the method: to micromanipulate one single filamentous bacterium and directly identify it further without previous cultivation.

In activated sludge from Aalborg East WWTP, *Betaproteobacteria* constituted a high fraction (40–50%) of all bacteria present, and most were present in large microcolonies (Nielsen *et al*., 2004). By applying various more specific gene probes for ammonia oxidizing bacteria, *Azoarcus, Zoogloea, Thauera* and *Candidatus* 'Accumulibacter phosphatis' belonging to *Rhodocyclus* in a preliminary screening it became clear that there were some unidentified microcolonies belonging to the *Betaproteobacteria.* Therefore, these microcolonies were micromanipulated for further phylogenetic analysis.

Suspensions of FISH-labelled activated sludge were compared with traditional FISH, and no differences in signal intensities were observed. Subsequently, the FISH in suspension was used for micromanipulation of microcolonies using a glass capillary needle. A homogenization of the sludge sample before hybridization and micromanipulation was important to make a rough separation of the flocs. Micromanipulation was performed on microcolonies from sludge hybridizing to the BONE23A probe. It was generally difficult to manipulate single colonies without contaminants from single cells in the sample. It was, however, possible to get a highly enriched sample. A total of 20 microcolonies were micromanipulated. Polymerase chain reaction products resulting from RT-PCR on micromanipulated microcolonies were obtained for 14, of which three (samples A, B, C) were cloned. Two to three types of DGGE band representing different sequences were achieved from each PCR product, indicating that several species were obtained by means of the micromanipulation. Plasmids representing the different DGGE bands from sample A, B and C were then sequenced.

Phylogenetic trees based on aligned nucleic data were estimated by using distance matrix, parsimony, and maximum likelihood. The methods applied resulted in congruent tree topologies, and in Fig. 1 a maximum likelihood tree is shown. Sequences obtained from sample A affiliated to the genus *Aquaspirillum* in the family *Neisseriaceae* (sequence AquaspiA was 99.3% similar to *Pseudomonas lanceolata*), *Escherichia coli*, and a sequence rather distantly to clone OPB90 from a Yellowstone hot spring. Sample B sequences affiliated to the genus *Aquaspirillum* (sequence AquaspiB was 99.0% similar to *Pseudomonas lanceolata*), and one sequence grouped with *Peptostreptococcus*-like organisms. Sample C also consisted of the genus *Aquaspirillum*-related bacteria (AquaspiC was 99.1% similar to *Pseudomonas lanceolata*) and a sequence very similar to *Caulobacter*. Thus, even though the micromanipulation was performed on bacteria hybridizing to an oligonucleotide probe for a subgroup of the *Betaproteobacteria,* sequences from other groups were also obtained. However, in all three samples very similar Aquaspi-sequences were present (99.3–99.5% identical), and they were closely related to *Pseudomonas lanceolata* and *Aquaspirillum delicatum* in the genus *Aquaspirillum*, according to the nomenclature used on the new RDP preview release (Cole *et al*., 2003). Only one other sequence (AY050604, uncultured bacterium clone GOUTB19) from a recent study on microbial diversity in an *in situ* reactor system treating monochlorobenzene-contaminated groundwater (Alfreider *et al*., 2002), was closely related to the sequences obtained in this study (98.9–99.1% similarity to AquaspiA-C).

Probe design and test

The sequences closely related to the genus *Aquaspirillum* in the family *Neisseriaceae* were used to design an oligonucleotide probe (Aqs997), which matches these very similar sequences perfect in theory (AquaspiA, AquaspiB and AquaspiC); *Aquaspirillum delicatum* ATCC14667;

