Applied and Environmental Microbiology, Mar. 2008, p. 1517–1526 Vol. 74, No. 5
0099-2240/08/$08.00+0 doi:10.1128/AEM.02274-07

Amyloid-Like Adhesins Produced by Floc-Forming and Filamentous Bacteria in Activated Sludge[▽]

Poul Larsen, Jeppe Lund Nielsen, Daniel Otzen,† and Per Halkjær Nielsen*

Department of Biotechnology, Chemistry, and Environmental Engineering, Aalborg University, Sohngaardsholmsvej 57, DK-9000 Aalborg, Denmark

Received 6 October 2007/Accepted 26 December 2007

Amyloid proteins (fimbriae or other microbial surface-associated structures) are expressed by many types of bacteria, not yet identified, in biofilms from various habitats, where they likely are of key importance to biofilm formation and biofilm properties. As these amyloids are potentially of great importance to the floc properties in activated sludge wastewater treatment plants (WWTP), the abundance of amyloid adhesins in activated sludge flocs from different WWTP and the identity of bacteria producing these were investigated. Amyloid adhesins were quantified using a combination of conformationally specific antibodies targeting amyloid fibrils, propidium iodide to target all fixed bacterial cells, confocal laser scanning microscopy, and digital image analysis. The biovolume fraction containing amyloid adhesins ranged from 10 to 40% in activated sludge from 10 different WWTP. The identity of bacteria producing amyloid adhesins was determined using fluorescence in situ hybridization with oligonucleotide probes in combination with antibodies or thioflavin T staining. Among the microcolony-forming bacteria, amyloids were primarily detected among *Alpha-* and *Betaproteobacteria* and *Actinobacteria*. A more detailed analysis revealed that many denitrifiers (from *Thauera*, *Azoarcus*, *Zoogloea*, and *Aquaspirillum*-related organisms) and *Actinobacteria*-related polyphosphate-accumulating organisms most likely produced amyloid adhesins, whereas nitrifiers did not. Many filamentous bacteria also expressed amyloid adhesins, including several *Alphaproteobacteria* (e.g., *Meganema perideroedes*), some *Betaproteobacteria* (*e.g.*, *Aquaspirillum*-related filaments), *Gammaproteobacteria* (*Thiothrix*), *Bacteroidetes*, *Chloroflexi* (e.g., Eikelboom type 1851), and some foam-forming *Actinobacteria* (e.g., *Gordonia amarae*). The results show that amyloid adhesins were an abundant component of activated sludge extracellular polymeric substances and seem to have unexpected, divers functions.

Among the most important factors for activated sludge wastewater treatment plants (WWTPs) to operate well is the solid-liquid separation, which includes flocculation, clarification, settling, and dewatering (44). The outcome of these steps is to a large extent determined by the number of filamentous bacteria that may cause bulking (poor settling) if present in large numbers and the characteristics of the activated sludge flocs (37, 44, 69). Particularly for the flocs, the amount, composition, and properties of the extracellular polymeric substances (EPS) are assumed to be very important for flocculation and dewatering (40, 68). The EPS fraction consists mainly of a mixture of proteins, polysaccharides, lipids, nucleic acids, and humic substances (44). Proteins are the major component, but the detailed composition has not yet been adequately described.

Recently, we found that proteinaceous amyloid adhesins are an abundant component of the EPS fraction in biofilms from different habitats among these activated sludge flocs (32). Bacterial amyloids are usually thin fibrils, insoluble, and extremely resistant to various denaturants (8). This is primarily due to the chemical structure of the amyloid proteins, which are folded as beta sheets and stacked perpendicularly to the long fibril axis (60). They share many similarities to amyloid fibrils, which are known to cause neurodegenerative diseases such as Parkinson's and Alzheimer's disease in humans (6). Although many different bacteria are presumably able to produce amyloid adhesins (32), detailed descriptions of the size, amount, and properties of bacterial amyloids are restricted to pure culture studies of mainly *Escherichia coli*, *Salmonella* species, and the gram-positive *Streptomyces coelicolor* (48). Amyloid adhesins (named curli) produced by *E. coli* are 4 to 12 nm wide and 0.1 to 10 μm long (6, 15).

The bacterial amyloids in activated sludge and other biofilms are expressed by a broad range of phylogenetically distant species in the phyla *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, and *Actinobacteria* (32). Generally, the function of amyloid fibrils is assumed to be related to enhanced adhesion to surfaces (49) and biofilm formation, but they may also increase resistance to chlorine (55) and resistance to chemical and enzymatic digestion (45). The function in activated sludge flocs is still unknown but may be related to the aggregation of microorganisms internally in microcolonies, whereas it is more uncertain what the function is in the filamentous bacteria, which were also shown to produce amyloids (32). Activated sludge flocs are often described as having a strongly and loosely bound fraction of cells and EPS (23, 57), so amyloids might be a good candidate for contributing to the high stability of the strongly bound fraction. It is, however, still unknown which species produce amyloids and what roles they have among the various functional groups in activated sludge and biofilm systems,

* Corresponding author. Mailing address: Department of Biotechnology, Chemistry, and Environmental Engineering, Aalborg University, Sohngaardsholmsvej 57, DK-9000 Aalborg, Denmark. Phone: 45 99408503. Fax: 45 96350558. E-mail: phn@bio.aau.dk.

† Present address: Interdisciplinary Nanoscience Centre, Department of Molecular Biology, Aarhus University, Gustav Wieds Vej 10C, DK 8000 Aarhus C, Denmark.

▽ Published ahead of print on 11 January 2008.

such as nitrifiers, denitrifiers, polyphosphate-accumulating organisms (PAO), glycogen-accumulating organisms (GAO), and filamentous bacteria.

Detection of sessile bacteria producing amyloids can be performed by staining with thioflavin T (ThT) or by labeling with antibodies targeting a generic conformational epitope on amyloidal proteins (32). Antibodies have been found to be very specific for labeling of amyloid adhesins (32, 47), whereas ThT suffers from some nonspecificity, since it can also bind to cellulose and DNA (19, 51). As most bacteria in environmental biofilms are still uncultured, a combination of ThT or antibodies with fluorescence in situ hybridization (FISH) and oligonucleotide probes can be used to identify bacteria producing amyloid adhesins (32). This approach is particularly well suited for activated sludge systems, as most abundant bacteria can now be identified by culture-independent methods to species or genus level and thus are detectable by available oligonucleotide probes (28).

The aim of this study was to investigate the extent of amyloid adhesins in a range of activated sludge treatment plants and to identify the phylogenetic affiliation of microcolony-forming and filamentous bacteria producing amyloids by culture-independent methods.

MATERIALS AND METHODS

Sample collection and preparation. Samples of activated sludge were collected from the aeration tanks of 43 different WWTPs treating both municipal and industrial wastewater. The samples were chosen from a large collection of activated sludge samples used in various other studies for FISH analysis. For each oligonucleotide probe tested together with antibody, two samples with high abundance of the target organisms (typically, 2 to 10% of the biomass) were chosen from the sample collection. Samples used with oligonucleotide probes targeting gram-negative bacteria were fixed with 4% paraformaldehyde for 3 h at 4°C, followed by washing in sterile-filtered (0.22-μm-pore-size polycarbonate filter) tap water. After the final washing step, the samples were resuspended in phosphate-buffered solution (PBS)–ethanol (diluted 1:1) and stored at −20°C. Samples used to target gram-positive bacteria were fixed with 50% ethanol and stored at −20°C. We observed the same degree of antibody labeling of a fresh sample and a 2-year-old fixed sample. For the quantitative analysis of the fraction of microorganisms producing amyloid adhesin in sludge, 10 out of the 43 WWTP samples were chosen. These samples were taken from five plants with and five plants without biological phosphorus removal. All sludge samples tested were gently homogenized to a level where the probe-defined microcolonies and filamentous bacteria were exposed as free floating in the bulk liquid or exposed on the surface of the activated sludge flocs. This ensured that problems with the access of antibodies to the EPS-embedded microcolonies were minimized.

