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Development of an rRNA-Targeted Oligonucleotide Probe Specific for the Genus *Acinetobacter* and Its Application for In Situ Monitoring in Activated Sludge

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Enhanced biological phosphate removal in an anaerobic-aerobic activated sludge system has generally been ascribed to members of the genus *Acinetobacter*. A genus-specific 16S rRNA-targeted oligonucleotide probe was developed to investigate the role of *Acinetobacter* spp. in situ. Nonisotopic dot blot hybridization to 66 reference strains, including the seven described *Acinetobacter* spp., demonstrated the expected probe specificity. Fluorescent derivatives were used for in situ monitoring of *Acinetobacter* spp. in the anaerobic and aerobic compartments of a sewage treatment plant with enhanced biological phosphate removal. Microbial community structures were further analyzed with oligonucleotide probes specific for the alpha, beta, or gamma subclasses of the class *Proteobacteria*, for the *Cytophaga-Flavobacterium* cluster, for gram-positive bacteria with a high G+C DNA content, and for all bacteria. Total cell counts were determined by 4′,6-diamidino-2-phenylindole staining. In both the anaerobic and the aerobic basins, the activated sludge samples were dominated by members of the class *Proteobacteria* belonging to the beta subclass and by gram-positive bacteria with a high G+C DNA content. *Acinetobacter* spp. constituted less than 10% of all bacteria. For both basins, the microbial community structures determined with molecular techniques were compared with the compositions of the heterotrophic saprophytic microbiota determined with agar plating techniques. Isolates on nutrient-rich medium were classified by whole-cell hybridization with rRNA-targeted probes and fatty acid analysis. Cultivation on nutrient-rich medium favored the growth of members of the gamma subclass of *Proteobacteria* and selected against the growth of members of the beta subclass of *Proteobacteria* and gram-positive bacteria with a high G+C DNA content; 33% of the cultured bacteria from the anaerobic basin and 32% from the aeration basin were identified as *Acinetobacter* spp. The addition of small amounts of iron salts for chemical phosphate precipitation had no influence on the constitution of the microbial consortia. Enrichment of the return sludge with 20 mg of acetic acid per liter for 3 days significantly increased the relative abundance of gram-positive bacteria with a high G+C DNA content but had no effect on the numbers of *Acinetobacter* spp. The dominance of gram-positive bacteria with a high G+C DNA content and the presence of polyphosphate inclusions in these bacteria indicate that they may play a major role in biological phosphate removal.

Bacteria belonging to the genus *Acinetobacter* are nonmotile, gram-negative, strictly aerobic, oxidase-negative, and catalase-positive saprophytes which are not pigmented. On the basis of 16S rRNA sequence data, the genus *Acinetobacter* belongs to the gamma subclass of the class *Proteobacteria* (41). From DNA-DNA hybridization studies, it can be concluded that the genus *Acinetobacter* currently consists of seven described species and 13 additional DNA-DNA similarity groups (genospecies) (7, 8, 37). The members of the genus *Acinetobacter* are ubiquitous organisms that play an important role in medical and environmental scenarios. They are normal inhabitants of human skin and may cause opportunistic and nosocomial infections. Acinetobacters are also found in soil, water, and sewage (38). Here their occurrence has been linked to enhanced biological phosphate removal (EBPR) (17, 21, 36). However, there is a considerable discrepancy between the numbers of acinetobacters isolated from activated sludge by conventional plate count methods and estimations inferred from direct examinations by chemotaxonomic and immunochemical techniques of wastewater prior to cultivation. On the basis of conventional cultivation methods, acinetobacters represent a major part of the bacteria present in EBPR systems (e.g., 10, 12, 17), whereas chemotaxonomic and immunochemical data indicate that they may be present only in rather low numbers (4, 11, 19).