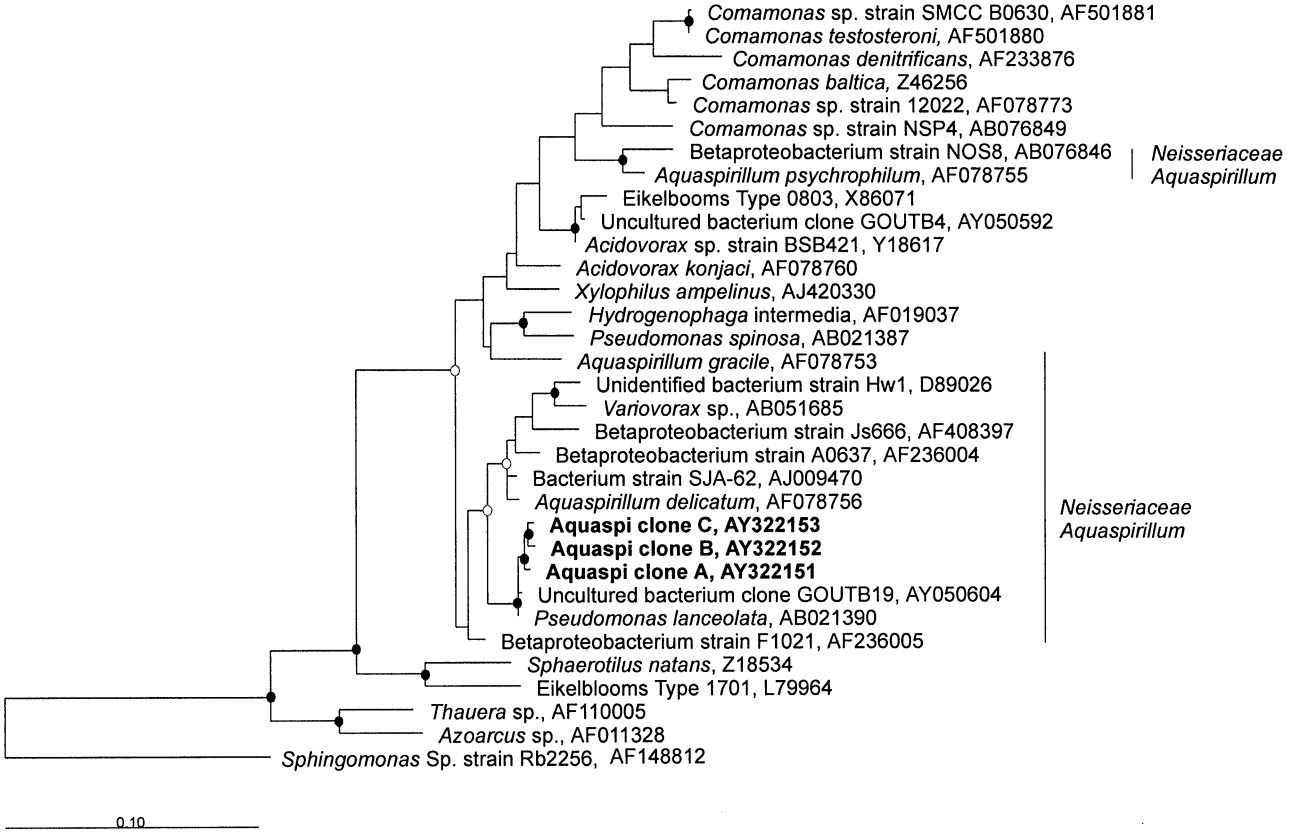

Fig. 1. A 16S rRNA tree showing the phylogenetic affiliation of the micromanipulated microcolonies (AquaspiA, AquaspiB and AquaspiC from samples A, B and C). The tree was calculated using the maximum-likelihood method using default settings in ARB. Branch points supported (bootstrap values >75%) by all inference methods used are indicated by solid circles, and open circles indicate those supported by most methods. The scale bar corresponds to 10% estimated sequence divergence.

Pseudomonas lanceolata AB021390; *Comamonas testosteroni* AF50188; *Comamonas* sp. AF501881; and a few uncultured bacteria: S-*-Aquaspi-997-a-A-18 (Aqs997), 5′-CTC TGG TAA CTT CCG TAC-3′. *Escherichia coli* numbering is used (Brosius *et al*., 1981).

Six reported sequences affiliated in the *Comamonadaceae* and *Neisseriaceae* family (*Pseudomonas spinosa* AB021387; *Hydrogenophaga* sp. AF019037; *Aquaspirillum gracile* AF078753; *Acidovorax konjaci* AF078760; *Comamonas* sp. AF078773; and *Comamonas baltica* Z46256) have one central mismatch to Aqs997, making the gene probe less specific than intended. Two competitors were designed to exclude the binding of probe Aqs997 to cells with one mismatch, comp1Aqs997: 5′-CTC TGG CAA CTT CCG TAC-3′ and comp2Aqs997: 5′-CTC TGG TCA CTT CCG TAC-3′. All other known sequences have a weighted mismatch above 1.5. Compared with probe Cte (cited in Loy *et al*., 2003), which target above 100 sequences of *Comamonas* spp., *Acidovorax* spp., *Hydrogenophaga* spp., and *Aquaspirillum* spp., probe Aqs997 has a much more narrow target. Not all targets of Aqs997 are targeted by probe Cte, e.g. Cte has one mismatch to *Aquaspirillum delicatum,* whereas Aqs997 matches perfectly. Aquaspi-sequences obtained in this study have theoretically perfect match with both the Aqs997 and the Cte oligonucleotide probes. However, more positive cells were observed with the Aqs997 probe than the Cte probe when hybridized to sludge from Aalborg East WWTP. The Aquaspi-sequences obtained by micromanipulation of microcolonies hybridizing to the BONE23A oligonucleotide probe have also theoretical perfect match with this probe, which is also the case for *Pseudomonas lanceolata.* On the contrary, *Aquaspirillum delicatum* has one mismatch to the BONE23A probe.