Pure cultures. Pure cultures of *Haliscomenobacter hydrossis* (DSM 1100) and *Sphaerotilus natans* (DSM 6575) were acquired from Deutsche Sammlung von Mikroorganismen (DSMZ), and *Zoogloea ramigera* Itzigzohn (ATCC 19544) was acquired from the American Type Culture Collection (ATCC). The media employed were DSMZ 134, DSMZ 51, and ATCC 1858 for *H. hydrossis*, *S. natans*, and *Z. ramigera*, respectively. Further details about growth conditions and medium description of the cultures are available from the DSMZ and ATCC. Paraformaldehyde-fixed samples of *Chloroflexi* morphotype 1851 strain EU25 (30) and *M. perideroedes* strain Gr1 (62) were used to support staining/labeling results from activated sludge samples.

Detection of amyloid adhesins in activated sludge samples. Detection of amyloid adhesins was performed using conformationally specific antibodies (32). The protocol was the same on fresh and fixed samples, apart from the addition of 0.05% sodium azide to the blocking buffer when fresh samples were used. Briefly, two conformationally specific antibodies, WO1 and WO2 (47), were used. The sludge samples were homogenized using a glass tissue grinder (Thomas Scientific), centrifuged (9,500 × *g* for 5 min), and resuspended to an optical density at 650 nm of approximately 1 in PBS containing 1% (wt/vol) gelatin as a blocking buffer. The samples were preincubated at 37°C for 1 h, after which antibodies WO1 or WO2 and Tween 20 were added to a final concentration of 10 nM and 0.05% (wt/vol), respectively. The samples were incubated for 2 h at 37°C. The primary antibody was removed by centrifugation (9,500 × *g* for 5 min), and the pellets were washed once in 80 μl of PBS containing gelatin (1%, wt/vol) and 0.1% (vol/vol) Triton X-100. Incubation with a 1:256 dilution of fluorescein isothiocyanate-conjugated secondary immunoglobulin M antibody (Sigma), 0.025% Tween 20, and PBS containing gelatin (1%, wt/vol) was carried out for 1 h at 37°C. Finally, the samples were washed three times as described above but without gelatin in the washing buffer. After the final washing step, the samples were resuspended in 80 μl of PBS and stored at 4°C until microscopy. Antibody staining of fresh samples was supplemented with ThT staining (32).

FISH. FISH was performed using 16S rRNA-targeted oligonucleotide probes (Tables 1 and 2) hybridized in suspended samples (14). Paraformaldehyde-fixed sludge samples were gently homogenized using a glass tissue grinder (Thomas Scientific). A total of 40 μl of homogenized sludge was pelleted by centrifugation for 5 min at 9,500 × *g*, washed, and resuspended in 80 μl of hybridization buffer. The sample was vortexed and pelleted again, followed by resuspension in 80 μl of hybridization buffer with the oligonucleotide probes used (final concentration, 50 ng of probe ml^{-1}). Oligonucleotide probes were labeled with 5(6)-carboxyfluorescein-*N*-hydroxy-succinimide ester (FLUOS) or with the sulfoindocyanine dyes (Cy3 and Cy5) (Biomers, Germany).

Combination of antibody and propidium iodide. Samples labeled with primary and secondary antibodies were stained with propidium iodide by mixing equal volumes of sludge sample with a stock solution of propidium iodide (60 μM) (Molecular Probes, The Netherlands), followed by a 15-min incubation in the dark. Staining with propidium iodide was performed only on prefixed samples to ensure penetration of propidium iodide.

For quantification of amyloid adhesins in multispecies biofilms, the combination of propidium iodide and antibody proved to be superior to the combination of DAPI (4′,6′-diamidino-2-phenylindole) and antibody (32) in two ways. First, it avoided a labor-intensive relocation step, and, second, it ensured a higher quality of the images by using the same focal layer on the confocal laser scanning microscope (CLSM). Each sample was tested in replicates, and 10 images were acquired on each replicate.

Combination of antibody and FISH. The activated sludge samples hybridized with oligonucleotide probes were fixed once again in 4% paraformaldehyde overnight. After this step the samples were washed twice in PBS, followed by labeling with primary and secondary antibodies as described above. This combination of FISH and antibody labeling is a slight modification of previous studies (32). The protocol used in this study leads to increased fluorescence signals from oligonucleotide probes and less interference from blocking agents.

Microscopy. A CLSM (LSM 510 META; Carl Zeiss) equipped with an Ar ion laser (458 and 488 nm) and two HeNe lasers (543 and 633 nm) was used to record digital images for quantification of the antibody-positive fraction of the activated sludge samples. Images recorded as documentation of positive antibody labeling of different probe-defined groups were recorded using either this CLSM or a Zeiss Axioscop 2 epifluorescence microscope. The settings for the CLSM were calibrated on a negative control sample with only the secondary antibody.

RESULTS

Abundance of bacteria producing amyloid adhesins. Amyloid adhesins in activated sludge originating from full-scale WWTPs were quantified by antibody and propidium iodide staining. As the process configuration of the treatment plants affects the microbial community composition and therefore also potentially the abundance of amyloids, five treatment plants with N removal and five plants with both N removal and enhanced biological phosphorus removal were selected. The amyloid-positive fraction in the different plants ranged from 10% to 40% of the total biovolume with consistent and similar results, using the two independent primary antibodies WO1 and WO2 (Fig. 1). In general, plants with biological phosphorus removal showed a higher level of binding of both antibodies (20 to 40% of the total biovolume). In all treatment plants, it was observed that certain microcolonies in the flocs produced amyloids, but filamentous bacteria were the dominant amyloid producers. Amyloids were primarily detected directly on the surface of the microorganisms, often as a thin layer or "cloud" outside the bacterial cells stained by propidium iodide

TABLE 1. Identity of microcolony-forming bacteria in activated sludge samples examined with combination of oligonucleotide probes, antibody, and ThT

Functional group	Overall phylogenetic group	Phylogenetic affiliation	Gene probe(s)	Reference(s)	Antibody binding (WWTP 1/2)[a]	ThT binding (WWTP 1/2/3)[b]
All phylogenetic groups[c]	*Alphaproteobacteria*	*Alphaproteobacteria*	ALF 968	42	+	ND
	Betaproteobacteria	*Betaproteobacteria*	BET42a	36	+	+
	Gammaproteobacteria	*Gammaproteobacteria*	GAM 42a	36	−	−
	Deltaproteobacteria	*Deltaproteobacteria*	SRB385+SRB385Db	50	−	ND
	Firmicutes	*Firmicutes*	LGC354mix	38	−	−
	Actinobacteria	*Actinobacteria*	HGC69a	52	+	+
Denitrifiers	*Betaproteobacteria*	*Aquaspirillum*-related bacteria	Aqs997	64	−/−	+/+/+
	Betaproteobacteria	*Zoogloea ramigera*	ZRA	54	−/−	+/ND/ND
	Betaproteobacteria	*Thauera* spp.	Thau646	31	−/−	−/+/ ND
	Betaproteobacteria	Most *Azoarcus*	Azo644	17	−/−	+/−/−
PAO	*Betaproteobacteria*	*Rhodocyclus*-related PAO	PAOmix: PAO462+PAO651+ PAO846	10	−/−	−/−
	Actinobacteria	*Actinobacterial* PAO	Actino-221+c1Actino-221+c2Actino-221, Actino-658+c1Actino-658+c2Actino-658	26	−/−	−/+/+
GAO	*Alphaproteobacteria*	*Defluviicoccus* spp.	DF2Mix: DF988+DF1020+ H966 +H1038	39	−/−	
	Gammaproteobacteria	*Competibacter*-related GAO	GAOmix: GAOQ989+GB_G2	9, 27	−/−	−/−
Nitrifiers	*Betaproteobacteria*	*Betaproteobacterial* ammonia-oxidizing bacteria	Nso190, Nso1225	41	−/−	−/−
	Betaproteobacteria	*Nitrosomonas oligotropha* lineage	Cluster 6a	1	−/−	−/−
	Nitrospira	*Nitrospira*	Ntspa662	11	−/−	−/−

[a] −, no binding of antibody to probe-defined group; +, binding of antibody to probe-defined group determined in a sample from one WWTP.
[b] ND, not determined.
[c] Tested in only one sample.

(Fig. 2a and b). Some of the amyloid adhesins were also detected in the EPS fractions of the sludge, where the amyloids could not be directly linked to microorganisms. This appeared both as amorphous aggregates and some elongated tubes, probably partly degraded filamentous bacteria or their sheaths.

