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Phylogenetic Analysis and In Situ Identification of Bacteria in Activated Sludge

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The bacterial community structure of activated sludge of a large municipal wastewater treatment plant was investigated by use of the rRNA approach. Almost-full-length genes coding for the small-subunit rRNA (rDNA) were amplified by PCR and subsequently cloned into the pGEM-T vector. Clones were screened by dot blot hybridization with group-specific oligonucleotide probes. The phylogenetic affiliations of clones were compared with the results obtained with the original sample by in situ hybridization with fluorescently labeled, rRNA-targeted oligonucleotide probes and found to be in general agreement. Twenty-five 16S rDNA clones were fully sequenced, 11 were almost fully (>80%) sequenced, and 27 were partially sequenced. By comparative sequence analyses, the majority of the examined clones (35 of 67) could be affiliated with the beta subclass of the class *Proteobacteria*. The gamma and alpha subclasses of *Proteobacteria* were represented by 13 and 4 clones, respectively. Eight clones grouped with the epsilon group of *Proteobacteria*, and five clones grouped with gram-positive bacteria with a low DNA G+C content. The 16S rDNA of two clones showed similarity with 16S rDNA genes of members of the phyla *Chlamydiae* and *Planctomyces*. 16S rRNA-targeted oligonucleotide probes were designed and used for the enumeration of the respective bacteria. Interestingly, potentially pathogenic representatives of the genus *Arcobacter* were present in significant numbers (4%) in the activated sludge sample examined. Pairs of probes targeted to the 5′ and 3′ regions were used for detection of chimeric sequences by in situ hybridization. Two clones could be identified as chimera by applying such a pair of probes.

Microorganisms present in activated sludge plants have always been of central interest to microbiologists. Numerous cultivation-based studies have been performed to isolate and identify bacteria from activated sludge plants (for examples, see references 7, 17 and 39). They provided interesting insights into the microbial diversity. However, it has been increasingly appreciated that plating or most-probable-number techniques are always selective and can therefore not yield sufficient documentation of the true community structure. Consequently, in the last decade, several attempts have been made to analyze the bacterial community structure of activated sludge by direct methods. Quinone profiles (22), polyamine patterns (5), and immunofluorescence (9, 13, 21, 23, 36, 57) were used successfully. However, all of these approaches were somehow limited since they still relied strongly on prior cultivation of the microorganisms of interest. Either pure cultures were needed to produce specific antibodies or reference data from pure cultures were required to interpret chemical compounds or profiles.

Today, the rRNA approach is a powerful tool to analyze in a cultivation-independent way the structure of microbial communities (4). There have been two types of rRNA-based studies on activated sludge in the last few years. Group- and genus-specific oligonucleotide probes were used to directly analyze the community structure of activated sludge by in situ hybridization (31, 59, 60). These studies indicated dominance of the beta subclass of the class *Proteobacteria* (59). Whereas members of this group were underestimated by cultivation techniques, members of the gamma subclass of *Proteobacteria*, most notably the genus *Acinetobacter*, were overestimated. Manz et al. (31) used the subclass-specific probes to demonstrate differences in the community structure between municipal and industrial activated sludge plants.

The probes used in these studies were still based on publicly available rRNA sequences and therefore mainly on sequences of cultured bacteria. Other groups have used direct retrieval and subsequent comparative analysis of sequences of genes coding for 16S rRNA (16S rDNA) to analyze the microbial diversity present in activated sludge (8, 46). Whereas Schuppler et al. (46) focused on clones from the group of gram-positive bacteria with a high DNA G+C content, Bond et al. (8) partially sequenced almost 100 clones each from two laboratory-scale sequencing batch reactors with strong differences in their phosphate-removing capabilities. Most 16S rDNA clones in this study were affiliated with the corresponding genes of the beta subclass of *Proteobacteria*, and only a few 16S rDNA clones had high similarity to the 16S rDNA of *Acinetobacter* spp. The results were in line with earlier findings by oligonucleotide probing (59, 60). Bond et al. (8) tried to use the relative abundance of the clones in the gene libraries for a comparison of the community structures of the two reactors. They found significantly more clones related to *Rhodocyclus* (β1 group of *Proteobacteria*) (65) in the reactor with high phosphate removal than in that with low phosphate removal. However, it has been argued (4, 19, 49) that clone frequencies should not be regarded as a valid means to determine true community structure, since different bacterial species contain different numbers of rRNA operons (i.e., 1 to 14) per genome (14, 43, 66). Furthermore, biases may occur in the efficacy of cell lysis (37), DNA extraction and purification (27, 34, 52), PCR amplification (11, 32, 41, 42, 62), or cloning (41). The combination of the two different rRNA-based techniques, direct rRNA retrieval followed by hybridization with probes

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based on the retrieved sequences, permits the analysis of the extent of these biases (4) and would therefore be the best way to characterize the community structure of activated sludge.

We here report results obtained with this combined approach on activated sludge from a large two-stage municipal activated sludge plant. The sample was taken from the first basin receiving a high load of organic waste. Recently, we have reported the initial results of this study (1), which were based on a limited number of 16S rDNA clones which could be affiliated to 16S rDNA genes of the β1 subgroup of *Proteobacteria*. Simultaneous in situ visualization of up to seven distinct bacterial genotypes was used to corroborate that the high diversity found in the clone library was indeed present in the complex activated sludge community. This report presents numerous additional 16S rDNA sequences retrieved from the same sample and the in situ visualization and enumeration of the corresponding bacteria.

MATERIALS AND METHODS

Sampling. Grab samples of mixed liquor were collected from aeration basin 1 of a municipal sewage plant (Munich-Grosslappen, Germany; 2 million population equivalents [population equivalent = 60 g of biological oxygen demand day^{-1}]). For in situ hybridization, the activated sludge sample was fixed for 3 h with paraformaldehyde as described before (2). The samples were stored in a 1:1 mixture of phosphate-buffered saline (130 mM sodium chloride, 10 mM sodium phosphate buffer [pH 7.2]) and 96% ethanol at −20°C. The cell concentration in the storage buffer was the same as that in the activated sludge sample. Activated sludge was also fixed by the addition of ethanol to a final concentration of 50%. In situ hybridizations with probes EUB338 and HGC69a were performed on such ethanol-fixed samples, whereas paraformaldehyde-fixed activated sludge samples were used for probing gram-negative bacteria. For PCR amplification of the 16S rDNA, unfixed aliquots of activated sludge were frozen at −20°C.

Membrane filtration and staining with DAPI. Total cell counts were determined by membrane filtration and staining with 4′,6-diamidino-2-phenylindole (DAPI) as described before (59).

Oligonucleotide probes. Oligonucleotides were synthesized with an aminolinker [6-(trifluoracetylamino)-hexyl-(2-cyanoethyl)-(*N*,*N*-diisopropyl)phosphoramidite] at the 5′ end (MWG-Biotech, Ebersberg, Germany). All probe sequences, their hybridization conditions, and references are given in Table 1. Labeling with carboxytetramethylrhodamine-5-isothiocyanate (CT; Molecular Probes, Eugene, Oreg.) or 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide-ester (Boehringer GmbH, Mannheim, Germany) and purification of the oligonucleotide-dye conjugates were performed as described before (3). Probes UNIV1392, ARCH915, EUK516, EUB338, ALF1b, CF319a, BONE23a, ACA23a, LDI23a, SNA23a, and AER66 were also labeled with digoxigenin as described by Zarda et al. (67). Hybridization conditions for the new oligonucleotide probes were optimized as described previously (30).

In situ hybridization and probe-specific cell counts. Fixed sludge samples were immobilized on glass slides by air drying. In situ hybridizations were performed at 46°C for 90 min in a hybridization buffer containing 0.9 M NaCl, the percent formamide shown in Table 1, 20 mM Tris-HCl (pH 7.4), and 0.01% sodium dodecyl sulfate (SDS). Probe concentrations were 5 ng/μl. Probes BET42a, GAM42a, BONE23a, LDI23a, SNA23a, and T25-220 were used with competitor oligonucleotides as described earlier (30, 58). Hybridization mixtures were removed, and the slides were washed for 15 min in buffer containing 20 mM Tris-HCl (pH 7.4), a millimolar concentration of NaCl, and 0.01% SDS at 48°C. The stringency of the washing step was probe dependent. It was adjusted by changing the NaCl concentration in the washing buffer. The concentrations were 900, 225, 80, 40, and 7 mM for probes hybridized at 0, 20, 35, 45, and 70% formamide, respectively. Washing buffer was removed with distilled water, and the slides were air dried. For the combination of probes T25-220 and T25-1028, no simultaneous hybridization was possible due to different stringencies. The combination was realized by starting with the hybridization and washing of the probe with the higher thermal stability (T25-220); this was followed by subsequent hybridization and washing of the probe requiring lower stringency (T25-1028). The slides were examined with an Axioplan microscope (Zeiss, Oberkochen, Germany) with filter sets 01 (for DAPI staining), 09, and 15. For each probe, more than 10,000 cells stained with the probe EUB338 were enumerated. Color photomicrographs were taken with Kodak Panther 1600X films, whereas black and white photomicrographs were done with Kodak Tmax400 films. Exposure times were 0.01 to 0.06 s for phase-contrast micrographs and 8 to 30 s for epifluorescence micrographs.