Probe-binding profiles of the pure culture (*Aquaspirillum delicatum* ATCC14667) and Aalborg East sludge samples showed that probe Aqs997 hybridized well up to 35% formamide (results not shown). When the competitor probes were used in combination with Aqs997 probe no differences were observed indicating that very few of the bacteria with one mismatch to probe Aqs997 were present in the sludges investigated. Applying the new probe in activated sludge from Aalborg East WWTP revealed that, indeed, 20–30% of the total number of bacteria in the

sludge sample were targeted by the Aqs997 probe, particularly microcolonies (Fig. 2A–F). Most Aqs997-positive bacteria showed a quite distinct morphology, being relatively large, coccoid cells with a diameter of 1–1.5 μm (Fig. 2A). The size of the microcolonies varied a lot. Most were 10–15 μm in diameter, but colonies of up to 40 μm were observed. Some of the microcolonies were not as dense as observed with other colonies indicating presence of varying amounts of exopolymers. Interestingly, in approximately 50% of the Aqs997-positive microcolonies, other bacteria were observed in between the cells forming mixed co-culture in the microcolonies. Some of the other bacteria were positive with the probe for the *Betaproteobacteria*. In addition, the specific probe also targeted a filamentous bacterium and some single cells with a slightly different morphology than cells within the microcolonies.

An extra oligonucleotide probe targeting the Aquaspi-sequences at position 460 was designed but did not hybridize to the sludge samples and isolates tested. This position is well known to be a rather inaccessible position for probe binding on the bacteria studied by Behrens *et al*. (2003).

Oligonucleotide probes were also designed, when possible, to target the sequences obtained from the micromanipulation, which were not related to the *Betaproteobacteria*. However, the probes hybridized to very few microcolonies and single cells in the activated sludge from Aalborg East WWTP, so no further work on these was performed. It was not possible to design a specific oligonucleotide probe for *Caulobacter*, which is one of the bacteria suggested to contribute to floc formation (Winkler and Cox, 1980). *Escherichia coli* ATCC25922 was used as a non-target organism for the probes (having four mismatches to the Aquaspi-sequences) and positive hybridization signals were never observed.

FISH survey in different WWTPs

Probe Aqs997 hybridized to microcolonies in all activated sludges tested (Table 1). The plants all had nitrification-denitrification, and in particular in the six WWTPs treating domestic wastewater a high number of Aqs997-positive bacteria were found, ranging between 10 and 30% of all bacteria. Less, but still many were observed in the industrial WWTPs. The majority of the bacteria hybridizing to the new gene probe were growing in microcolonies with the same characteristic morphology as that observed in Aalborg East WWTP. Some of the microcolonies consisting of Aqs997-positive cells were mixed with other bacteria, so far unidentified, especially in plants receiving domestic wastewater. Some of these bacteria belonged to the *Betaproteobacteria*. Microcolonies consisting of bacteria hybridizing with the probe for *Thauera* were observed in all treatment plants investigated, whereas *Zoogloea* was mostly found in the industrial WWTPs.

Table 1. Screening of bacteria in nine different wastewater treatment plants with the new oligonucleotide probe, Aqs997.

Plant	Type wastewater	Process design[a]	Aqs997 (%)
1 Aalborg East[b]	Domestic	C, N, DN, CP, BP	20–30
2 Aalborg West	Domestic	C, N, DN, CP, BP	20–30
3 Egå	Domestic	C, N, DN, BP	20–30
4 Horsens	Domestic	C, N, DN, CP	20–30
5 Aalbæk	Domestic	C, N, DN, CP	10–20
6 Aabybro	Domestic	C, N, DN, CP	10–20
7 Daka Loesning	Industrial	C, N, DN	5–10
8 Daka Randers	Industrial	C, N, DN	5–10
9 Daka Ringsted	Industrial	C, N, DN	5–10

a. C = carbon removal; N = nitrification; DN = denitrification, BP = biological phosphorus removal; CP = chemical phosphorus removal.
b. Aalborg East WWTP, where the micromanipulation and further identification of BONE23A-positive microcolonies in this study was performed.