Identity of bacteria producing amyloid adhesins in microcolonies. The identity of amyloid-producing microorganisms was first investigated using the broad oligonucleotide probes in combination with WO1, revealing some microcolony-forming bacteria among the *Alpha-* and *Betaproteobacteria* and *Actinobacteria*. Subsequently, a selection of probes targeting microcolony-forming nitrifiers, denitrifiers, PAO, and GAO were investigated for amyloid adhesins. However, all these specific probe-defined groups tested negative for amyloid adhesins by antibody labeling in all samples investigated (Table 1). The combination of antibody and oligonucleotide probes worked without problems in most cases. Only for probe Nsv443 targeting *Nitrosospira* spp. and probe NmV targeting *Nitrosococcus mobilis* was the FISH signal lost when it was used in combination with antibodies (results not shown).

In order to verify these results, the less specific dye ThT was also combined with FISH and applied to the most abundant probe-defined groups. Initially, each probe-defined group was analyzed in two WWTPs where they were abundant. If the results were different in these plants, one to two more plants were investigated to verify the results. ThT-positive bacteria were identified among the denitrifiers and the actinobacterial PAO, whereas all ammonium and nitrite-oxidizing nitrifiers were found to be ThT negative (Table 1). Among the denitrifiers, a ThT-positive subpopulation of *Aquaspirillum*-related bacteria was detected in all WWTPs (Fig. 2c). This population of coccoid cells formed characteristic spherical microcolonies. *Thauera* and *Azoarcus* tested both ThT positive and negative in the WWTPs investigated, indicating that these oligonucleotide probes target different subpopulations in different WWTPs or that these organisms express amyloid adhesins only in some cases (Fig. 2f shows an example of a ThT-positive *Thauera* microcolony). *Zoogloea* microcolonies were present in only some of the WWTPs and in low abundance, which complicated in situ analysis of this probe-defined group. Therefore, a pure culture of *Z. ramigera* was tested with ThT staining showing that *Zoogloea* was highly positive close to the cell membrane but negative in the large space between the cells in the characteristic finger-like microcolonies. The same ThT signal distribution was found on *Zoogloea* microcolonies when they were tested in situ (Fig. 2d). When the pure culture was tested with antibodies, no binding was found, indicating that

TABLE 2. Identity of filamentous bacteria in activated sludge samples examined with the combination of oligonucleotide probes and antibody

Overall phylogenetic group	Phylogenetic affiliation	Gene probe	Reference(s)	Antibody binding[b]	
				WWTP1	WWTP2
Class *Alphaproteobacteria*	"*Candidatus* Meganema perideroedes"	Meg983+Meg1028	62	+	+
	"*Candidatus* Monilibacter batavus"	MC2-649	58	−	−
	"*Candidatus* Sphaeronema italicum"	Nost993+Helper1010	29	+[c]	+[c]
	"*Candidatus* Alysiosphaera europaea"	Noli-644	58	+	+[c]
	"*Candidatus* Alysiomicrobium bavaricum"	PPx1002	29	−[c]	−
Class *Betaproteobacteria*	*Aquaspirillum*-related bacteria	Aqs997	64	+	+
	Sphaerotilus natans	SNA23a	65	−	+[c,d]
Class *Gammaproteobacteria*	*Thiothrix nivea*	TNI	65	−	+[a]
Phylum *Bacteroidetes*	*Cytophaga-Flavobacterium*	CFB319a+b	35	+	−
	Members of family *Flavobacteriaceae*	CFB 563	66	+	−
	Environmental isolates; most members of the class *Bacteriodetes*	CFB719	66	+	−
	Members of the family *Saprospiraceae*	Sap-309	56	−	−
	Haliscomenobacter hydrossis	HHY	65	−	−
Phylum *Chloroflexi*	Phylum *Chloroflexi*	CFXmix: CFX1223+GNSB941	4, 16	+	+
	Chloroflexi subdivision 3	CFX109	4	+	+
	Chloroflexi subdivision 1a-1b	CFX784	4	+	ND
	Eikelboom morphotype 1851	Chl1851	2	+	+
Candidate phylum TM7	Most of Candidate division TM7	TM7-905	18	−[c]	−[c]
Phylum *Actinobacteria*	*Microthrix sp.*	MPA-all-1410	34	−[c]	−[c]
	Skermania piniformis	Spin1490	13	−	−[a]
	Gordonia amarae	Gor596	12	−	−[a]

[a] For some filamentous bacteria, the combination of antibody and FISH did not work, but some of these filamentous bacteria could be targeted and identified by FISH alone. Labeling the same sample with antibody alone enabled identification of these filamentous bacteria by comparison with the morphology of filamentous bacteria in the FISH-probed sample.

[b] −, no binding of antibody to probe-defined group; +, binding of antibody to probe-defined group; ND, not determined because of poor binding of oligonucleotide probe and no possibility of morphological identification.

[c] Identified only on the basis on morphology.

[d] Only empty sheaths were positive. Filaments containing FISH-positive bacteria were negative.

the amyloids were masked by other exopolymers or that ThT stained nonamyloidal material. The ThT-positive actinobacterial PAO were identified with probe Actino-658 targeting bacteria with rod morphology (Fig. 2e). Tetrad-forming PAO targeted with probe Actino-221 were not positive with ThT in any of the WWTPs investigated.

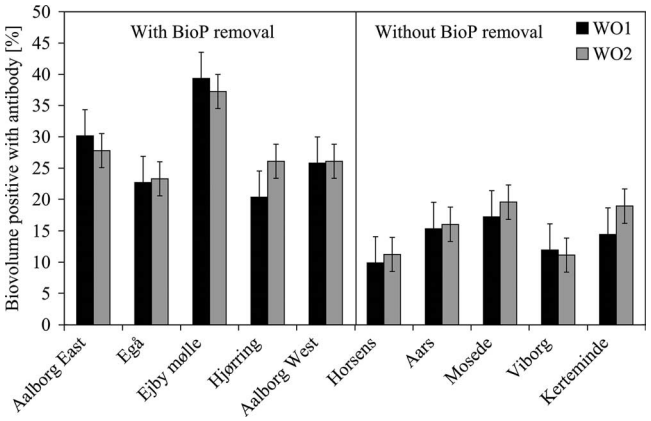

FIG. 1. Fraction of microorganisms positive with antibodies (WO1 and WO2) in activated sludge from 10 different WWTPs. Values were determined by dual staining with antibody and propidium iodide. The error bars indicate the standard error. BioP, biological phosphorus.

Identity of filamentous bacteria producing amyloid adhesions. Many filamentous bacteria producing amyloid adhesins were observed belonging to several phylogenetic groups, such as *Alpha-* and *Betaproteobacteria*, *Bacteroidetes*, *Chloroflexi*, and *Actinobacteria* (mycolata) (Table 2). The FISH signal was highly variable between each filament, which made quantification of the fraction of positive filaments within each probe-defined group impossible. Therefore, the result in Table 2 for each sample is given as positive or negative, indicating whether bacteria clearly positive with the oligonucleotide probe were also positive with antibody WO1.