Amplification and cloning of the 16S rRNA gene. Aliquots of the activated sludge sample were used directly without prior treatment for PCR amplification of almost-full-length bacterial 16S rRNA gene fragments. The nucleotide sequences of the primers were 5′-AGAGTTTGATYMTGGCTCAG-3′ (*Escherichia coli* 16S rDNA positions 8 to 27 [10]) and 5′-CAKAAAGGAGGTGATC C-3′ (*E. coli* 16S rDNA positions 1529 to 1546 [10]). Amplification was performed with a Hybaid OmniGene temperature controller (MWG-Biotech) as follows. One microliter of activated sludge, 50 pmol each of the appropriate primers, 200 μmol of each deoxyribonucleoside triphosphate, 10 μl of 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl [pH 8.3], 15 mM $MgCl_2$, 0.1% [wt/vol] gelatin), 10 μl of 25 mM $MgCl_2$, and 3 U of thermostable *Taq* polymerase (Promega, Madison, Wis.) were added to a 0.5-ml test tube. The total volume was adjusted to 100 μl with sterile water. The mixture was overlaid with 70 μl of mineral oil (Sigma, Deisenhofen, Germany). After initial heating to 94°C for 3 min, 30 cycles consisting of 94°C (1 min), 50°C (2 min), and 72°C (3 min) were performed. The retrieved amplificates were analyzed by electrophoresis in 1% (wt/vol) agarose gel and staining with ethidium bromide. The amplificates were purified with the Magic Prep kit (Serva, Heidelberg, Germany) and subsequently ligated into the pGEM-T vector cloning system (Promega) as described in the manufacturer's instructions. The ligation product was transformed into competent cells supplied in the pGEM-T vector cloning kit. Detection occurred through blue-white screening with IPTG (isopropy-β-D-thiogalactopyranoside) and X-Gal (5-chloro-4-bromo-3-indolyl-β-D-galactopyranoside). The presence of correctly sized inserts was checked by restriction analysis with *Not*I followed by agarose gel electrophoresis. Plasmids were extracted with the QIAprep-spin kit (Diagen, Hilden, Germany), and inserts were detected by agarose gel electrophoresis with subsequent ethidium bromide staining.

Southern blot. Plasmid DNAs were transferred to uncharged nylon membranes (QIAbrane; Diagen) by use of a vacuum blot apparatus (VacuGene; Pharmacia, LKB Biotechnology, Uppsala, Sweden) and immobilized by UV irradiation (two 15-W UV tubes) at a distance of 30 cm for 2 min.

Gene library screening. Filters with blotted plasmid DNA were prehybridized for 1 h at 46°C with 15 ml of solution containing 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 4% blocking reagent, 0.2% *N*-lauroylsarcosine, and 0.04% SDS. Hybridization was performed at 46°C for at least 4 h with a solution of 0.1% *N*-lauroylsarcosine, 4% blocking reagent, 0.01% SDS, 0.9 M NaCl, the percent formamide shown in Table 1, and 5 pmol of digoxigenin-labeled oligonucleotide probes. After hybridization, the filters were washed twice at 48°C for 15 min with 20 ml of washing buffer (20 mM Tris-HCl [pH 8.0], 0.01% SDS, and a millimolar concentration of NaCl). The stringency of the washing step was again adjusted by lowering the sodium chloride concentration (30). Probe-conferred digoxigenin molecules were detected as described in the manufacturer's (Boehringer) guidelines, by using antidigoxigenin antibodies coupled with alkaline phosphatase and the chemiluminescent substrate disodium 3-(4-methoxyspiro{1,2-dioxetan-3,2′(5′ chloro) tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenylphosphate (CSPD). Chemiluminescence was documented by exposing membranes to X-ray films (X-Ray 90; Agfa Gevaert, München, Germany).

16S rDNA sequencing. 16S rDNA clones were sequenced on one strand (only in the case of obvious ambiguities on both strands) with a direct blotting electrophoresis system (GATC 1500; MWG-Biotech) and a LICOR automated sequencer (MWG-Biotech). Cycle sequencing protocols of the chain termination technique (12) were applied as described in the manufacturer's instructions (Boehringer; Amersham-Buchler, Braunschweig, Germany) with digoxigenin-labeled and infrared dye-labeled primers.

Data analysis. Sequences were added to the 16S rRNA sequence database of the Technical University Munich by use of the program package ARB (48). The tool ARB_EDIT was used for sequence alignment. The alignment was checked by eye and corrected manually. The 16S rRNA-based phylogenetic trees were based on the results of distance matrix analyses of all available 16S rRNA primary structures for *Proteobacteria*. The topologies of the different trees were evaluated by performing maximum parsimony and maximum likelihood analyses of the full data set and subsets, respectively. Only sequences that were at least 90% complete were used for treeing. Alignment positions at which fewer than 50% of the sequences of the entire data set shared the same residues were excluded from the calculations. The phylogenetic positions of organisms presented by partial sequences were roughly reconstructed by applying the parsimony criteria without changing the overall tree topology.

Detection of chimera. Several tests were applied to detect chimeric sequences. (i) We checked every sequence manually during alignment. On this level, chimeric sequences can be identified on the basis of signatures. (ii) Sections of 300 bases from both ends of a sequence were separately analyzed by use of the parsimony criteria. The two ends of a chimera would likely be assigned to different phylogenetic groups. (iii) Phylogenetic trees were checked for clones with a unique branching site or unrealistically long branch length. (iv) The Check_Chimera program of the Ribosomal Database Project (28) was applied for every sequence. This was possible since most sequences exceeded 1,000 bases in length. (v) Finally, based on some clone sequences, two different fluorescently labeled oligonucleotide probes, targeting sites at the 5′ and the 3′ regions of the same sequence, were constructed. A chimera can be excluded with high probability if both probes bind to one cell population during in situ hybridization of the original sample, resulting in the double staining with the two different fluorescent dyes.

Nucleotide sequence accession numbers. Partial and full 16S rDNA sequences of the 16S rDNA clones were submitted to GenBank under the accession numbers Z93951 to Z94012.

TABLE 1. Oligonucleotide probes

Probe	Specificity	Sequence (5′-3′) of probe	Target[a] site (rRNA positions)	[%] FA[b] in situ	[%] FA[c] dot blot	Reference
UNIV1392	Universal	ACGGGCGGTGTGTRC	16S (1392–1406)	0	0	35
ARCH915	Archaea	GTGCTCCCCCGCCAATTCCT	16S (915–934)	0	0	47
EUK516	Eucarya	ACCAGACTTGCCCTCC	16S (502–516)	0	0	4
EUB338	Bacteria	GCTGCCTCCCGTAGGAGT	16S (338–355)	0	0	3
ALF1b	Alpha subclass of *Proteobacteria*, several members of delta subclass of *Proteobacteria*, most spirochetes	CGTTCGYTCTGAGCCAG	16S (19–35)	20	40	30
BET42a	Beta subclass of *Proteobacteria*	GCCTTCCCACTTCGTTT	23S (1027–1043)	35	NU[d]	30
GAM42a	Gamma subclass of *Proteobacteria*	GCCTTCCCACATCGTTT	23S (1027–1043)	35	NU	30
CF319a	Cytophaga-flavobacterium cluster of cytophaga-flavobacterium-bacteroides phylum	TGGTCCGTGTCTCAGTAC	16S (319–336)	35	50	29
HGC69a	Gram-positive with high G+C DNA content	TATAGTTACCACCGCCGT	23S (1901–1918)	35	NU	44
BONE23a	β1 group of *Proteobacteria*	GAATTCCATCCCCCTCT	16S (663–679)	35	45	1
BTWO23a	Competitor for BONE23a	GAATTCCACCCCCCTCT	16S (663–679)	35	45	1
ACA23a	*Acinetobacter* spp.	ATCCTCTCCCATACTCTA	16S (652–669)	35	45	60
AER66	*Aeromonas* spp.	CTACTTTCCCGCTGCCGC	16S (66–83)	35	45	24
LDI23a	*Leptothrix discophora* and other members of β1 group of *Proteobacteria*	CTCTGCCGCACTCCAGCT	16S (649–666)	35	45	58
SNA23a	*Sphaerotilus natans* and other members of the β1 group of *Proteobacteria*	CATCCCCCTCTACCGTAC	16S (656–673)	45	50	58
CTE23a	Competitor for SNA23a	TTCCATCCCCCTCTGCCG	16S (659–676)	45	NU	45
ARC94	*Arcobacter* spp.	TGCGCCACTTAGCTGACA	16S (94–111)	20	NU	This study
ARC1430	*Arcobacter* spp.	TTAGCATCCCCGCTTCGA	16S (1430–1447)	20	NU	This study
ACA652b	Some unknown *Acinetobacter*-like species	ATCCTCTCCAATACTCTA	16S (652–669)	20	NU	This study
T3-86	T3	GCCACACGCCACCAGGAT	16S (86–103)	35	NU	1
T25-1028	T25, T19	UGUAUCGGCUCUCUUUCG	16S (1028–1045)	20	NU	This study
T25-220	T25 and other clones of cluster I of the β1 group of *Proteobacteria*, not T19	ATCGGCCGCTCCGATCGC	16S (220–237)	70	NU	This study
COP220	Competitor for T25-220	ATCGGCCGCTCCAATCGC	16S (220–237)	70	NU	This study
AVO220	Competitor for T25-220	ATCGGCCGCTCCGTTCGC	16S (220–237)	70	NU	This study
T28-649	T28, T29, *Sphingomonas capsulata*	CCTCTCCAAGATTCCAGTC	16S (649–667)	20	NU	This study
T28-1435	T28, T29	CTGAGTTAGCGCAACGCC	16S (1435–1452)	20	NU	This study
T52Chi-228	T52, some members of the β1 group of *Proteobacteria*	AATCTGCCATCGGCCGCT	16S (228–245)	0	NU	This study
T52Chi-1426	T52, *Acinetobacter* spp., *Methylococcus* spp.	ACTACCTACTTCTGGTGC	16S (1426–1443)	0	NU	This study