Autoradiography

Microautoradiography was applied to study the physiology of bacteria targeted by the Aqs997 probe in sludge from Aalborg East WWTP. A mixture of amino acids was added to the sludge under conditions with oxygen, nitrate, or nitrite as electron acceptor and under anaerobic conditions without any added electron acceptor. Substrate uptake by Aqs997-positive microcolonies and single cells were evident with oxygen, nitrate, and nitrite as electron acceptor but not under anaerobic conditions. An example is shown in Fig. 2G–J, where the silver grains on top of many Aqs997-positive cells indicate substrate uptake under denitrifying conditions and thus illustrate a denitrifying potential of this bacterium. Labelled acetate, ethanol, glucose, lactate, and propionate was also tested but no substrate uptake was found by Aqs997-positive cells under any conditions tested (See Table 2). However, other bacteria in the flocs were found to take up the tested substrates, thus serving as positive controls.

Discussion

Evaluation of method

The combination of micromanipulation of FISH-labelled filaments or microcolonies in activated sludge samples with RT-PCR for further identification was successful. A great advantage of using FISH-labelled samples is that the identity is known to a certain extent, e.g. to belonging to the Beta 1 group of *Betaproteobacteria*, which can be tested after sequencing so the number of oligonucleotide

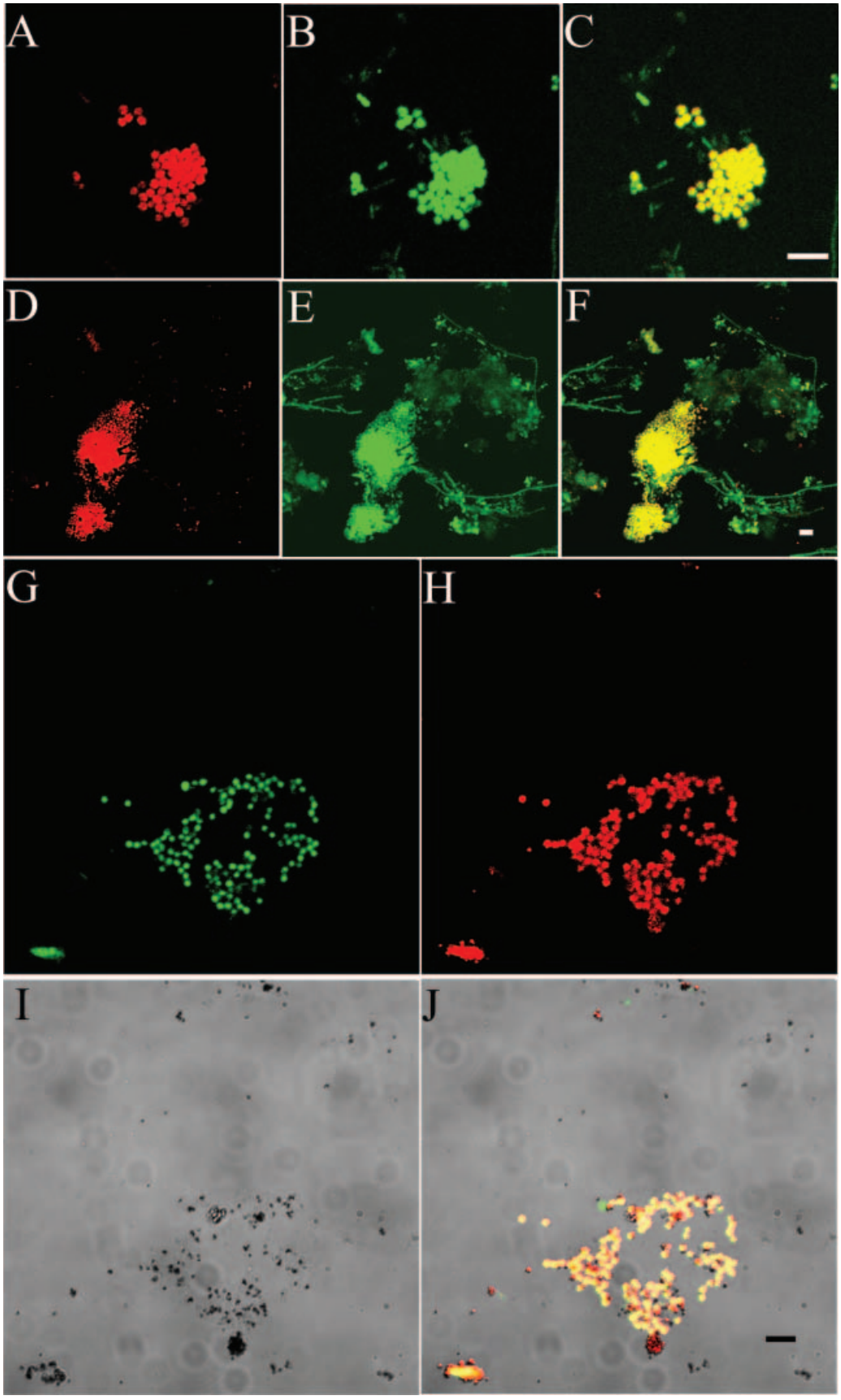
A
B
C
D
E
F
G
H
I
J

Table 2. Uptake of radiolabelled substrates by *Aquaspirillum*-related bacteria (Aqs) or other floc-forming bacteria (Floc) with oxygen, nitrate and nitrite as electron acceptor, and under anaerobic conditions in Aalborg East WWTP. -: no uptake, + : uptake observed.

	Oxygen		Nitrate		Nitrite		Anaerobic	
	Aqs	Floc	Aqs	Floc	Aqs	Floc	Aqs	Floc
Acetate	–	+	–	+	–	–	–	+
Propionate	–	+	–	+	–	–	–	+
Lactate	–	+	–	+	–	–	–	+
Amino acid mixture	+	+	+	+	+	+	–	+
Ethanol	–	+	–	+	–	–	–	+
Glucose	–	+	–	+	–	–	–	+

probes to be designed and tested can be minimized. The applied method was more successful on filamentous bacteria than on microcolonies, as the filaments were generally easier to separate from the floc material. It was not possible to obtain microcolonies consisting of only one single species, but only highly enriched samples from the micromanipulated microcolonies. In some cases this was due to the presence of several species within the microcolony, in others probably contamination during the micromanipulation procedure. This might vary according to sludge type and bacteria species, and according to our experience some sludges and microcolonies are more suitable than others for micromanipulation. This simple method could probably be improved, but as it was generally impossible to micromanipulate single, pure microcolonies, colonies from several micromanipulations can be pooled followed by RT-PCR to minimize the further work.