Within the *Alphaproteobacteria*, antibody-positive filaments were identified among *M. perideroedes*, "*Candidatus* Sphaeronema italicum," and "*Candidatus* Alysiosphaera europaea." The amyloids of *M. perideroedes* were almost exclusively found on the tips of the filaments and never on the sheath (Fig. 3a). Interestingly, in some cases the antibody also bound to the septum dividing the two outermost cells at the tip but rarely between cells in the middle of the filament. This indicates either that the production of amyloid adhesins primarily took place by the cells at the end of the filaments or that the relatively large primary immunoglobulin M antibody was un-

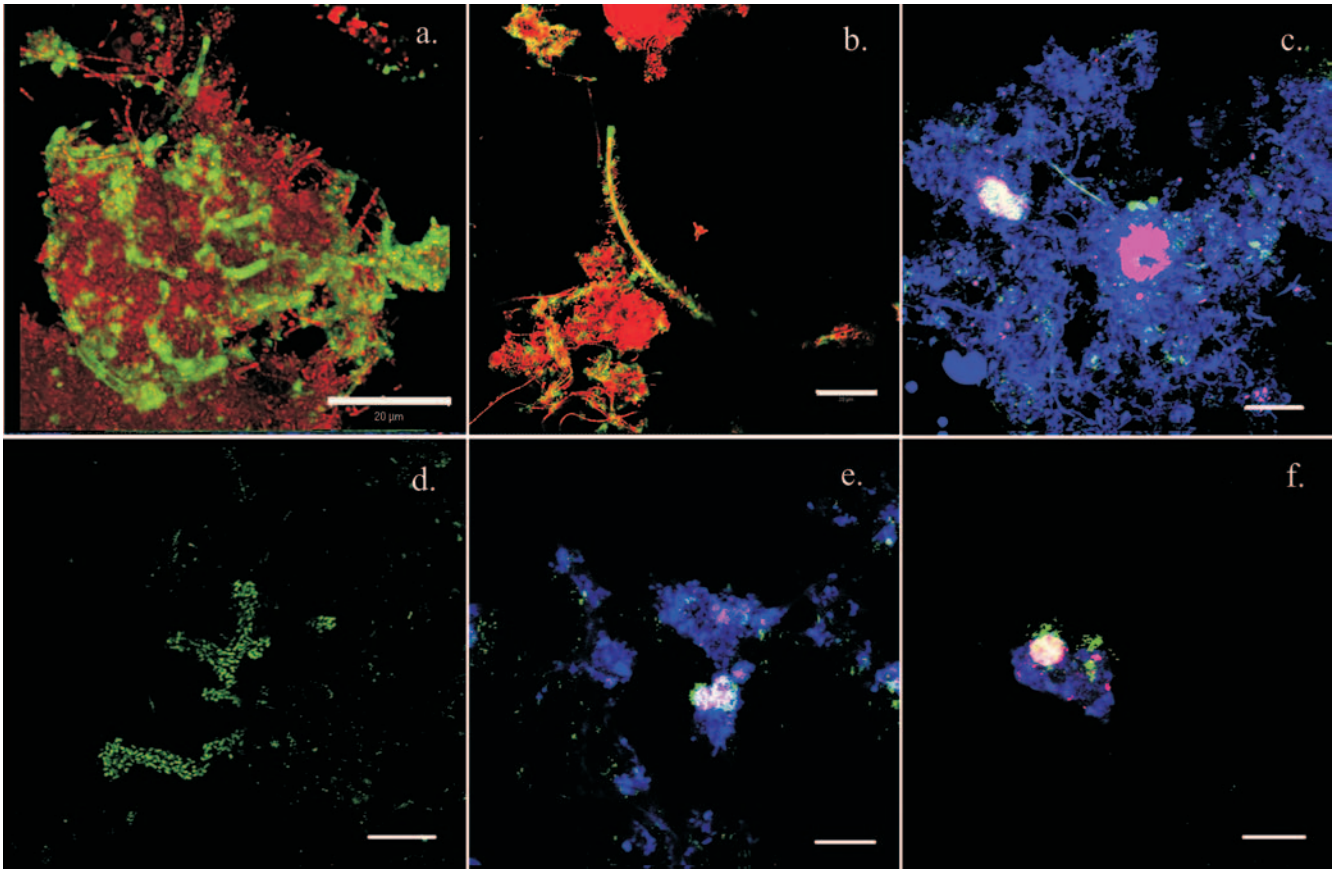

FIG. 2. Detection of amyloid adhesins in activated sludge by antibody or ThT staining in combination with propidium iodide (a and b) or oligonucleotide probes (c to f). Double staining with antibody WO1 (green) and propidium iodide (red) on paraformaldehyde-fixed sludge. (c) Simultaneous labeling of *Aquaspirillum*-related organisms with ThT (green) and oligonucleotide probes (Aqs997, red; EUBmix, blue). (d) ThT staining (green) of *Zoogloea* microcolony with finger-like morphology. (e) Simultaneous labeling of *Actinobacteria*-related PAO with ThT (green) and oligonucleotide probes (Actino658, red; EUBmix, blue). (f) Simultaneous labeling of *Thauera* with ThT (green) and oligonucleotide probes (Thau646, red; EUBmix, blue). Scale bar, 20 μm.

able to penetrate through the sheath. To test these hypotheses, ThT staining was applied since the ThT molecule is much smaller than the antibody. A fresh sludge sample with *M. perideroedes* was stained and gave a very bright signal on all cells (Fig. 3d). This shows that *M. perideroedes* most likely contained amyloids on the surface of all cells underneath a sheath that prevented penetration and binding of antibodies. Among filaments of "*Ca*. Sphaeronema italicum" and "*Ca*. Alysiosphaera europaea," approximately half were amyloid positive and half were negative in the same samples, suggesting that the oligonucleotide probe targeted different subspecies or that only some of the bacteria expressed amyloids at the time of fixation. Antibody was detected on the entire surface of the filament of "*Ca*. Sphaeronema italicum," whereas "*Ca*. Alysiosphaera europaea" showed antibody binding only at the tips. It was not possible to get fresh samples of these species for ThT staining.

Among the *Betaproteobacteria*, filamentous *Aquaspirillum*-related bacteria detected by probe Aqs997 were positive in both samples. Some of the filaments had attached cells that hybridized with probe Sap309 targeting the family *Saprospiraceae*, but these did not show any presence of amyloids. Probe-defined *S. natans* was difficult to investigate as the sludge samples mainly contained many empty sheaths, presumably from this organism, and these were amyloid positive (Table 2). However, this could not be verified as no amyloids could be detected in a pure culture of *S. natans* either with antibodies or with ThT.

The gammaproteobacterial *Thiothrix* showed a positive signal with antibody in one sample and was negative in the other sample. In the sample containing antibody-positive *Thiothrix*, the positive signal was detected only at the tips of the filaments forming rosettes. With ThT staining a positive signal was found on all the cells underneath the sheath. In some places, a single cell under the sheath was ThT negative although it was covered by a sheath, indicating that the amyloids were bound to the cells and not to the sheath (Fig. 3b and e).

In the phylum *Bacteroidetes*, most of the probes tested showed amyloid-positive filamentous bacteria. The probes for the family *Flavobacteriaceae* and the class *Bacteroidetes* revealed filaments with amyloids on the filament sheaths (Fig. 3g). Only the family *Saprospiraceae*, which includes the species *H. hydrossis*, did not express amyloid adhesins. A pure culture of *H. hydrossis* was also tested with both antibody and ThT without revealing the presence of any amyloids.