A possible way to clarify these contradictory results is via the application of reliable in situ detection methods. Immunofluorescence studies have been carried out, but the specific-antibody technique has severe limitations; i.e., the specificity of antibodies is generally restricted to the species or subspecies level (6) and, consequently, perhaps not all acinetobacters are detected. More recently, fluorescence-labeled, rRNA-targeted oligonucleotide probes were used for the microscopic identification of bacteria (2, 13). In contrast to that of immunoprobes, the specificity of oligonucleotide probes is freely adjustable to different phylogenetic levels ranging from the subspecies level to the kingdom level (35).

In the present study, the in situ bacterial community structures of activated sludge in an anaerobic-aerobic treatment plant with EBPR were characterized by use of oligonucleotide probes specific for the genus *Acinetobacter* and others specific

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TABLE 1. Listing of studied strains and arrangement of extracted nucleic acids on the membrane

Position	Organism	Strain[a]
A 1	*Azospirillum amazonense*	DSM 2787^T
2	*Azospirillum brasiliense*	DSM 1690^T
3	*Azospirillum halopreferens*	DSM 3675^T
4	*Rhodospirillum rubrum*	DSM 107
5	*Agrobacterium tumefaciens*	ATCC 23308^T
6	*Bradyrhizobium japonicum*	DSM 30131^T
7	*Magnetospirillum gryphiswaldense*	DSM 6361^T
8	*Rhizobium meliloti*	DSM 30135^T
9	*Rhodopseudomonas palustris*	DSM 123^T
10	*Pseudomonas diminuta*	DSM 1635
11	*Paracoccus denitrificans*	DSM 65^T
12	*Rhodobacter capsulatus*	DSM 1710^T
B 1	*Thiobacillus acidophilus*	DSM 700^T
2	*Zoogloea ramigera*	WS 1610
3	*Comamonas testosteroni*	DSM 50244^T
4	*Sphaerotilus natans*	DSM 565
5	*Aquaspirillum metamorphum*	DSM 1837^T
6	*Alcaligenes eutrophus*	DSM 531^T
7	*Alcaligenes faecalis*	ATCC 8750^T
8	*Chromobacterium violaceum*	DSM 30191^T
9	*Acinetobacter calcoaceticus*	LMG 1046^T
10	*Pseudomonas cepacia*	DSM 50181
11	*Alteromonas putrefaciens*	DSM 50426
12	*Leucothrix mucor*	DSM 2157^T
C 1	*Aeromonas hydrophila*	WS 1406
2	*Vibrio anguillarum*	NCIMB 2129
3	*Escherichia coli*	DSM 30083^T
4	*Enterobacter aerogenes*	WS 1292
5	*Enterobacter cloacae*	WS 1293
6	*Erwinia carotovora*	WS 1394
7	*Proteus vulgaris*	WS 1356
8	*Serratia marcescens*	WS 1359
9	*Pseudomonas aeruginosa*	DSM 50071^T
10	*Pseudomonas alcaligenes*	DSM 50342^T
11	*Pseudomonas fluorescens*	DSM 10090
12	*Pseudomonas pseudoalcaligenes*	LMG 1225^T
D 1	*Corynebacterium glutamicum*	DSM 20300^T
2	*Micrococcus luteus*	CCM 169^T
3	*Rhodococcus rhodochrous*	DSM 43008
4	*Brevibacterium linens*	DSM 20425^T
5	*Brevibacterium ketoglutamicum*	DSM 20165
6	*Pimelobacter simplex*	DSM 20130^T
7	*Propionibacterium freudenreichii*	DSM 20271^T
8	*Flavobacterium ferrugineum*	DSM 30193^T
9	*Cytophaga johnsonae*	GBF Cy j1
10	*Myxococcus fulvus*	GBF Mx f2
11	*Myxococcus virescens*	GBF Mx v4
12	*Pseudomonas putida*	DSM 291^T
E 1	*Bacillus cereus*	DSM 31^T
2	*Bacillus subtilis*	ATCC 6633
3	*Clostridium acetobutylicum*	NCIMB 8052^T
4	*Clostridium stercorarium*	NCIMB 11754^T
5	*Pectinatus frisingensis*	DSM 20465
6	*Enterococcus faecium*	DSM 20477^T
7	*Enterococcus faecalis*	DSM 20478^T
8	*Lactobacillus casei*	LMG 9091
9	*Lactococcus lactis*	DSM 20481^T
10	*Lactococcus cremoris*	DSM 20069^T
11	*Staphylococcus aureus*	DSM 20231^T
12	*Streptococcus salivarius*	DSM 20560^T
F 1	*Acinetobacter haemolyticus*	LMG 996^T
2	*Acinetobacter junii*	LMG 998^T
3	*Acinetobacter johnsonii*	LMG 999^T
4	*Acinetobacter lwoffii*	LMG 1029^T
5	*Acinetobacter baumannii*	LMG 1041^T
6	*Acinetobacter radioresistens*	LMG 10613^T