Several other methods of sorting bacteria exist, e.g. micromanipulation using a Bactotip (Fröhlich and König, 1999) and cell fishing based on biotinylated polyribonucleotide probes (Stoffels *et al*., 1999). In complex samples, only flow cytometry has been used successfully to sort bacteria (Wallner *et al*., 1997). The advantages of micromanipulation compared with flow cytometry are that the sorting can be based on morphology in case no relevant oligonucleotide probe exists, the sensitivity is higher, it is possible to isolate microcolonies despite the 3D-structure of the cell aggregate, and that the method is rather simple and the costs are low.

Identity and significance of microcolony-forming bacteria

The identified bacterium represented by the sequences AquaspiA-C was closely related to *Pseudomonas lanceolata* and *Aquaspirillum delicatum* in the family *Neisseriaceae* and a clone GOUTB19 described by Alfreider *et al*. (2002). The clone GOUTB19 was present in one out of 87 sequences obtained in a study of a reactor treating polluted ground water. The nomenclature we have used is in accordance with the new RDP preview release (Cole *et al*., 2003) and Bergey's manual (Garrity *et al*., 2002). The only difference is that in the new RDP preview release *Pseudomonas lanceolata* is placed within the *Betaproteobacteria* in the genus *Aquaspirillum,* whereas in Bergey's manual it affiliates with the *Gammaproteobacteria*. In the older version of RDP, *Aquaspirillum delicatum* and *Pseudomonas lanceolata* are both placed in the *Acidovorax* group within the *Betaproteobacteria.*

The experiments using MAR showed that most *Aquaspirillum*-related bacteria were active under both aerobic and anoxic conditions with nitrate and nitrite as electron acceptor suggesting that they are denitrifiers with a capability to grow under such conditions. This is in agreement with other recent studies showing that other bacteria related to the family *Neisseriaceae* and *Comamonadaceae* are denitrifiers. Denitrifiers have been isolated from activated sludge several times (e.g. Gumaelius *et al*., 2001; Khan and Hiraisi, 2001; Khan *et al*., 2002; Hiraishi and Khan, 2003). A few of these strains are relatively closely related to the *Aquaspirillum*-related sequences obtained in our study (e.g. 94.8% similarity with a *Aquaspirillum psycrophilum*-related strain NOS8 from Khan *et al*., 2002).