Most filamentous bacteria belonging to the phylum *Chlo-*

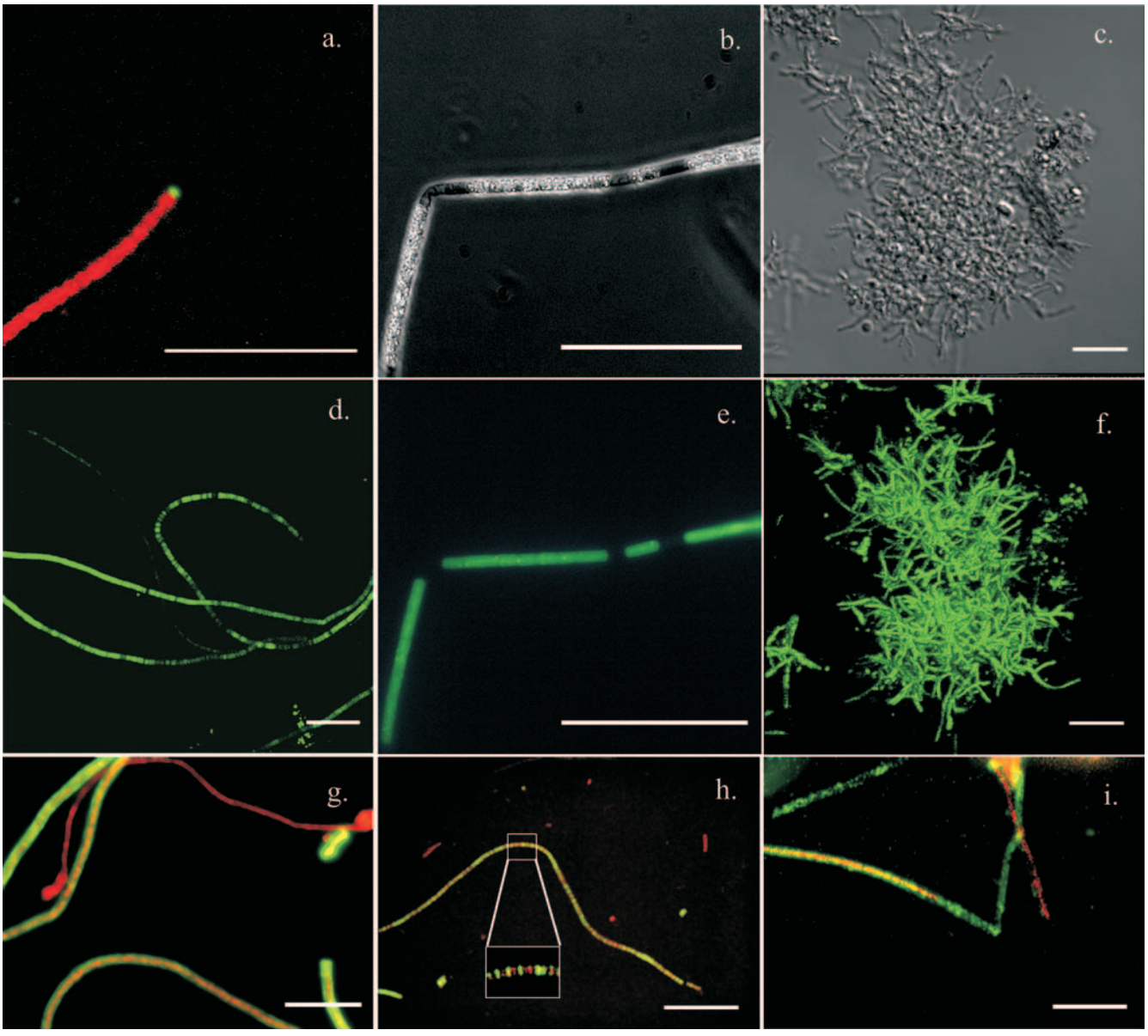

FIG. 3. Detection of amyloid adhesins on filamentous bacteria in activated sludge by antibody and ThT staining. (a) Simultaneous labeling of *M. perideroedes* filaments with antibody (green) and oligonucleotide probes (red). (b) Bright-field micrograph of *Thiothrix* with sulphur inclusions. (c) Differential interference contrast microscopy of *G. amarae*. (d) Labeling of *M. perideroedes* in sludge with ThT. (e) ThT staining (green) of the same view shown in panel b. (f) ThT staining (green) of the same view shown in panel c. (g to i) Simultaneous labeling of filaments with antibody (green) and oligonucleotide probes (red): phylum *Bacteroidetes* (g), phylum *Chloroflexi* (h), and phylum *Chloroflexi* subdivision 3 (i). Scale bar, 20 μm.

roflexi expressed amyloids. This was observed using both the broad and the more specific oligonucleotide probes. *Chloroflexi* filaments belonging to Eikelboom type 1851 (positive with the specific probe Chl1851) had amyloids. A pure culture strain of this type 1851 (strain EU25) also tested amyloid positive. Filaments from the broader subdivision 3 (probe CFX109), which were negative with probe Chl1851, were also amyloid positive. Many filamentous bacteria belonging to this subdivision expressed amyloids along the entire filament, despite the fact that, in some cases, the filaments showed regions without any cells or signals from the oligonucleotide probe. This suggested that a sheath with amyloids persisted after death of the cells (Fig. 3i). The combination of probe CFX784 and antibodies was unsuccessful, so a direct link between the production of amyloid adhesins and *Chloroflexi* subdivision 1a-1b was impossible. However, as some amyloid-positive filamentous bacteria were targeted by the overall probe for *Chloroflexi* but had a morphology different from type 1851, it is very likely that representatives of *Chloroflexi* subdivision 1a-1b also produced amyloids. Most of the *Chloroflexi* filaments had attached epiflora that hybridized with probe Sap309 targeting the family *Saprospiraceae*, but these did not show any amyloid presence.

Some filamentous *Chloroflexi* without attached growth, only detected with the broad *Chloroflexi* probe, were primarily amyloid positive at the septa (Fig. 3h).

Among filamentous gram-positive bacteria, the production of amyloid adhesins was investigated by branched mycolata and the unbranched "*Candidatus* Microthrix parvicella" in the phylum *Actinobacteria*. "*Ca.* Microthrix parvicella" did not produce any amyloids. The production of amyloid adhesins by mycolata was tested on probe-defined *Gordonia amarae* and *Skermania piniformis*. Both groups stained negative with antibodies, so a fresh sample from a plant with foam problems containing both *S. piniformis* and *G. amarae* was further tested by ThT staining. Contrary to the antibody staining, ThT staining showed that all branched organisms in the sample (thus both *S. piniformis* and *G. amarae*) produce amyloids but that these were masked by other surface components (Fig. 3c and f). The gram-positive filamentous bacteria belonging to the candidate phylum TM7 (primarily Eikelboom type 0041) did not express any amyloids.

DISCUSSION

The structure of the microbial communities in activated sludge plants with nutrient removal is now relatively well described (28), making it possible to target most of the abundant populations with FISH, using genus or species-specific oligonucleotide probes. This allows a detailed study of the presence of amyloids in the individual populations under the actual in situ conditions. Recently, it was shown that amyloid adhesins are abundant in natural biofilms, including activated sludge, by combining FISH with monoclonal antibodies specific to a generic epitope on amyloid fibrils, a method that proved to be highly specific and reliable (32, 47). In this study, however, it was found in some cases that the amyloids were apparently masked by other EPS components quenching the antibody binding. ThT staining proved to be a useful tool for the analysis of amyloid adhesin layers quenched for detection with antibodies. This means that amyloid adhesins can be analyzed in at least two functional forms in different populations: surface-exposed amyloids (can bind antibody) as described for *E. coli* and other *Enterobacteriaceae* (73) and amyloids in a sublayer integrated into other EPS components or perhaps into the cell wall (no antibody binding but ThT positive). The latter type has not been described before, and the two types of amyloids may have different functions for the bacteria, as will be discussed below. A drawback of application of ThT is its low specificity compared to the antibodies, which means that the bacteria staining positive only with ThT can be designated only as potential producers of amyloid protein. However, for the *Gordonia* species we have been able to purify "hidden" amyloids underneath an EPS layer, indicating that most of the ThT-positive signals are truly amyloids (P. L. Jensen, P. Larsen, P. H. Nielsen, and D. Otzen, unpublished results).

Distribution of amyloid adhesins in activated sludge. In the 10 different WWTPs investigated, the amyloidal proteins constituted 10 to 40% of the cell area, based on positive staining with propidium iodide, highlighting that amyloids are an important EPS component in activated sludge. The higher fraction of amyloid adhesins in treatment plants with biological phosphorus removal than in plants with only N removal seemed primarily to be associated with a relatively larger fraction of amyloid-producing filamentous bacteria. In many plants of this type, filaments belonging to *Chloroflexi* and *Aquaspirillum* are very common (4, 30, 63). Microcolonies and non-cell-bound amyloids were also present in flocs from all plants, but were, as detected by antibodies, not as abundant. However, this fraction must be regarded as a minimum fraction due to the shielding provided by other EPS components against antibody binding.

Identity of amyloid-producing populations in activated sludge. The most abundant probe-defined populations in nutrient-removing activated sludge plants were investigated for the presence of amyloids in at least two different WWTPs, where previous investigations had shown that the probe-defined populations were abundant. This approach ensured that their potential expression of amyloids was investigated in plants where they were actively growing, and the results can thus be taken as being representative of that particular probe-defined population. Surprisingly, none of the specific populations tested growing in microcolonies produced surface-exposed amyloids that could be labeled with antibody. Only some species hybridizing with the broad probes for *Alpha-* and *Betaproteobacteria* and *Actinobacteria* expressed surface-exposed amyloids, but they could not be identified further by specific probes. When most of the same microcolony-forming probe-defined populations were investigated with ThT for sublayers of amyloid adhesins, great diversity in the bacteria potentially producing amyloid adhesions was revealed. These were detected primarily among the denitrifiers: *Azoarcus*, *Thauera*, *Zoogloea*, and a subpopulation of *Aquaspirillum*-related bacteria. Gram-positive *Actinobacteria* also tested ThT positive. A characteristic microcolony-forming bacterium in activated sludge staining with ThT has previously been shown not be targeted by any of the broader probes. Using the specific probes, these microcolonies were identified as a subgroup of *Aquaspirillum*-related bacteria, which fits well with the fact that they did not hybridize with probe BET42a (64). In typical WWTPs with N and P removal, the microcolony-forming groups, which were ThT positive in this study but not antibody positive, comprise 30 to 50% of the total bacterial biovolume (28, 61). This indicates that sublayers of amyloidal material are an important EPS component for these microcolony-forming species, which generally belong to the strongest floc formers in activated sludge (3, 22, 24).