[a] ATCC, American Type Culture Collection, Rockville, Md.; CCM, Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; GBF, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany (H. Reichenbach); LMG, Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium; NCIMB, National Collection of Industrial and Marine Bacteria, Torry Research Station, Aberdeen, Scotland; WS, Bakteriologisches Institut der Süddeutsche Versuchs- und Forschungsanstalt für Milchwirtschaft, Technische Universität München, Freising-Weihenstephan, Germany.

for defined phylogenetic groups (the alpha, beta, and gamma subclasses of the class *Proteobacteria*, the *Cytophaga-Flavobacterium* cluster, and gram-positive bacteria with a high G+C DNA content). In particular, we monitored population changes upon (i) anaerobic stress, (ii) the addition of iron salts (for simultaneous chemical precipitation of phosphate), and (iii) the addition of acetic acid to the return sludge.

MATERIALS AND METHODS

Organisms and culture conditions. The organisms investigated in this study are listed in Tables 1 and 2. Cells were cultured as described in the catalogs of strains of the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; American Type Culture Collection, Rockville, Md., Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia; and Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium.

Sampling. Grab samples of mixed liquor were collected from the influent wastewater and the successive anaerobic, anoxic, and aeration stages of the municipal wastewater treatment plant in Hirblingen, Germany (30,000 population equivalents [PE]). This plant uses the Phoredox process (30) without

TABLE 2. Listing of *Acinetobacter* strains used for whole-cell hybridization

Genospecies	Organism	Strain
1	*Acinetobacter calcoaceticus*	LMG 1046^T
2	*Acinetobacter baumannii*	LMG 1041^T
3	*Acinetobacter* sp.	ATCC 19004
4	*Acinetobacter haemolyticus*	LMG 996^T
5	*Acinetobacter junii*	LMG 998^T
6	*Acinetobacter* sp.	ATCC 17979
7	*Acinetobacter johnsonii*	LMG 999^T
8	*Acinetobacter lwoffii*	LMG 1029^T
9	*Acinetobacter* sp.	ATCC 9957
10	*Acinetobacter* sp.	ATCC 17924
11	*Acinetobacter* sp.	ATCC 11171
12	*Acinetobacter radioresistens*	LMG 10613^T
13	*Acinetobacter* sp.	ATCC 17905
13	*Acinetobacter* sp.	ATCC 17903
14	*Acinetobacter* sp.	382 (8)
14	*Acinetobacter* sp.	71 (37)
15	*Acinetobacter* sp.	79 (8)
15	*Acinetobacter* sp.	151a (37)
16	*Acinetobacter* sp.	ATCC 17988
17	*Acinetobacter* sp.	942 (8)