[a] *E. coli* numbering (10).
[b] Percent (vol/vol) formamide in the in situ hybridization buffer.
[c] Percent (vol/vol) formamide in the dot blot hybridization buffer (only given when used).
[d] NU, not used for dot blot hybridization.

RESULTS

In situ analysis of the activated sludge sample. The activated sludge sample was first analyzed by DAPI staining and in situ hybridization. The results are shown in Fig. 1. The signals obtained with all oligonucleotide probes were strong and indicated high cellular rRNA contents. Enumeration of DAPI-stained cells (38) revealed a total cell count of 1.8×10^9/ml. Eighty-one percent of the cells visualized by DAPI could be detected with the bacterial probe EUB338. As in earlier studies of a similar wastewater treatment plant (Munich-Dietersheim) (59–61), the beta subclass of *Proteobacteria* dominated the activated sludge. Forty-one percent of all DAPI-stained cells hybridized with probe BET42a. Other members of the *Proteobacteria* hybridizing with probes for the alpha subclass (ALF1b) and the gamma subclass (GAM42a) constituted 8 and 12% of the cells, respectively. The probe CF319a, specific for most members of the cytophaga-flavobacterium group, bound to 12%, and probe HGC69a, which is complementary to a 23S rRNA signature of bacteria with a high DNA G+C content, bound to 13% of all DAPI-stained cells. The sum of the group-specific counts was, at 86%, higher than the percentage of cells hybridizing with the bacterial probe EUB338 (81%). As discussed earlier (31), this might be due to an underestimation of the total cell count as determined by DAPI staining. About 80% of the members of the beta subclass of *Proteobacteria* also hybridized to a probe for the β1 group (BONE23a), which visualized a total of 33% of the cells detected by DAPI. Within the β1 group of bacteria, those detected by probe SNA23a (targeting *Sphaerotilus natans* and other members of the β1 group of *Proteobacteria*) were, at 17% of total DAPI counts, more abundant than those detected by probe LDI23a (8%; targeting *Leptothrix discophora* and other members of the β1 group of *Proteobacteria*). Cells visualized with SNA23a were located predominantly in flocs, whereas bacteria which hybridized with probe LDI23a were mainly outside the aggregates. Bacteria hybridizing with probe ACA23a, complementary to a signature present in all validly described species of the genus *Acinetobacter*, made up 3% of the cells.

Construction and screening of a clone library. A clone library of 16S rDNA clones was constructed from the same sample as that described above. One hundred clones were selected at random. To rapidly evaluate the representativeness of this 16S rDNA library, the clones were screened with some of the group- and genus-specific oligonucleotide probes used for in situ hybridization. As the clones encompassed only the 16S rDNA, only 16S rRNA-targeted oligonucleotide probes

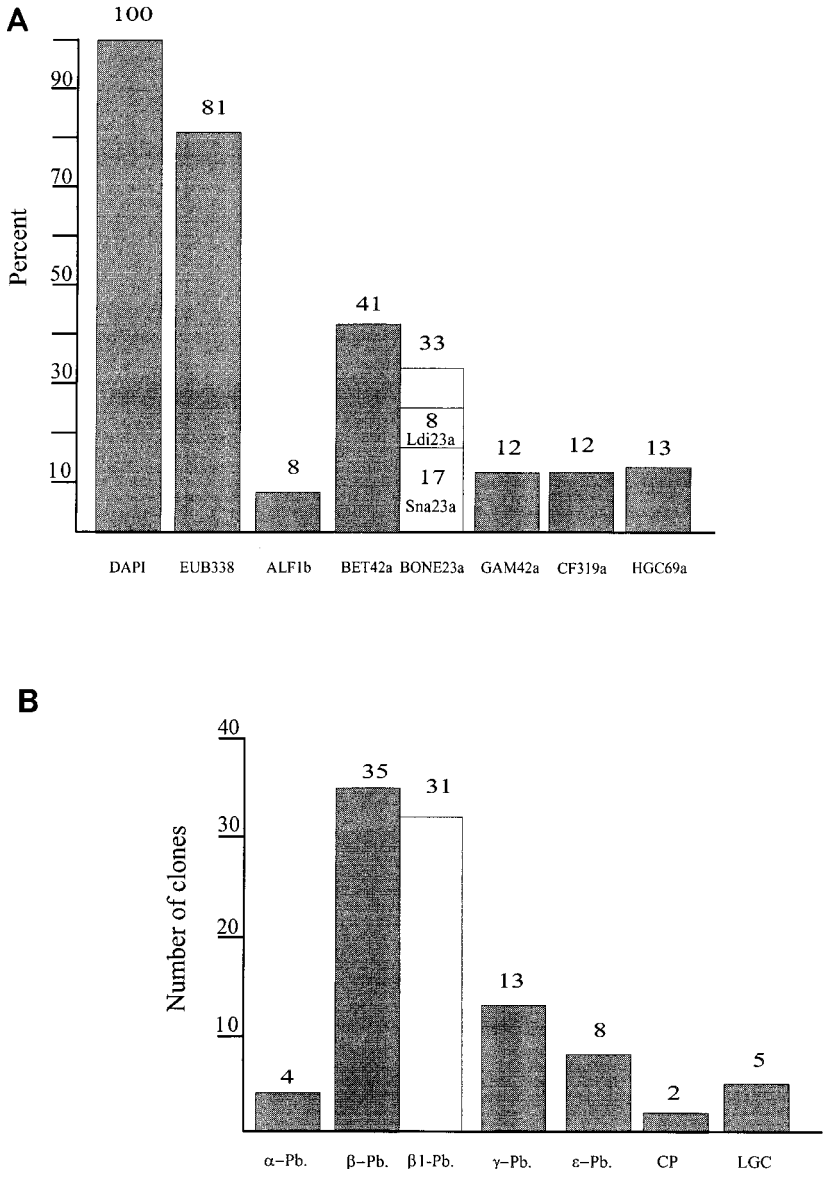

FIG. 1. Comparison of the community structure of the activated sludge sample as determined by in situ hybridization and the composition of the clone library. (A) In situ hybridization with oligonucleotide probes. The bar which represents hybridization with probe BONE23a (33%) also shows the results obtained from in situ hybridization with the probes SNA23a (17%) and LDI23a (8%). (B) Phylogenetic analysis of the clone library. α-Pb., alpha subclass of *Proteobacteria*; β-Pb., beta subclass of *Proteobacteria*; β1-Pb., β1 group of *Proteobacteria*; γ-Pb., gamma subclass of *Proteobacteria*; ε-Pb., epsilon subclass of *Proteobacteria*; CP, *Chlamydiae* and *Planctomyces* phyla; LGC, gram-positive bacteria with a low DNA G+C content.

could be used and not the 23S rRNA-targeted probes BET42a, GAM42a, and HGC69a.

Hybridization of 100 clones with the general rRNA probe UNIV1392 revealed positive signals with only 67 clones. Nine of these clones hybridized with probe ALF1b, and 31 hybridized with probe BONE23a. Thirteen of these 31 clones showed positive signals with probe LDI23a, and 7 showed positive signals with probe SNA23a. Six clones gave positive signals with ACA23a, and two gave positive signals with AER66, a probe targeting *Aeromonas* sp. The remaining 19 clones hybridized with probes UNIV1392 and EUB338 but with none of the tested group-specific probes. The results are given in Table 2.