The probe-defined filamentous bacteria investigated are all commonly encountered in WWTPs, where they form an integrated part of the flocs. However, if they are present in large numbers, they grow into the bulk water and prevent flocculation and settling (cause bulking), making them a nuisance (14, 21). Most of these probe-defined filamentous bacteria produced amyloid adhesins. Interestingly, in most phyla where filamentous organisms with amyloids were found, examples of closely related species with and without amyloids were observed. For example, the alphaproteobacterial *M. perideroedes*, "*Ca.* Sphaeronema italicum," and "*Ca.* Alysiosphaera europea" produced amyloids, whereas two other types ("*Candidatus* Monilibacter batavus" and "*Candidatus* Alysiomicrobium bavaricum") did not. The results from the probe-defined filamentous organisms were generally supported by pure culture studies (*M. perideroedes*, Eikelboom type 1851, and *H. hydros-*

sis), and only *S. natans* gave a different result. This strongly indicates that the production of amyloids is widely distributed among filamentous bacteria in several phyla but that not all species expressed amyloids, either because they did not have the capability or because growth conditions triggering the expression of amyloid adhesins were absent. For *E. coli* and *Salmonella* species, amyloid production is known to depend on a range of environmental factors such as temperature, osmolarity, nutrient limitation (nitrogen, phosphate, and iron), and oxygen (15, 46, 53).

Possible function of amyloid adhesions in activated sludge. The term adhesin is an overall term covering bacterial surface structures implicated in specific adhesion of bacteria to different surfaces, and different adhesins are specific for adhesion to target molecules in a lock-and-key fashion like enzymes and immunoglobulins (25). Many pure-culture studies performed on bacteria related to the family *Enterobacteriaceae* have also shown that the amyloid-like fibrils expressed under different environmental conditions are involved in bacterial adhesion 49); hence, the term amyloid adhesins was adopted (32). However, the results obtained in this study suggest that the function of amyloids in activated sludge is more diverse than hitherto expected.

Microcolony-forming bacteria expressing surface-exposed amyloid adhesins were not present to the same extent as groups producing amyloid sublayers (detected by ThT), indicating that adhesion within microcolonies is promoted by interaction between different surface molecules. This is in accordance with pure-culture studies performed on *Salmonella enterica* and *E. coli*, where amyloid fibers, cellulose, colanic acid, and other capsular polysaccharides are integrated to form a highly hydrophobic extracellular matrix under environmental stress (59, 67, 72). It also ties in with the suggestion that the amyloid is essential for biofilm formation and biofilm structure (49). Production of a variety of chemically different surface structures in parallel by ThT-positive bacteria in activated sludge will most likely increase their adhesion strength by a suite of different adhesion mechanisms, such as hydrophobic and electrostatic interactions and physical entanglement, leading to strong microcolonies. We have observed this, in fact, in recent studies (24, 33). Several filamentous bacteria with ThT-positive sublayers were also detected. *M. perideroedes*, *G. amarae*, and *S. piniformis* are known to be highly hydrophobic (20, 29, 43), which might be a consequence of the presence of amyloid-like material together with other substances such as mycolic acid for *G. amarae* and *S. piniformis*, in the same way that amyloidal chaplins are responsible for the surface hydrophobicity of filamentous streptomyces hyphae (7). For *Thiothrix*, antibody-positive filament tips were detected in a sample containing *Thiothrix* rosettes, which could be the fimbriae previously described on the tips of *Thiothrix* rosettes (70). However, the ThT staining also indicated the presence of amyloids underneath the sheath, and the function of these remains uncertain, as *Thiothrix* is usually hydrophilic (P. H. Nielsen, unpublished results).

The function of surface-exposed amyloids on the filamentous *Chloroflexi* and *Aquaspirillum* may be related to the presence of a very strong sheath or skeleton. These filamentous bacteria were often observed to have dead cells in the middle of the filament; but the sheath material kept the filament intact, and it was still antibody positive in these segments, confirming its relatively high mechanical and/or biological stability. They may thus be very important backbones in the floc and are also the most common filamentous bacteria in many N- and P-removing treatment plants (30). Interestingly, these two particular groups are also frequently observed with attached growth of other bacteria. This epiflora is primarily affiliated to the family *Saprospiraceae* (28), and the bacteria are highly specialized in protein hydrolysis (71). Whether they degrade the amyloids or use these for anchorage to the sheaths remains unknown.

In summary, this study clearly showed that amyloid adhesins constitute a relatively large fraction of EPS in activated sludge flocs and are expressed by a diverse range of probe-defined groups, growing in microcolonies and as filaments. The function of the amyloidal material seems surprisingly divers, including a role in internal adhesion within the microcolonies, strengthening the sheaths of filamentous bacteria important for the backbone of the flocs, and increasing surface hydrophobicity. Most likely, future studies will reveal additional functions.

ACKNOWLEDGMENTS

We thank R. Wetzel for providing antibodies.

The project was supported by the Danish Research Council (framework program "Activity and diversity in complex microbial systems") and Aalborg University.

REFERENCES

1. **Adamczyk, J., M. Hesselsoe, N. Iversen, M. Horn, A. Lehner, P. H. Nielsen, M. Schloter, P. Roslev, and M. Wagner.** 2003. The isotope array, a new tool that employs substrate-mediated labeling of rRNA for determination of microbial community structure and function. Appl. Environ. Microbiol. **69:** 6875–6887.
2. **Beer, M., E. M. Seviour, Y. Kong, M. Cunningham, L. L. Blackall, and R. J. Seviour.** 2002. Phylogeny of the filamentous bacterium Eikelboom type 1851, and design and application of a 16S rRNA targeted oligonucleotide probe for its fluorescence in situ identification in activated sludge. FEMS Microbiol. Lett. **207:**179–183.
3. **Biggs, C., P. Lant, and M. Hounslow.** 2003. Modelling the effect of shear history on activated sludge flocculation. Water Sci. Technol. **47:**251–257.
4. **Bjornsson, L., P. Hugenholtz, G. W. Tyson, and L. L. Blackall.** 2002. Filamentous *Chloroflexi* (green non-sulfur bacteria) are abundant in wastewater treatment processes with biological nutrient removal. Microbiology **148:** 2309–2318.
5. **Bossier, P., and W. Verstraete.** 1996. Triggers for microbial aggregation in activated sludge? Appl. Microbiol. Biotechnol. **45:**1–6.
6. **Chapman, M. R., L. S. Robinson, J. S. Pinkner, R. Roth, J. Heuser, M. Hammar, S. Normark, and S. J. Hultgren.** 2002. Role of *Escherichia coli* curli operons in directing amyloid fiber formation. Science **295:**851–854.
7. **Claessen, D., R. Rink, W. Jong, J. Siebring, P. Vreugd, F. G. H. Boersma, L. Dijkhuizen, and H. A. B. Wösten.** 2003. A novel class of secreted hydrophobic proteins is involved in aerial hyphae formation in *Streptomyces coelicolor* by forming amyloid-like fibrils. Genes Dev. **17:**1714–1726.
8. **Collinson, S. K., L. Emody, K. H. Muller, T. J. Trust, and W. W. Kay.** 1991. Purification and characterization of thin, aggregative fimbriae from *Salmonella enteritidis*. J. Bacteriol. **173:**4773–4781.
9. **Crocetti, G. R., J. F. Banfield, J. Keller, P. L. Bond, and L. L. Blackall.** 2002. Glycogen-accumulating organisms in laboratory-scale and full-scale wastewater treatment processes. Microbiology **148:**3353–3364.
10. **Crocetti, G. R., P. Hugenholtz, P. L. Bond, A. Schuler, J. Keller, D. Jenkins, and L. L. Blackall.** 2000. Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. Appl. Environ. Microbiol. **66:**1175–1182.
11. **Daims, H., J. L. Nielsen, P. H. Nielsen, K. H. Schleifer, and M. Wagner.** 2001. In situ characterisation of *Nitrospira*-like nitrite-oxidizing bacteria in wastewater treatment plants. Appl. Environ. Microbiol. **67:**5273–5283.
12. **de los Reyes, F. L., W. Ritter, and L. Raskin.** 1997. Group-specific small-subunit rRNA hybridization probes to characterize filamentous foaming in activated sludge systems. Appl. Environ. Microbiol. **63:**1107–1117.
13. **Eales, K. L., J. L. Nielsen, E. M. Seviour, P. H. Nielsen, and R. J. Seviour.** 2006. The in situ physiology of *Skermania piniformis* in foams in Australian activated sludge plants. Environ. Microbiol. **8:**1712–1720.