The closest isolated relatives to the Aquaspi- sequences, *Aquaspirillum delicatum* and *Pseudomonas lanceolata,* are heterotrophs, grow under aerobic conditions, but are unable to perform full denitrification (can reduce nitrate to nitrite) (Leifson, 1962; Krieg, 1984; Pot

Fig. 2. FISH micrographs showing two microscopic fields (A–C and D–F) of activated sludge from Aalborg East WWTP revealing the clustering and abundance of Aqs997-positive cells. Panels G–J show the MAR-FISH micrographs of a microcolony consisting of both MAR-positive and MAR-negative *Aquaspirillum*-related cells (I) incubated under anaerobic conditions with nitrate as electron acceptor and with a mixture of ^{3}H-labelled amino acids. Panels A, D and H show the sludge hybridized with Cy3-labelled Aqs997 probe, and panels B, E and G show the hybridization with FLUOS-labelled EUBmix. Panel C and F are superimposed images of A+B and C+D respectively, whereas panel J is the overlay of panels G–I. The scale bars correspond to 5 μm.

et al., 1991). Clone GOUTB19 and GOUTB4 from Alfreider *et al*. (2002) were found in the monochlorobenzene-contaminated groundwater flowing out from a reactor. In the *Neisseriaceae* family denitrifying isolates are known, e.g. strain NOS8, NSP4 and KSP2 from Khan *et al*. (2002), which were isolated under denitrifying conditions using poly(3-hydroxybutyrate-Co-3-hydroxyvalerate) as substrate. However, most relatives to the Aquaspi-sequences are still undescribed species and clones (See Fig. 1).

So far, the dominant denitrifiers in nutrient removal plants treating municipal wastewater are unknown. In industrial wastewater treatment plants, bacteria belonging to the genera *Azoarcus*, *Thauera,* and *Zoogloea* are abundant and believed to be the dominant denitrifiers (Juretschko *et al*., 2002; Wagner and Loy, 2002). Our study strongly suggests that bacteria belonging to the genus *Aquaspirillum* were dominant denitrifiers in the municipal treatment plants investigated. They were present in lower numbers in the WWTPs treating industrial wastewater, where *Thauera* and *Zoogloea* were often found in large numbers and were thus likely to be the dominating denitrifyers. It will be interesting to reveal why this difference in the dominant population exists. It might be related to the incoming wastewater composition where municipal wastewater is relatively similar among the treatment plants, at least in Denmark, whereas industrial wastewater can be very different from plant to plant. The capability to consume amino acids could indicate that they are important in the degradation of proteins in the wastewater. However, there was also a difference in the phosphorus removing operation of the plants, so although the *Aquaspirillum*–related bacteria did not exhibit typical phosphorus accumulating capabilities with uptake of organic substrate under anaerobic conditions, the growth conditions in these treatment plants may affect the dominance. Future studies must be carried out to enlighten this.

It was interesting to observe that some microcolonies consisting of Aqs997-positive cells were mixed with other, so far unidentified, bacteria. As these types of colonies are as abundant as the pure microcolonies and present in all plants investigated, this could indicate that some sort of interaction takes place.

The new oligonucleotide probe developed, Aqs997, seems rather specific and targets particularly microcolony-forming bacteria consisting of characteristic coccoid cells. However, a few filamentous bacteria and a few single cells were also targeted by probe Aqs997. If the different morphotypes represented by a filamentous morphotype, and the characteristic coccid cells primary found as microcolonies but also as single cells correspond to different species it might be necessary to develop 23S rRNA probes to reveal this difference. However, the MAR experiments indicated that it is the same species as the substrate uptake patterns were very similar for the various morphotypes. Other bacteria are known also to grow as filaments and single cells depending on the growth conditions (e.g. Foot *et al*., 1992; Gumaelius *et al*., 2001).