Phylogenetic analysis of the clone library. The strategy was to determine full-length or almost-full-length 16S rDNA sequences for at least one member of each group of related clones. Accordingly, 25 clones were fully sequenced, 11 were almost fully sequenced (>80%), and 27 rDNA inserts were partially sequenced (290 to 1,200 bases). The phylogenetic analysis revealed five clusters within the β1 group of *Proteobacteria* (Fig. 2). Similarities of the fully or almost fully analyzed clones of cluster IV (T30, T54, and T60) to known bacteria ranged from 93.6% (T60 to *Comamonas testosteroni*) to 95.1% (T30 to *Comamonas testosteroni*). Members of cluster III (T3, T14, T67, T73, and T99) revealed a high sequence similarity to *Rhodoferax fermentans* (T67, 96.4%). According to Fig. 2, clusters I (T19, T25, T35, T41, T47, T49, T70, T71, T83, T90, and T98), II (T20, T22, T33, T36, T59, T69, and T87), and V (T15 and T65) represent new branches within the β1 group of *Proteobacteria* for which no sequences have been determined to date. The similarities within these clusters were between 96.7

and 99.9%. Since some clones have very high similarity values (the similarity matrix is not given due to the large amount of data), not all β1 group-related clones are shown in Fig. 2.

Six clone sequences were related to the 16S rDNA genes of members of the genus *Acinetobacter*. Similarity values ranged from 91.6 to 99.5% (Table 3). Whereas clone T17 was almost identical (99.5% similarity) to the 16S rDNA of *Acinetobacter johnsonii*, the two closely related sequences of clones T23 and T58 and the sequence of clone T48 were relatively separate from the main cluster of *Acinetobacter* (Fig. 3). Eight 16S rDNA clones could be assigned to the 16S rRNA-encoding genes of the genus *Arcobacter* (Fig. 4). All of them had the greatest similarity to *Arcobacter cryaerophilus* (98.3 to 99.5%) (Table 4). Two clones could be assigned to the 16S rDNA of representatives of the *Chlamydiae* and *Planctomyces* phyla but had, at 73 to 74%, quite low similarities (Table 2). Several 16S rDNA clones turned out to be affiliated to the 16S rDNA genes of gram-positive bacteria with a low DNA G+C content. Two of the clones (T21 and T37) were related to the 16S rDNA genes typical for cluster XI (similarity to *Clostridium sticklandii*, 89.3%) and cluster XVI (similarity to *Eubacterium biforme*, 98.4%) of the genus *Clostridium* (16), whereas the clones T44, T46, and T79 could be affiliated to the 16S rDNA genes of the genera *Streptococcus* and *Erysipelothrix*, respectively (similarity values, 94.8 and 96.1%, respectively).

Chimera. In this study, the rDNA sequences of clones T42 and T52, which were almost identical (98.8% similarity), were identified as chimeric. The breakpoint could be mapped to a site between 16S rRNA positions 330 and 340 (10). Bases 1 to 330 of clones T42 and T52 were 99.4% similar to that of clone T20, which was affiliated with the 16S rDNA genes of bacteria belonging to the β1 group of *Proteobacteria*. Bases 340 to 1528 of clones T42 and T52 had 97.2% similarity to the 16S rDNA of *Acinetobacter junii*, a gamma subclass proteobacterium. Two oligonucleotide probes, T52-277 and T52-1425, were developed to confirm the chimeric character of the sequences by in situ hybridization. Simultaneous in situ hybridization with differently labeled probes (probe T52-227, with CT; probe T52-1435, with fluorescein) resulted in strong probe-conferred signals, but detected cells were either red or green, not both. A double exposure with a rhodamine- and fluorescein-specific filter is shown in Fig. 5A. No cells hybridizing with both probes and consequently appearing as yellow can be seen. This is strong evidence for the chimeric character of clones T42 and T52.

In situ identification based on clone sequences related to the 16S rDNA of members of the genus *Acinetobacter*. Seven clone sequences were affiliated with the 16S rDNA of members of the genus *Acinetobacter*. Comparative data analysis revealed that two of the *Acinetobacter*-related sequences (T23 and T58) showed a single central mismatch to the binding site of probe ACA23a earlier designed to be specific for all described members of the genus *Acinetobacter* (60). A new probe, ACA652b, was designed making use of this mismatch. It was applied in combination with ACA23a to differentiate the respective cell populations (Fig. 5B). ACA652b bound to chains of small coccoid cells with diameters of approximately 1 μm. ACA652b-positive cells made up about 2% of the DAPI counts. Simultaneous in situ hybridization of probe ACA652b with probe GAM42a corroborated affiliation of the detected population with the gamma subclass of *Proteobacteria*.

In situ identification of the β1 group of *Proteobacteria*. Eleven of the 67 clones examined formed cluster I of the β1 group of *Proteobacteria*. As an example, clone T25 was selected for in situ visualization and enumeration. Two probes were constructed. One targeted the 5′ end (T25-220) and should visualize all members of cluster I (Fig. 2) with the exception of clone T19. It had at least one single mismatch to other members of the β1 group of *Proteobacteria*. For better discrimination, this probe was used in combination with two unlabeled competitor oligonucleotides, AVO220 and COP220. A mixed population of big and small rods was detected. The second oligonucleotide probe for T25 was targeted near the 3′ end (T25-1028) and should detect only clones T25 and T19. Therefore, a simultaneous in situ hybridization with both differently labeled oligonucleotide probes should reveal only cells which could be assigned to clone T25. The combined successive in situ hybridization with probes T25-220 (CT labeled) and T25-1028 (fluorescein labeled) yielded double-stained cells, which appear yellow upon double exposure (Fig. 5C). These cells were big rods and appeared with an abundance of 4% of total DAPI counts. Additionally, the simultaneous binding of both oligonucleotide probes to one cell population confirms the nonchimeric character of clone T25. The cell population detected with both T25 probes showed a characteristic probe-conferred fluorescence that was for most cells stronger at the ends of the rods.

In situ identification based on clone sequences related to the 16S rDNA of the alpha subclass of *Proteobacteria*. The sequences of two almost identical clones, T28 and T29, could be assigned to the rRNA sequences of the alpha subclass numbers of the *Proteobacteria*. They had a similarity of 96% to the sequence of *Sphingomonas capsulata*. Two oligonucleotide probes, T28-649 (*E. coli* positions 649 to 667 [10]) and T28-1435 (*E. coli* positions 1435 to 1452 [10]), were designed and successfully used for in situ hybridization. They both detected one population of short rods which appeared mainly in groups and made up approximately 3% of the total cells (Fig. 5D). Probe T28-649 is also complementary to the 16S rRNA of *Sphingomonas capsulata*, but probe T28-1435 targets only the sequence of clones T28 and T29. All cells detected with T28-649 also had the T28-1435 signature.

In situ identification of *Arcobacter* in activated sludge. Comparative sequence analysis affiliated eight clones with rRNA sequences obtained from members of the genus *Arcobacter*. The genus is part of the epsilon subclass of *Proteobacteria*. Two oligonucleotide probes, ARC94 and ARC1430, were designed specifically for the genus. They were evaluated on pure cultures of *Arcobacter* spp. Probes ARC94 (*E. coli* positions 94 to 111 [10]) and ARC1430 (*E. coli* positions 1430 to 1447 [10]) yielded strong signals with whole paraformaldehyde-fixed cells (data not shown). Simultaneous in situ hybridization with both differently labeled oligonucleotide probes showed ARC94-positive cells to be a subgroup of the ARC1430-positive cells (Fig. 5E). About 15% of the cells detected by ARC1430 were not detected by probe ARC94. Cells positive for both ARC94 and ARC1430 formed a morphologically homogeneous population of slender curved rods, which frequently formed loose spirals. These made up 4% of all cells in the activated sludge examined. With a diameter of 0.8 μm and a length of 2 to 3 μm, they had the typical morphology of arcobacters.