14. **Fuchs, B. M., G. Wallner, W. Beisker, I. Schwippl, W. Ludwig, and R. Amann.** 1998. Flow cytometric analysis of the in situ accessibility of *Escherichia coli* 16S rRNA for fluorescently labeled oligonucleotide probes. Appl. Environ. Microbiol. **64:**4973–4982.
15. **Gerstel, U., and U. Romling.** 2001. Oxygen tension and nutrient starvation are major signals that regulate agfD promoter activity and expression of the multicellular morphotype in *Salmonella typhimurium*. Environ. Microbiol. **3:**638–648.
16. **Gich, F., J. Garcia-Gil, and J. Overmann.** 2001. Previously unknown and phylogenetically diverse members of the green nonsulfur bacteria are indigenous to freshwater lakes. Arch. Microbiol. **177:**1–10.
17. **Hess, A., B. Zarda, D. Hahn, A. Haner, D. Stax, P. Hohener, and J. Zeyer.** 1997. In situ analysis of denitrifying toluene- and m-xylene-degrading bacteria in a diesel fuel-contaminated laboratory aquifer column. Appl. Environ. Microbiol. **63:**2136–2141.
18. **Hugenholtz, P., G. W. Tyson, R. I. Webb, A. M. Wagner, and L. L. Blackall.** 2001. Investigation of candidate division TM7, a recently recognized major lineage of the domain bacteria with no known pure-culture representatives. Appl. Environ. Microbiol. **67:**411–419.
19. **Ilanchelian, M., and R. Ramaraj.** 2004. Emission of thioflavin T and its control in the presence of DNA. J. Photochem. Photobiol. A **162:**129–137.
20. **Iwahori, K., T. Tokutomi, N. Miyata, and M. Fujita.** 2001. Formation of stable foam by the cells and culture supernatant of *Gordonia* (*Nocardia*) *amarae*. J. Biosci. Bioeng. **92:**77–79.
21. **Jenkins, D., M. G. Richard, and G. T. Daigger.** 2003. Manual on the causes and control of activated sludge bulking, foaming, and other solid separation problems, 3rd ed. IWA Publishing, London, United Kingdom.
22. **Jorand, M. F., F. Zartarian, M. F. Thomas, J. C. Block, M. J. Y. Bottero, G. Villemin, V. Urbain, and J. Manem.** 1995. Chemical and structural (2D) linkage between bacteria within activated sludge flocs. Water Res. **29:**1639–1647.
23. **Keiding, K., and P. H. Nielsen.** 1997. Desorption of organic macromolecules from activated sludge: effect of ionic composition. Water Res. **31:**1665–1672.
24. **Klausen, M. M., T. R. Thomsen, J. L. Nielsen, L. H. Mikkelsen, and P. H. Nielsen.** 2004. Variations in microcolony strength of probe-defined bacteria in activated sludge flocs. FEMS Microbiol. Ecol. **50:**123–132.
25. **Klemm, P., and M. A. Schembri.** 2000. Bacterial adhesins: function and structure. Int. J. Med. Microbiol. **290:**27–35.
26. **Kong, Y., J. L. Nielsen, and P. H. Nielsen.** 2005. Identity and ecophysiology of uncultured actinobacterial polyphosphate-accumulating organisms in full-scale enhanced biological phosphorus removal plants. Appl. Environ. Microbiol. **71:**4076–4085.
27. **Kong, Y., S. L. Ong, W. J. Ng, and W. T. Liu.** 2002. Diversity and distribution of a deeply branched novel proteobacterial group found in anaerobic-aerobic activated sludge processes. Environ. Microbiol. **4:**753–757.
28. **Kong, Y., Y. Xia, J. L. Nielsen, and P. H. Nielsen.** 2007. Structure and function of the microbial community in a full-scale enhanced biological phosphorus removal plant. Microbiology **153:**4061–4073.
29. **Kragelund, C., Y. Kong, J. van der Waarde, K. Thelen, D. Eikelboom, V. Tandoi, T. R. Thomsen, and P. H. Nielsen.** 2006. Ecophysiology of different filamentous *Alphaproteobacteria* in industrial wastewater treatment plants. Microbiology **152:**3003–3012.
30. **Kragelund, C., C. Levantesi, A. Borger, K. Thelen, D. Eikelboom, V. Tandoi, Y. Kong, J. van der Waarde, J. Krooneman, S. Rossetti, T. R. Thomsen, and P. H. Nielsen.** 2007. Identity, abundance and ecophysiology of filamentous *Chloroflexi* species present in activated sludge treatment plants. FEMS Microbiol. Ecol. **59:**671–682.
31. **Lajoie, C. A., A. C. Layton, I. R. Gregory, G. S. Sayler, D. E. Taylor, and A. J. Meyers.** 2000. Zoogloeal clusters and sludge dewatering potential in an industrial activated-sludge wastewater treatment plant. Water Environ. Res. **72:**56–64.
32. **Larsen, P., J. L. Nielsen, M. S. Dueholm, R. Wetzel, D. Otzen, and P. H. Nielsen.** 2007. Amyloid adhesins are abundant in natural biofilms. Environ. Microbiol. **9:**3077–3090.
33. **Larsen, P., P. S. Eriksen, M. A. Lou, T. R. Thomsen, Y. Kong, J. L. Nielsen, and P. H. Nielsen.** 2006. Floc-forming properties of polyphosphate accumulating organisms in activated sludge. Water Sci. Technol. **54:**257–265.
34. **Levantesi, C., S. Rossetti, K. Thelen, C. Kragelund, J. Krooneman, D. Eikelboom, P. H. Nielsen, and V. Tandoi.** 2006. Phylogeny, physiology and distribution of "*Candidatus* Microthrix calida," a new *Microthrix* species isolated from industrial activated sludge wastewater treatment plants. Environ. Microbiol. **8:**1552–1563.
35. **Manz, W., R. Amann, W. Ludwig, M. Vancanneyt, and K. H. Schleifer.** 1996. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum *Cytophaga-Flavobacter-Bacteroides* in the natural environment. Microbiology **142:**1097–1106.
36. **Manz, W., R. Amann, W. Ludwig, M. Wagner, and K. H. Schleifer.** 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of *Proteobacteria*—problems and solutions. Syst. Appl. Microbiol. **15:**593–600.
37. **Martins, A. M. P., K. Pagilla, J. J. Heijnen, and M. C. M. Van Loosdrecht.** 2004. Filamentous bulking sludge—a critical review. Water Res. **38:**793–817.
38. **Meier, H., R, Amann, W. Ludwig, and K. H. Schleifer.** 1999. Specific oligonucleotide probes for in situ detection of a major group of gram-positive bacteria with low DNA G+C content. Syst. Appl. Microbiol. **22:**186–196.
39. **Meyer, R. L., A. M. Saunders, and L. L. Blackall.** 2006. Putative glycogen-accumulating organisms belonging to the *Alphaproteobacteria* identified through rRNA-based stable isotope probing. Microbiology **152:**419–429.
40. **Mikkelsen, L. H., and K. Keiding.** 2002. Physico-chemical characteristics of full scale sewage sludges with implications to dewatering. Water Res. **36:**2451–2462.
41. **Mobarry, B. K., M. Wagner, V. Urbain, B. E. Rittmann, and D. A. Stahl.** 1996. Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl. Environ. Microbiol. **62:**2156–2162.
42. **Neef, A.** 1997. Anwendung der in situ-einzelzell-identifizierung von bakterien zur populationsanalyse in komplexen mikrobiellen Biozönosen. Ph.D. thesis. Technische Universität München, Munich, Germany.
43. **Nielsen, J. L., L. H. Mikkelsen, and P. H. Nielsen.** 2001. In situ detection of cell surface hydrophobicity of probe-defined bacteria in activated sludge. Water Sci. Technol. **43:**97–103.
44. **Nielsen, P. H.** 2002. Activated sludge: the floc, p. 54–61. *In* G. Bitton (ed.), Encyclopedia of environmental microbiology. Wiley, Chichester, England.
45. **Oh, J., J. G. Kim, E. Jeon, C. H. Yoo, J. S. Moon, S. Rhee, and I. Hwang.** 2007. Amyloidogenesis of type III-dependent hairpins from plant pathogenic bacteria. J. Biol. Chem. **282:**13601–13609.
46. **Olsen, A., A. Jonsson, and S. Normark.** 1989. Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli*. Nature **338:**652–655.
47. **O'Nuallain, B., and R. Wetzel.** 2002. Conformational Abs recognizing a generic amyloid fibril epitope. Proc. Natl. Acad. Sci. USA **99:**1485–1490.
48. **Otzen, D., and P. H. Nielsen.** 23 November 2007. We find them here, we find them there: functional bacterial amyloid. Cell Mol. Life. Sci. doi: 10.1007/s00018-007-7404-4.
49. **Prigent-Combaret, C., G. Prensier, T. T. Le Thi, O. Vidal, P. Lejeune, and C. Dorel.** 2000. Developmental pathway for biofilm formation in curli-producing *Escherichia coli* strains: role of flagella, curli and colanic acid. Environ. Microbiol. **2:**450–464.
50. **Rabus, R., H. Wilkes, A. Schramm, G. Harms, A. Behrends, R. Amann, and F. Widdel.** 1999. Anaerobic utilization of alkylbenzenes and n-alkanes from crude oil in an enrichment culture of denitrifying bacteria affiliating with the beta-subclass of *Proteobacteria*. Environ. Microbiol. **1:**145–157.
51. **Raj, C. R., and R. Ramaraj.** 2001. Emission of thioflavin T and its off-on control in polymer membranes. Photochem. Photobiol. **74:**752–759.
52. **Roller, C., M. Wagner, R. Amann, W. Ludwig, and K. H. Schleifer.** 1994. In situ probing of gram-positive bacteria with high DNA G+C content using 23S-ribosomal-RNA targeted oligonucleotides. Microbiology **140:**2849–2858.
53. **Romling, U., W. D. Sierralta, K. Eriksson, and S. Normark.** 1998. Multicellular and aggregative behaviour of *Salmonella typhimurium* strains is controlled by mutations in the *agfD* promoter. Mol. Microbiol. **28:**249–264.
54. **Rossello-Mora, R. A., M. Wagner, R. Amann, and K. H. Schleifer.** 1995. The abundance of *Zoogloea ramigera* in sewage treatment plants. Appl. Environ. Microbiol. **61:**702–707.
55. **Ryu, J. H., and L. R. Beuchat.** 2005. Biofilm formation by *Escherichia coli* O157:H7 on stainless steel: effect of exopolysaccharide and curli production on its resistance to chlorine. Appl. Environ. Microbiol. **71:**247–254.
56. **Schauer, M., and M. W. Hahn.** 2005. Diversity and phylogenetic affiliations of morphologically conspicuous large filamentous bacteria occurring in the pelagic zones of a broad spectrum of freshwater habitats. Appl. Environ. Microbiol. **71:**1931–1940.
57. **Sheng, G. P., H. Q. Yu, and X. Y. Li.** 2006. Stability of sludge flocs under shear conditions: roles of extracellular polymeric substances (EPS). Biotechnol. Bioeng. **93:**1095–1102.
58. **Snaidr, J., C. Beimfohr, C. Levantesi, S. Rossetti, J. van der Waarde, B. Geurkink, D. Eikelboom, M. Lemaitre, and V. Tandoi.** 2002. Phylogenetic analysis and in situ identification of "*Nostocoida limicola*"-like filamentous bacteria in activated sludge from industrial wastewater treatment plants. Water Sci. Technol. **46:**99–104.
59. **Snyder, D. S., D. Gibson, C. Heiss, W. Kay, and P. Azadi.** 2006. Structure of a capsular polysaccharide isolated from *Salmonella enteritidis*. Carbohydr. Res. **341:**2388–2397.
60. **Sunde, M., L. C. Serpell, M. Bartlam, P. E. Fraser, M. B. Pepys, and C. C. F. Blake.** 1997. Common core structure of amyloid fibrils by synchrotron X-ray diffraction. J. Mol. Biol. **273:**729–739.
61. **Thomsen, T. R., Y. Kong, and P. N. Nielsen.** 2007. Ecophysiology of abundant denitrifying bacteria in activated sludge. FEMS Microbiol. Ecol. **60:**370–382.
62. **Thomsen, T. R., L. L. Blackall, M. A. de Muro, J. L. Nielsen, and P. H. Nielsen.** 2006. *Meganema perideroedes* gen. nov., sp nov., a filamentous *Alphaproteobacterium* from activated sludge. Int. J. Syst. Evol. Microbiol. **56:**1865–1868.
63. **Thomsen, T. R., C. Kragelund, and P. H. Nielsen.** 2006. Identity, abundance and physiology of *Aquaspirillum*-related filamentous bacteria in activated sludge. Water Sci. Technol. **54:**237–245.
64. **Thomsen, T. R., J. L. Nielsen, N. B. Ramsing, and P. H. Nielsen.** 2004.