The *Aquaspirillum*-related bacteria were the most abundant *Betaproteobacteria* in Aalborg East WWTP, and as bacteria belonging to this group have previously exhibited very strong floc-forming properties (Nielsen *et al*., 2004), it is likely that it was in fact the *Aquaspirillum*-related bacteria that were responsible for these properties. It is known that *Zoogloea* and *Thauera* produce extreme water containing exopolymers that can give settling and dewatering problems (Lajoie *et al*., 2000) in a way that is rarely seen in municipal treatment plants with N-removal. In any case, as dominant bacteria in these nutrient removal plants, they will strongly influence the floc properties, and this will be investigated further in future studies.

Experimental procedures

Activated sludge sampling

The experiments were carried out with activated sludge from Aalborg East and Grindsted WWTP, Denmark. Aalborg East WWTP has carbon removal, nitrification, denitrification, chemical and biological phosphorus removal and a mean cell residence time (sludge age) of 20–30 days. Grindsted WWTP is described by Nielsen *et al*. (2000). The activated sludge used for fluorescence *in situ* hybridization and micromanipulation was fixed according to Amann (1995), and sludge used for microautoradiography was collected and treated as described by Nielsen and Nielsen (2002b).

FISH

Fluorescence *in situ* hybridization (FISH) was performed according to Amann (1995). The following oligonucleotide probes were used in the preliminary screening of microcolony forming bacteria related to the *Betaproteobacteria* in activated sludge from Aalborg East WWTP: NEU and Nso190 (ammonia-oxidizing bacteria), Azo644 (targeting most *Azoarcus*), PAO651 *(*specific for *Candidatus* 'Accumulibacter phosphatis'), ZRA (*Zoogloea ramigera*), BONE23A (Beta 1 group of *Betaproteobacteria*), BTWO23A (Beta 2 group of *Betaproteobacteria*), and Bet42a (*Betaproteobacteria*). Further information about the probes can be found in probeBase (Loy *et al*., 2003). The probe Thau646 (*Thauera* spp.) is described by Lajoie *et al*. (2000).

Suspension of FISH-labelled activated sludge was conducted using a modified version of the protocol published by Fuchs *et al*. (1998). Ethanol fixed sludge from Aalborg East WWTP was gently homogenized using a glass tissue grinder (Thomas Scientific®). Forty microlitres of the suspended sludge was pelleted by centrifugation for 5 min at 1924 *g* and resuspended in 40 µl hybridization buffer with the used oligo-

nucleotide probes (final concentration 50 ng probe μl^{-1}). After hybridization for two hours at 46°C, the sludge was pelleted for 5 min at 1924 *g* and washed with preheated washing buffer for 15 min at 48°C. The following probes were used in suspension of FISH: EUB338, EUB338-II and EUB338-III (all *Bacteria*) (Daims *et al*., 1999), BONE23A (Beta 1 group of *Betaproteobacteria*) and BTWO23A (Beta 2 group of *Betaproteobacteria*) as competitor for BONE23A (Loy *et al*., 2003) and Meg983 (*Meganema perideroedes*) (Thomsen *et al*., 2004).

Micromanipulation

Glass slides were covered with 1% agarose, and 5 μl suspension of FISH-labelled activated sludge was placed on the solidified agarose. A Skerman micromanipulator (Skerman, 1968) was mounted on a long distance 33 × objective on an Axioskop 2 plus microscope (Carl Zeiss), and the desired FISH-labelled microcolony was micromanipulated using a glass capillary needle and directly transferred to a PCR tube containing 5 μl DEPC-treated water (deionized, high quality, molecular biology grade water). The setup was tested on filamentous bacteria abundant in an industrial WWTP in Grindsted, Denmark.

Reverse transcriptase PCR (RT-PCR)

Superscript One-Step RT-PCR with Platinum Taq (Invitrogen Life Technologies) was applied for cDNA synthesis and subsequent PCR according to the manufacturer's protocols using a gene-specific primer. PCR was performed using the primers 27F and 1390R (Lane, 1991). Only micromanipulations on ethanol fixed samples were successfully used for RT-PCR, whereas paraformaldehyde was found to inhibit the reverse transcriptase.

Cloning

Fresh PCR amplificates were purified using a QIAquick PCR Purification Kit (Qiagen GmbH). The amplificates were ligated into a pCR®4-TOPO® vector and transformed into ONE SHOT *Escherichia coli* cells following the manufacturer's directions (Invitrogen). Plasmids of 20 randomly selected clones were then recovered using a QIAprep spin miniprep kit (Qiagen GmbH).