DISCUSSION

The rRNA approach to microbial ecology is based on the sequencing and probing of rRNA or rRNA genes. Our study of the microbial community of a municipal activated sludge basin started with probing. Fluorescently labeled oligonucleotide probes were used for the in situ enumeration of important phylogenetic groups. On this level, the community structure in the sample from the Munich-Grosslappen plant was quite similar to a sample taken from a second large municipal plant, in Munich-Dietersheim (59). These plants receive similar waste-

TABLE 2. Hybridization results, length of the sequenced part, and phylogenetic affiliation of the clones

Clone no.	Hybridization with oligonucleotide probes	Length of sequenced part (bases)	Phylogenetic relationship[a]	
			Species	% Similarity
T3	UNIV1392, EUB338, BONE23a, SNA23a	1,529	*Rhodoferax fermentans*	96.3
T10	UNIV1392, EUB338, BONE23a	1,020	*Anoxobacterium dechloratium*	97.3
T14	UNIV1392, EUB338, BONE23a, SNA23a	481	*Rhodoferax fermentans*	97.7
T15	UNIV1392, EUB338,	729	*Variovorax paradoxus*	96.6
T16	UNIV1392, EUB338	487	*Azoarcus* species	89.3
T17	UNIV1392, EUB338, ACA23a	1,532	*Acinetobacter* species	97.2
T18	UNIV1392, EUB338	797	*Arcobacter cryaerophilus*	98.4
T19	UNIV1392, EUB338, BONE23a, LDI23a	1,521	*Rhodoferax fermentans*	95.2
T20	UNIV1392, EUB338, BONE23a	1,526	*Variovorax paradoxus*	95.5
T21	UNIV1392, EUB338	717	*Eubacterium biforme*	98.4
T22	UNIV1392, EUB338, BONE23a	1,417	*Variovorax paradoxus*	95.2
T23	UNIV1392, EUB338	1,534	*Acinetobacter calcoaceticus*	96.4
T24	UNIV1392, EUB338, ALF1b	1,513	*Arcobacter cryaerophilus*	98.5
T25	UNIV1392, EUB338, BONE23a, LDI23a	1,519	*Rhodoferax fermentans*	95.2
T26	UNIV1392, EUB338	917	*Coxiella burnetti*	87.3
T27	UNIV1392, EUB338, ACA23a	1,532	*Acinetobacter junii*	97.6
T28	UNIV1392, EUB338, ALF1b	1,481	*Sphingomonas capsulata*	96.6
T29	UNIV1392, EUB338, ALF1b	1,184	*Sphingomonas capsulata*	96.0
T30	UNIV1392, EUB338, BONE23a, SNA23a	1,388	*Comamonas testosteroni*	95.1
T31	UNIV1392, EUB338	1,513	*Arcobacter cryaerophilus*	99.3
T32	UNIV1392, EUB338	1,367	*Coxiella burnetii*	86.5
T33	UNIV1392, EUB338, BONE23a	1,529	*Rhodoferax fermentans*	94.1
T34	UNIV1392, EUB338	1,538	*Burkholderia solanaceum*	92.5
T35	UNIV1392, EUB338, BONE23a, LDI23a	869	*Rhodoferax fermentans*	94.9
T36	UNIV1392, EUB338, BONE23a	994	*Variovorax paradoxus*	94.6
T37	UNIV1392, EUB338	1,182	*Clostridium sticklandii*	89.3
T39	UNIV1392, EUB338, ACA23a	957	*Acinetobacter calcoaceticus*	97.5
T40	UNIV1392, EUB338	1,059	*Arcobacter cryaerophilus*	99.1
T41	UNIV1392, EUB338, BONE23a, LDI23a	1,519	*Rhodoferax fermentans*	95.2
T42	UNIV1392, EUB338, ACA23a	1,528	*Acinetobacter junii*	93.6
T43	UNIV1392, EUB338	651	*Arcobacter cryaerophilus*	98.3
T44	UNIV1392, EUB338	1,543	*Streptococcus bovis*	95.5
T46	UNIV1392, EUB338, ALF1b	837	*Streptococcus bovis*	94.8
T47	UNIV1392, EUB338, BONE23a, LDI23a	1,526	*Rhodoferax fermentans*	94.9
T48	UNIV1392, EUB338, ACA23a	1,419	*Acinetobacter junii*	93.2
T49	UNIV1392, EUB338, BONE23a, LDI23a	320	*Variovorax paradoxus*	99.3
T50	UNIV1392, EUB338, BONE23a, LDI23a	ND[b]	ND	
T52	UNIV1392, EUB338, ACA23a	1,528	*Acinetobacter junii*	92.8
T54	UNIV1392, EUB338, BONE23a, SNA23a	292	*Comamonas testosteroni*	99.3
T55	UNIV1392, EUB338, ALF1b	1,513	*Arcobacter cryaerophilus*	99.6
T57	UNIV1392, EUB338	1,189	*Acinetobacter junii*	97.3
T58	UNIV1392, EUB338	1,539	*Acinetobacter calcoaceticus*	96.5
T59	UNIV1392, EUB338, BONE23a	1,299	*Variovorax paradoxus*	95.3
T60	UNIV1392, EUB338, BONE23a	1,522	*Comamonas testosteroni*	93.6
T62	UNIV1392, EUB338, ALF1b	1,412	*Chlamydia trachomatis*	73.4
T65	UNIV1392, EUB338, BONE23a	1,259	*Variovoarx paradoxus*	96.7
T67	UNIV1392, EUB338, BONE23a, SNA23a	1,328	*Rhodoferax fermentans*	96.4
T68	UNIV1392, EUB338, AER66	790	*Aeromonas hydrophila*	99.4
T69	UNIV1392, EUB338, BONE23a	700	*Comamonas testosteroni*	92.7
T70	UNIV1392, EUB338, BONE23a, LDI23a	1,518	*Variovorax paradoxus*	95.0
T71	UNIV1392, EUB338, BONE23a, LDI23a	1,497	*Variovorax paradoxus*	95.2
T73	UNIV1392, EUB338, BONE23a, SNA23a	856	*Rhodoferax fermentans*	96.8
T74	UNIV1392, EUB338, ALF1b	ND	ND	
T76	UNIV1392, EUB338, BONE23a	ND	ND	
T78	UNIV1392, EUB338	1,499	*Chlamydia trachomatis*	74.1
T79	UNIV1392, EUB338	1,529	*Erysipelothrix rhusiopathiae*	96.1
T81	UNIV1392, EUB338	957	*Arcobacter cryaerophilus*	99.0
T83	UNIV1392, EUB338, BONE23a, LDI23a	1,495	*Variovorax paradoxus*	95.2
T85	UNIV1392, EUB338, ALF1b	ND	ND	
T87	UNIV1392, EUB338, BONE23a	365	*Variovoarx paradoxus*	91.0
T88	UNIV1392, EUB338, AER66	1,540	*Aeromonas hydrophila*	99.4
T90	UNIV1392, EUB338, BONE23a, LDI23a	540	*Rhodoferax fermentans*	92.5
T92	UNIV1392, EUB338	1,094	*Spirillum volutans*	85.8
T95	UNIV1392, EUB338, ALF1b	1,022	*Arcobacter cryaerophilus*	99.5
T96	UNIV1392, EUB338, BONE23a, LDI23a	945	*Rhodoferax fermentans*	95.5
T98	UNIV1392, EUB338, BONE23a, LDI23a	1,319	*Rhodoferax fermentans*	94.2
T99	UNIV1392, EUB338, BONE23a, SNA23a	940	*Rhodoferax fermentans*	97.9

[a] The phylogenetic relationship of the sequenced part of the clones was analyzed. A closely related species together with the sequence similarity is given.
[b] ND, not determined.