Micromanipulation and further identification of FISH-labelled microcolonies of a dominant denitrifying bacterium in activated sludge. Environ. Microbiol. **6:**470–479.

65. **Wagner, M., R. Amann, P. Kampfer, B. Assmus, A. Hartmann, P. Hutzler, N. Springer, and K. H. Schleifer.** 1994. Identification and in situ detection of gram-negative filamentous bacteria in activated sludge. Syst. Appl. Microbiol. **17:**405–417.
66. **Weller, R., F. O. Glockner, and R. Amann.** 2000. 16S rRNA-targeted oligonucleotide probes for the in situ detection of members of the phylum *Cytophaga-Flavobacterim-Bacteroides*. Syst. Appl. Microbiol. **23:**107–114.
67. **White, A. P., D. L. Gibson, S. K. Collinson, P. A. Banser, and W. W. Kay.** 2003. Extracellular polysaccharides associated with thin aggregative fimbriae of *Salmonella enterica* serovar Enteritidis. J. Bacteriol. **185:**5398–5407.
68. **Wilen, B. M., B. Jin, and P. Lant.** 2003. Relationship between flocculation of activated sludge and composition of extracellular polymeric substances. Water Sci. Technol. **47:**95–103.
69. **Wilen, B. M., M. Onuki, M. Hermansson, D. Lumley, and T. Mino.** 2006. Influence of flocculation and settling properties of activated sludge in relation to secondary settler performance. Water Sci. Technol. **54:**147–155.
70. **Williams, T. M., R. F. Unz, and J. T. Doman.** 1987. Ultrastructure of *Thiothrix* spp. and Type-021N bacteria. Appl. Environ. Microbiol. **53:**1560–1570.
71. **Xia, Y., Y. Kong, and P. H. Nielsen.** 2007. In situ detection of protein-hydrolyzing microorganisms in activated sludge. FEMS Microbiol. Ecol. **60:**156–165.
72. **Zogaj, X., M. Nimtz, M. Rohde, W. Bokranz, and U. Romling.** 2001. The multicellular morphotypes of *Salmonella typhimurium* and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. Mol. Microbiol. **39:**1452–1463.
73. **Zogaj, X., W. Bokranz, M. Nimtz, and U. Romling.** 2003. Production of cellulose and curli fimbriae by members of the family *Enterobacteriaceae* isolated from the human gastrointestinal tract. Infect. Immun. **71:**4151–4158.