Screening of clones by denaturing gradient gel electrophoresis (DGGE)

Polymerase chain reaction products were obtained from the selected plasmids using the primers 341F-GC (Muyzer *et al*., 1993) and 907r (Lane, 1991). Denaturing gradient gel electrophoresis was performed using the D-Gene System (Bio-Rad, Herlev, Denmark) according to the manufacturer's guidelines, using polyacrylamide gels with a denaturing gradient of 30–70%. Gels were run for 15 h at 100 V in 1× TAE buffer at a temperature of 60°C, and were stained in 100 ng μl^{-1} SYBR Gold (Molecular Probes). Plasmids showing different band patterns were chosen for sequencing.

Sequencing

Polymerase chain reaction amplification was performed on diluted plasmids using M13 primers. Sequence reactions were performed using 0.5 μl primers (10 pmol μl^{-1}), 5 μl DYEnamic ET dye terminator sequencing kit (Amersham Biosciences) and 2–7 μl purified PCR product in each set-up. After amplification the reactions were loaded on a MegaBACE DNA sequencer (Amersham Biosciences).

Phylogeny and oligonucleotide probe design

Near complete (1363 bases) 16S rDNA sequences were compiled and aligned using the automatic nucleic acid aligner in the ARB software package (http://www.arb-home.de), and alignments were refined manually. Checks for chimeric sequences were conducted using the CHECK_CHIMERA program of Ribosomal Database Project (http://rdp.cme.msu.edu) and the program BELLEROPHON developed by Hugenholtz and Huber (2003). Only unambiguously aligned sequences were used for calculation of trees by distance matrix, parsimony, and maximum likelihood approaches using default settings in the ARB software. Further phylogenetic analysis was performed using PAUP* version 5.0b (written by David Swofford) as described in Hugenholtz *et al*. (2001). A distance matrix was calculated using default settings in PAUP* version 5.0b.

Oligonucleotide probes were designed using the probe design/match tools of the ARB software package. Oligonucleotides were 5′-labelled with 5(6)-carboxyfluorescein-*N*-hydroxy-succinimide ester (FLUOS) or with sulphoindocyanine dyes (Cy5 and Cy3) (Thermo Hybaid, Ulm, Germany). Probe-binding profiles were performed using hybridization buffers containing 0% to 70% formamide at 5–10% increments on fixed activated sludge samples from Aalborg East WWTP, *Aquaspirillum delicatum* ATCC14667 (having perfect match to oligonucleotide probe Aqs997), and *Escherichia coli* ATCC25922 (chosen as non-target organism with four mismatches to the target site). Quantification of signal intensity was performed using image analysis. The most stringent conditions, which gave a bright signal with the presumed target cells and the pure culture, were chosen as optimal hybridization conditions for the oligonucleotide probes and used in the subsequent survey.

The FISH survey was conducted on different activated sludge samples fixed according to the procedures described for Gram-negative and Gram-positive bacteria by Amann (1995). The samples were immobilised on gelatine-coated slides, dehydrated in series of ethanol and subsequently hybridized.

Autoradiography

Microautoradiography (MAR) experiments were performed according to the procedure described by Nielsen and Nielsen (2002b). The incubations were carried out using 2 ml diluted activated sludge (1 g SS l^{-1}). A [^{3}H]-labelled amino acid mixture (16 amino acids, ICN Biomedicals) was used. The final concentration of each amino acid was adjusted to 0.1 mM by adding unlabelled amino acids. The amount of radioactivity

of each amino acid varied from 6.8 to 28.8 μmCi/2 ml. [^{3}H]-labelled acetate, ethanol, glucose, lactate, and [^{14}C]-propionate (Amersham Biosciences) were adjusted to a final concentration of 1 mM and 10 μmCi/2 ml. The potential use of nitrate and nitrite as electron acceptor was tested using a preincubation step with unlabelled substrate for 2 h before the labelled substrate was added (Andreasen and Nielsen, 2000). A preincubation step was also included under anaerobic conditions. An incubation time with labelled substrates of 3 h was used under aerobic, anoxic conditions with nitrate and nitrite and under anaerobic conditions. Nitrate and nitrite concentrations were 2 mM and 0.5 mM in anoxic incubations respectively. As a control for chemography, sludge was pasteurized at 70°C for 10 min just before the incubation. After incubation the activated sludge was fixed and prepared as described under the FISH procedure before applying the photographic emulsion (Lee *et al*., 1999).

Nucleotide accession numbers

The GenBank accession numbers for the 16S rRNA sequences reported in this paper are AY322151- AY322153.

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