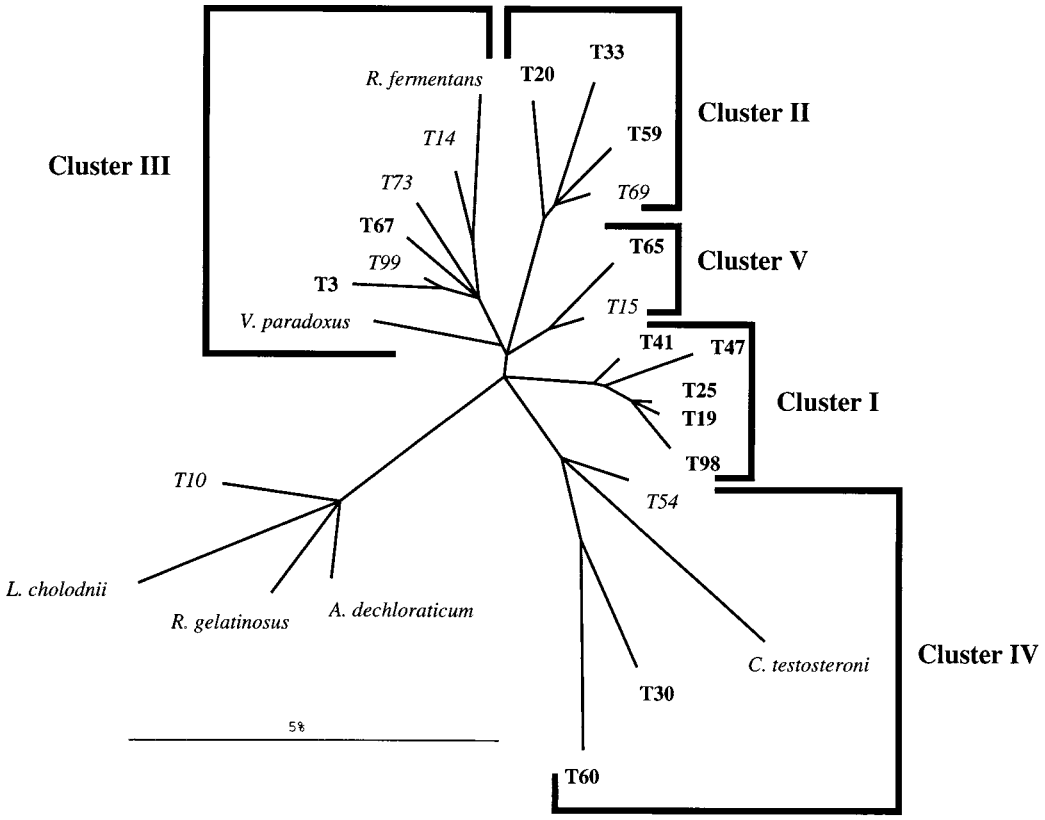

FIG. 2. Phylogenetic tree showing the relationships of the organisms represented by the examined clones and their closest relatives among the β1 group members of *Proteobacteria*. The tree is based on the results of distance matrix analyses of all available 16S rRNA primary structures for *Proteobacteria*. The topology of the tree was evaluated by performing maximum parsimony and maximum likelihood analyses of the full data set and subsets, respectively. Only sequences that were at least 90% complete were used for treeing (these clones are indicated in bold type). Alignment positions at which less than 50% of sequences of the entire data set have the same residues were excluded from the calculations. The phylogenetic positions of organisms presented by partial sequences were roughly reconstructed by applying the parsimony criteria without changing the overall tree topology. Multifurcations indicate that a common branching order was not significantly supported by applying different treeing methods. The five detected clusters are numbered from I to V. The bar indicates 5% estimated sequence divergence.

waters with relatively low inputs from industry or food processing and have been characterized by the dominance of the beta subclass of *Proteobacteria* over the last 4 years. However, it is important to realize that when group-specific probes based on cultured bacteria are used, only those populations that are looked for will be detected. In contrast, if it is assumed that the primers used for PCR are fairly general, rDNA libraries should not be biased towards known groups since they rely on direct rRNA gene retrieval.

The initial in situ probing was performed mainly to evaluate in an early stage of the study the representativeness of an rDNA library constructed from the examined sample. When

TABLE 3. Similarity matrix for clones affiliated with the genus *Acinetobacter*

Organism	Similarity (%) to organism:														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. *A. lwoffi*	100	97.3	97.7	97.7	96.4	96.2	96.1	96.4	96.1	95.9	96.2	95.8	95.6	97.7	92.0
2. *A. calcoaceticus*	97.3	100	97.6	97.0	95.7	96.3	96.2	97.0	96.5	97.5	96.4	95.6	95.2	97.1	92.5
3. *A. haemolyticus*	97.7	97.6	100	98.6	96.8	97.2	97.1	96.6	96.2	96.3	96.4	96.7	96.7	98.6	93.0
4. *A. johnsonii*	97.7	97.0	98.6	100	97.2	96.5	96.4	97.1	96.8	97.2	96.8	96.8	96.8	99.5	93.2
5. *Acinetobacter* sp.	96.4	95.7	96.8	97.2	100	96.6	96.4	96.0	95.5	93.9	95.3	95.8	95.6	97.2	91.6
6. *A. baumanii*	96.2	96.3	97.2	96.5	96.6	100	99.9	97.5	96.4	94.0	96.6	97.1	97.3	96.4	92.6
7. *A. anitratus*	96.1	96.2	97.1	96.4	96.4	99.9	100	97.4	95.9	93.4	96.1	96.5	96.6	95.9	91.8
8. *A. junii*	96.4	97.0	96.6	97.1	96.0	97.5	97.4	100	96.3	97.2	96.6	97.6	97.3	96.9	93.2
9. T58	96.1	96.5	96.2	96.8	95.5	96.4	95.9	96.3	100	96.2	98.8	95.8	95.8	96.3	92.9
10. T39	95.9	97.5	96.3	97.2	93.9	94.0	93.4	97.2	96.2	100	97.3	95.2	94.8	96.4	91.0
11. T23	96.2	96.4	96.4	96.8	95.3	96.6	96.1	96.6	98.8	97.3	100	96.0	96.1	96.5	92.9
12. T27	95.8	95.6	96.7	96.8	95.8	97.1	96.5	97.6	95.8	95.2	96.0	100	99.1	96.6	92.9
13. T57	95.6	95.2	96.7	96.8	95.6	97.3	96.6	97.3	95.8	94.8	96.1	99.1	100	96.4	95.8
14. T17	97.7	97.1	98.6	99.5	97.2	96.4	95.9	96.9	96.3	96.4	96.5	96.6	96.4	100	92.8
15. T48	92.0	92.5	93.0	93.2	91.6	92.6	91.8	93.2	92.9	91.0	92.9	92.9	95.8	92.8	100

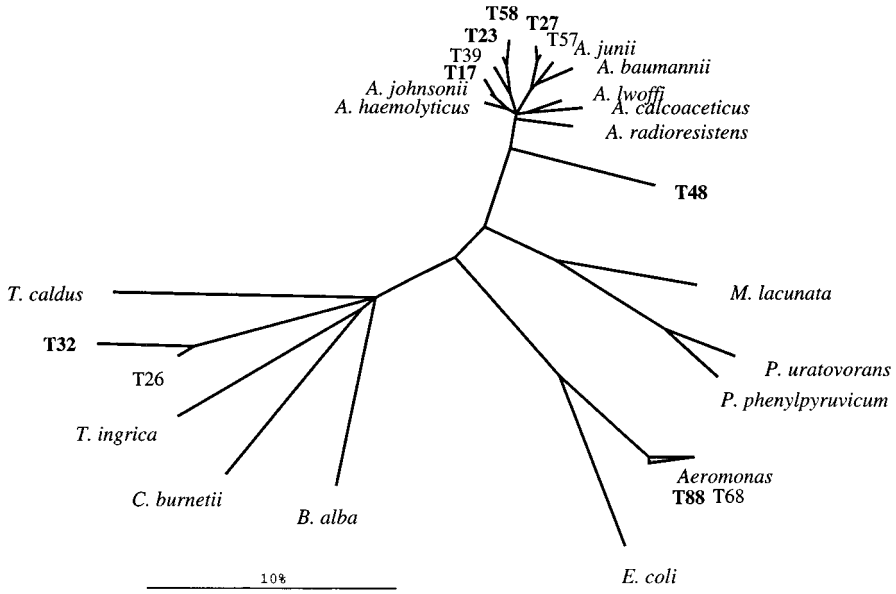

FIG. 3. Phylogenetic tree showing the relationships of the organisms represented by the examined clones and their closest relatives among the gamma subclass of *Proteobacteria*. For further details, see the legend to Fig. 2. The bar indicates 10% estimated sequence divergence.

the probe-based screening of the clones was compared to the results of the in situ probing of the original sample, good agreement was found at first sight. On a second look, however, discrepancies become clear. Within the dominant beta subclass of *Proteobacteria*, cells hybridizing with SNA23 are in situ, at 17%, more abundant than those hybridizing with LDI (8%). However, 13 clones had the LDI signature and only 7 had the SNA signature. Furthermore, none of the clones hybridized with the probe for the cytophaga-flavobacterium group, which was found to be quite abundant in situ. This is not surprising if the various biases that may occur in this multistep procedure are considered (11, 27, 32, 34, 37, 41, 42, 49, 52, 62). Despite these discrepancies, it was decided to continue with this clone library. One reason was the good representation of the important beta subclass; another was that 19 of the 67 clones hybridized only with the general rDNA probe UNIV1392 and the bacterial probe EUB338 but not any of the group-specific probes. These clones could originate from the rDNA of bacteria of groups that had not been tested for initially.

As for many other environmental rDNA libraries, none of our sequences was identical with already existing sequences in public databases. However, comparative sequence analysis revealed that most of the rDNA clone sequences were fairly close to 16S rRNA sequences of known bacteria. It is also important to note that the sequence analysis of 63 clones proved that 56 of the probe-based assignments or nonassignments were correct. Discrepancies occurred for probes ACA23a, which in two cases did not detect clones (T23 and T58) related to acinetobacters, ALF1b, which hybridized with five clones not affiliated to the alpha subclass of *Proteobacteria* (T24, T46, T55, T62, T95), and BONE23a, which did not detect a clone related to the β1 group of *Proteobacteria* (T15). Interestingly, none of the clones was related to the gram-positive bacteria with high DNA G+C contents, even though the abundance of cells of this group in situ was 13%. This discrimination could not be detected earlier by screening clones since the probe for this group, HGC69a, is targeted to the 23S rRNA and the clones were restricted to the 16S rDNA. Clones from this group were also absent from the two rDNA libraries studied by Bond et al. (8). It is clear from cultivation and in situ probing studies that this is an experimental artifact which is likely due to problems with lysis of the thick gram-positive cell walls and/or a less efficient amplification during PCR of the rDNA of gram-positive bacteria with high DNA G+C contents (49). The effects of DNA G+C content on PCR amplification have been described before (18, 42). In an attempt to retrieve rDNA of gram-positive bacteria with a high G+C content, Schuppler et al. (46) had to screen 3,000 clones by colony hybridization to obtain 27 clones with rDNA inserts from nocardioform actinomycetes and mycobacteria.