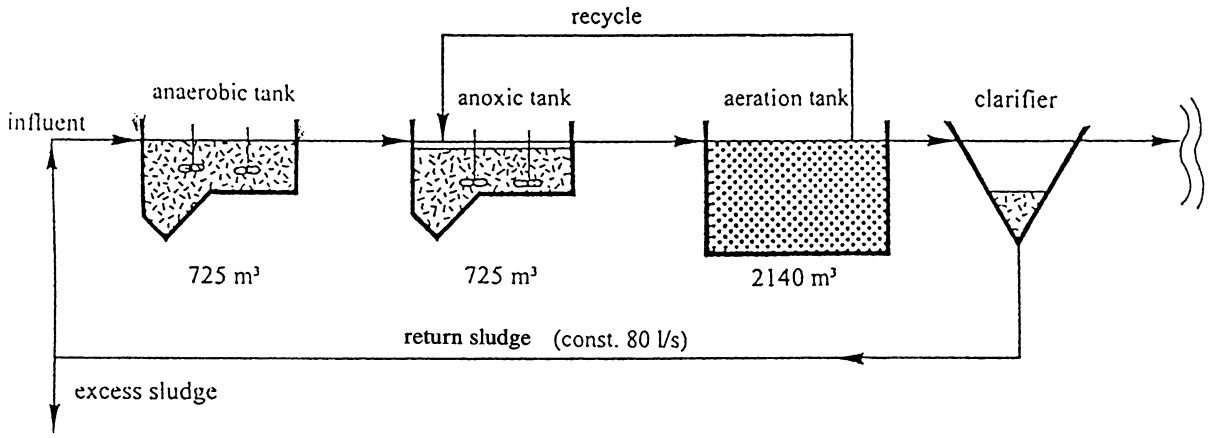


FIG. 1. Process schematic and reactor volumes of the Hirblingen, Germany, wastewater treatment plant.

a primary settling tank (Fig. 1). Filamentous bacterium type 1863 (15) in activated sludge samples from a large municipal sewage plant (München II, Gut Marienhof, Germany; 1 million PE) was studied. In addition, samples were collected from the aeration basin of another municipal sewage plant (München I, Grosslappen, Germany; 1 million PE) and from the anaerobic and aerobic reactors of the plant at Berlin-Ruhleben, Germany (1.3 million PE). For in situ hybridization, activated sludge samples were fixed with paraformaldehyde solution (4%) immediately after the samples were taken (1). For in situ detection of gram-positive bacteria with a high G+C DNA content, activated sludge samples were fixed by the addition of ethanol to a final concentration of 50%. This treatment allows the simultaneous detection of gram-positive bacteria with a high G+C DNA content and gram-negative bacteria by hybridization with a bacterial probe.

Phosphate determination. The P_i concentrations in the bulk liquid of the activated sludge were analyzed by on-line monitoring with a pump-colorimeter-analyzer (model 31500; Hach Co.) by the vanadomolybdophosphoric acid colorimetric method. The sample analyzed was separated from the activated sludge by ultrafiltration. Total phosphorus analyses of the wastewater and the activated sludge were carried out by previously described methods (14).

Plate counts and cultivation. Sludge samples were homogenized by treatment with an Ultraturrax blender (IKA-Werk; Janke and Kunkel, Staufen, Germany) for 4 min on ice after the addition of 2.8 g of sodium PP_i per liter. Serial dilutions in the range of 10^{-4} to 10^{-8} were plated in duplicate on TSA (Trypticase soy broth [BBL 11768], 30 g/liter; Bacto agar [Difco 0140-01], 15 g/liter; pH 7.2), and plates were scored after incubation at 22°C for 2 days. The statistical error was in the range of 6 to 18%. From the TSA plates, about 160 randomly chosen colonies were further cultivated for fatty acid analysis of whole-cell hydrolysates (Microbial Identification System [MIS]; MIDI Inc., Newark, Del.) and whole-cell probing with fluorescent oligonucleotides.

Staining techniques. Neisser staining of cells was done as described by Eikelboom and van Buijsen (16). Simultaneous staining of lipophilic and metachromatic granules in individual cells with Nile blue A and Neisser's methylene blue was performed as described by Rees et al. (31). Gram staining was carried out by the technique described by Eikelboom and van Buijsen (16).