Regarding the diversity in the rDNA library, it was quite unexpected that sequences related to *Arcobacter* spp. were quite abundant and were found in 9 of 67 clones examined (13%). On the basis of immunotyping, SDS-polyacrylamide gel electrophoresis, and DNA-rRNA and DNA-DNA hybridization investigations, the genus *Arcobacter* was recently amended to encompass a genetically separate group of *Campylobacter* spp. (54). *Arcobacter* spp. are part of the epsilon subclass of *Proteobacteria* and are considered to be pathogenic (63). In their rDNA libraries, Bond et al. (8) detected no sequences related to the epsilon subclass of *Proteobacteria*.

Based on the clone sequences, a second probing phase was initiated. In this phase, specific probes, usually two per sequence of interest, were hybridized with the activated sludge. The reason for this was twofold. First, we intended to use this approach for addressing the problem of chimera formation. Chimera are formed in different frequencies in rDNA-based analyses of microbial communities (6, 25, 27). Chimera indicate a rDNA diversity not present in the original sample. In this study, the simultaneous application of two differently fluorescently labeled oligonucleotides targeted to the 5′ and 3′ end of a 16S rDNA was used to demonstrate the chimeric character of clones T42 and T52. It also confirmed that clones T25 and T28 and T29 were not chimera. This strategy offers a straightforward approach to the analysis of chimera. The relatively low frequency of chimera in our library (only 2 of 63 clones examined) might be the result of using the activated sludge sample

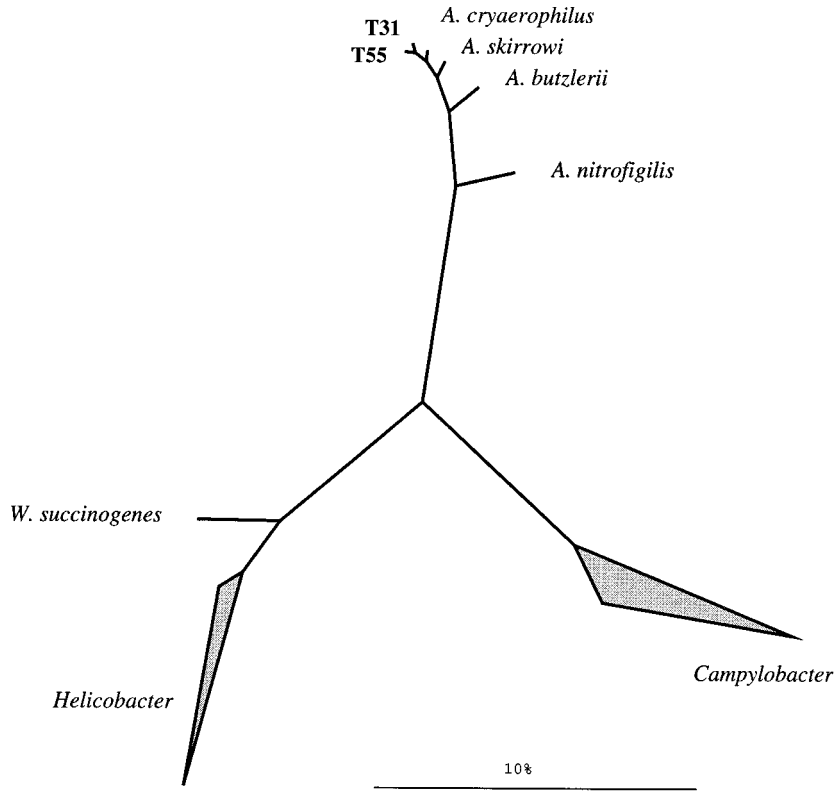

FIG. 4. Phylogenetic tree showing the relationships of the organisms represented by the examined clones and their closest relatives among the epsilon subclass of *Proteobacteria*. For further details, see the legend to Fig. 2. The bar indicates 10% estimated sequence divergence.

directly for PCR amplification. Shearing of DNA was thereby minimized.

In other cases, the simultaneous application of two differently labeled probes was used to increase the reliability of in situ identification (4). The importance of this strategy becomes clear from the in situ enumeration of arcobacters. According to the database, both probes should detect the same target group. However, one probe detected more cells than the other one. This clearly demonstrates that the activated sludge sample contains cells from yet another hitherto-unsequenced genotype that has just one of two signatures for the genus *Arcobacter*. Only those cells which bound both probes were counted and assigned with high probability to the genus *Arcobacter*. The binding of one probe to the unknown genotype could indicate relatively close phylogenetic relationships of an as-yet-undescribed population to members of the genus *Arcobacter*; it could, however, also be caused by the presence of this signature in an unrelated species.

Community structure of activated sludge. Preliminary results from the sample examined had already indicated a high genetic diversity of the β1 group of *Proteobacteria* in activated sludge (1). Five clusters of more closely related sequences were evident. In this earlier study, the focus was on a representative of cluster III, clone T3. The respective cells were present only in low numbers (≪1%). In this study, a sequence from cluster I (T25) could be assigned with two specific probes to

TABLE 4. Similarity matrix for clones affiliated with the genus *Arcobacter*

Organism	Similarity (%) to organism:												
	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *A. cryaerophilus*	100	98.6	97.4	94.2	92.1	99.5	99.1	99.2	99.6	99.0	98.5	98.4	98.3
2. *A. skirrowi*	98.6	100	97.1	94.2	92.4	98.2	97.6	98.2	98.5	98.0	97.6	97.8	96.9
3. *A. butzlerii*	97.4	97.1	100	94.5	92.5	97.2	96.7	96.8	97.2	96.1	97.4	95.5	95.3
4. *A. nitrofigilis*	94.2	94.2	94.5	100	93.9	92.9	91.8	93.4	93.8	92.2	93.7	90.4	91.6
5. *Arcobacter* sp.	92.1	92.4	92.5	93.9	100	90.9	90.0	91.6	92.0	90.0	91.9	88.6	89.2
6. T95	99.5	98.2	97.2	92.9	90.9	100	98.8	99.1	99.2	99.1	97.6	98.8	98.2
7. T40	99.1	97.6	96.7	91.8	90.0	98.8	100	99.3	99.4	98.6	97.7	98.3	98.9
8. T31	99.2	98.2	96.8	93.4	91.6	99.1	99.3	100	99.5	99.2	98.3	98.7	98.8
9. T55	99.6	98.5	97.2	93.8	92.0	99.2	99.4	99.5	100	99.1	98.5	98.9	98.8
10. T81	99.0	98.0	96.1	92.2	90.0	99.1	98.6	99.2	99.1	100	97.3	98.2	98.0
11. T24	98.5	97.6	97.4	93.7	91.9	97.6	97.7	98.3	98.5	97.3	100	96.9	96.3
12. T18	98.4	97.8	95.5	90.4	88.6	98.8	98.3	98.7	98.9	98.2	96.9	100	97.7
13. T43	98.3	96.9	95.3	91.6	89.2	98.2	98.9	98.8	98.8	98.0	96.3	97.7	100

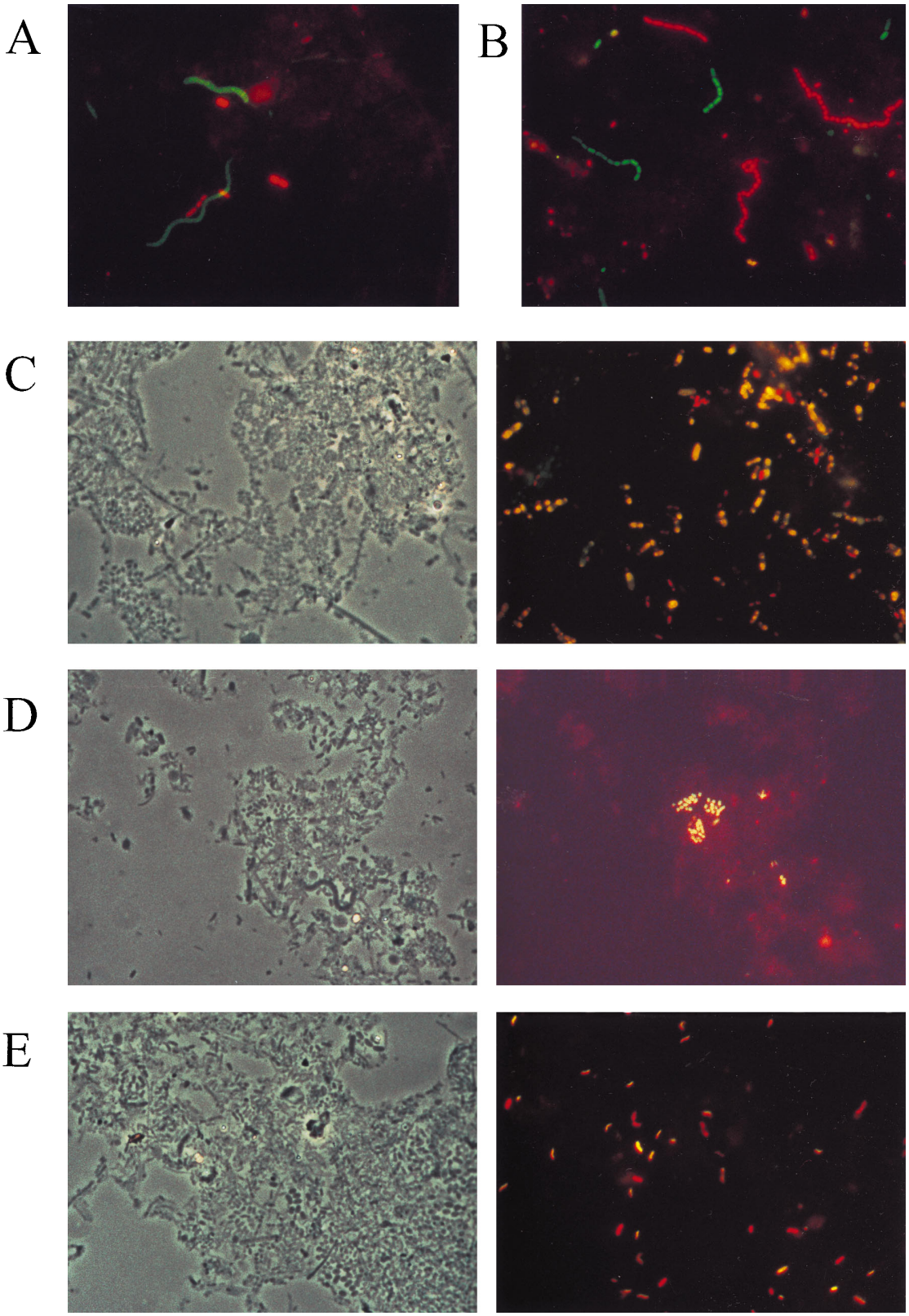

FIG. 5. Micrographs of activated sludge after in situ hybridization with fluorescently labeled oligonucleotide probes. (A) Chimera check by simultaneous in situ hybridization with probes T52Chi-228 (fluorescein labeled, green) and T52Chi-1426 (CT labeled, red). Note that the lack of cells detected in yellow shows that none of the detected bacteria binds both probes. Therefore, clones T52 and T42 are chimeric. (B) Simultaneous in situ hybridization with probes ACA23a (fluorescein labeled, green) and ACA652b (CT labeled, red). (C) In situ hybridization of activated sludge with probes T25-220 (CT labeled, red) and T25-1028 (fluorescein labeled, green). Phase-contrast (left) and epifluorescence (right) micrographs are shown for identical microscopic fields. (D) In situ hybridization of activated sludge with probes T28-649 (fluorescein labeled, green) and T28-1435 (CT labeled, red). Phase-contrast (left) and epifluorescence (right) micrographs are shown for identical microscopic fields. (E) In situ hybridization of activated sludge with two probes for *Arcobacter*. Phase-contrast (left) and epifluorescence (right) micrographs are shown for identical microscopic fields. Simultaneous hybridization with the probes ARC94 (fluorescein labeled, green) and ARC1430 (CT labeled, red) reveals a majority of bacteria which bind both probes (resulting in yellow). Note that some bacteria do not bind the oligonucleotide probe ARC94 and therefore appear only red.

cells present in significantly higher numbers (approximately 4%). Several factors indicate a significant role of this population in wastewater treatment. The cells are relatively large and contain high amounts of rRNA. The latter suggests a high metabolic potential. Furthermore, in several activated sludge samples from Munich-Grosslappen investigated since the initial sampling on 14 June 1994, the T25 population was always present in numbers well above 1% of the total count. Both in terms of biomass and number, T25 is an important member of the most dominant group in the activated sludge sample examined, the β1 group of *Proteobacteria*. β1 group numbers of the *Proteobacteria* encompass with the genera *Hydrogenophaga*, *Comamonas*, and *Acidovorax* several former pseudomonads and are well known for the utilization of a wide spectrum of carbon sources. This group seems to be present in Munich-Grosslappen with a wide array of genotypes. From this set, those genotypes best adjusted to the wastewater composition and the operational parameters at a given time will have a selective advantage and increase in abundance.

With a relative abundance of 3%, the T28- and T29-related cells also form a fairly large population. In the examined sample, about one of three cells hybridizing with the probe for the alpha subclass of *Proteobacteria* was assigned to this population. Based on the closest relationship, with a similarity value of 96% to *Sphingomonas capsulata*, clones T28 and T29 likely contain rDNA of a hitherto-unsequenced or even unknown species of the genus *Sphingomonas*. It is not surprising that sphingomonads make up such an important fraction of the alpha subclass of *Proteobacteria* in activated sludge. *Sphingomonas* spp. are relatively ubiquitous in environmental samples and are found, e.g., in soil, water, and sediments (20). They are able to degrade a broad range of different contaminants (20, 26, 33, 50, 64) and seem therefore to be perfectly suited for wastewater treatment.

Members of the genus *Acinetobacter* have long been known as typical bacteria in sewage. In cultivation-based studies, they were responsible for between 30 and 60% of all CFU. When their in situ abundance in wastewater treatment plants was determined based on a fluorescently labeled, rRNA-targeted probe specific for all validly described species of this genus, it was found that they occur only in numbers constituting between 3 and 9% (60). It was also shown that plating on nutrient-rich agar plates resulted in an overestimation of the abundance of acinetobacters in wastewater treatment plants. The results of our study now clearly show that hitherto-unknown genotypes of acinetobacter-related bacteria were present in the examined activated sludge. Comparative sequence analysis demonstrated that two of the clones (T23 and T58) were not detected by the probe for the genus *Acinetobacter* due to one central mismatch in the target region. Phylogenetically, both clones were clearly within the range of *Acinetobacter*. The relatively small cocci reacting with probe ACA652b were almost as abundant (2.5%) as the cocci detected by probe ACA23a (3%). Use of the combination of the two probes is recommended for in situ visualization of all acinetobacters. Regarding their physiological role, it has been speculated that members of the genus *Acinetobacter* might be responsible for enhanced biological phosphorus removal. It should be noted here that the examined sludge did not show enhanced biological phosphorus removal.

Potentially, the most interesting finding of this study is that of high numbers of bacteria related to the genus *Arcobacter*. This genus encompasses an aerotolerant group of former campylobacters. In situ hybridizations with two newly developed genus-specific probes indicated with high probability a relative abundance of 4% and consequently an absolute abundance of approximately 7×10^7/ml. Hitherto, the gram-negative, spiral members of the genus *Arcobacter* have been isolated from, e.g., root-associated sediment and roots of salt marsh plants (55), humans with enteritis (40) or diarrhea (51), and various other animal and human sources. *Arcobacter* spp. have generally been regarded as pathogenic because they have frequently been recovered from aborted embryos of several species of farm animals (63). The isolation of *Arcobacter* spp. from meat, poultry, water, and patients with enteritis has increased awareness of this organism as a potential food safety concern (15). We had to construct two new genus-specific oligonucleotide probes since those described by Wesley et al. (63) were only valid for the hitherto-known species *Arcobacter butzleri*, *Arcobacter skirrowii*, *Arcobacter cryaerophilus*, and *Arcobacter nitrofigilis* and missed a fifth sequence of an *Arcobacter* sp. which has recently been determined (53). The new probes detected nine clones retrieved from the activated sludge examined which had similarity values between 98.4 and 99.6% to *A. cryaerophilus*. The population detected with both probes is therefore with high probability composed of cells with a very high rRNA sequence similarity to the genus *Arcobacter*. However, this does not automatically imply that the population detected in the activated sludge is also pathogenic. The high number present in the activated sludge basin, which is essentially a bioreactor with biomass recycling, even suggests that the detected population is part of the autochthonous microbiota. Other well known enteric bacteria, e.g., members of *Bacteroides*, *Enterococcus*, or the *Enterobacteriaceae*, can be detected only in much lower numbers. Therefore, nonpathogenic environmental strains of *Arcobacter* might exist and be abundant in activated sludge. Even though cultivation-independent molecular methods have facilitated this surprising finding, it must be acknowledged that an analysis of the function and potential pathogenicity of these bacteria would require their isolation.

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