Cell fixation. Cells growing in the logarithmic phase at an optical density at 600 nm of between 0.5 and 0.8 were harvested by centrifugation (2 min, 5,000 × *g*) and washed in phosphate-buffered saline (PBS; 130 mM NaCl, 10 mM sodium phosphate buffer [pH 7.4]). Bacteria were fixed for 3 h in 3% paraformaldehyde–PBS (1), washed in PBS, and finally resuspended. Bacteria were stored in a 1:1 mixture of PBS and 96% ethanol at −20°C.

Extraction and analysis of fatty acids. Fatty acid analyses of whole-cell hydrolysates were performed according to the MIS. According to the "aerobe method" of the MIS for the determination of aerobic bacteria, cells were grown on TSA plates for 24 h at 28 ± 1°C. About 40 mg of wet cells was harvested from the quadrant with confluent growth, indicating the late logarithmic phase, which typically yields the most stable fatty acid composition. Weakly growing cells were plated in triplicate and grown for an additional period of time until confluent growth was obtained.

Fatty acid extraction from whole cells was performed according to the MIS with a 1:1 mixture of hexane and *methyl-tert*-butyl ether as the organic phase. The fatty acid composition was determined in a gas chromatograph (Hewlett Packard HP 5890 II) with a capillary inlet flow system (HP Ultra 2 fused-silica capillary column) and a flame ionization detector and with hydrogen as the carrier gas. After qualitative and quantitative calibration of the gas chromatograph with fatty acid standards, a library search was performed by use of a pattern recognition program yielding most likely matches between the library and the unknown fatty acid composition and a similarity index for each match. The library used here was TSBA version 3.7 (MIDI).

Membrane filtration and staining with DAPI. Membrane filtration and staining with 4′,6-diamidino-2-phenylindole (DAPI) were performed as described earlier (39). The method of dual staining of cells with DAPI and fluorescent oligonucleotides was modified from the method of Hicks et al. (18) so that cells were stained after in situ hybridization with DAPI (0.33 μg/ml) for 5 min.

Oligonucleotide probes. The following rRNA-targeted oligonucleotides were used: (i) ACA, complementary to a region of the 16S rRNA of *Acinetobacter calcoaceticus*; (ii) CF, complementary to a region of the 16S rRNA of members of the *Cytophaga-Flavobacterium* cluster; and (iii) HGC, complementary to a region of the 23S rRNA specific for all hitherto-sequenced gram-positive bacteria with a high G+C DNA content (unpublished data). Sequences and target sites are shown in Table 3. In addition, the following oligonucleotides were used: (i) ALF, BET, and GAM, complementary to

TABLE 3. Probe sequences, target sites, and formamide concentration in the hybridization buffer required for specific in situ hybridization

Probe	Sequence	Target site[a] (rRNA positions)	% Formamide
ACA	5′-ATCCTCTCCCATACTCTA-3′	16S (652–669)	35
CF	5′-TGGTCCGTGTCTCAGTAC-3′	16S (319–336)	35
HGC	5′-TATAGTTACCACCGCCGT-3′	23S (1901–1918)	25

[a] *Escherichia coli* numbering (9).

regions of 16S (ALF) or 23S (BET and GAM) rRNA conserved in the alpha, beta, and gamma subclasses of the class *Proteobacteria*, respectively (25); and (ii) EUB, complementary to a conserved region of bacterial 16S rRNA molecules (1).

Oligonucleotides were synthesized with a C6-TFA aminolinker [6-(trifluoroacetylamino)-hexyl-(2-cyanoethyl)-(*N*,*N*-diisopropyl)phosphoramidite] at the 5′- end (MWG Biotech, Ebersberg, Germany). Labeling with tetramethylrhodamine-5-isothiocyanate (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 by Amann et al. (2). Probes ACA and EUB were also labeled with digoxigenin as described by Zarda et al. (42).

Optimal hybridization conditions were determined for probes ACA, CF, and HGC as described by Manz et al. (25). For each probe, the optimal formamide concentration in the hybridization buffer is shown in Table 3.

In situ hybridization and probe-specific cell counts. Hybridization of fixed activated sludge samples (immobilized on glass slides) was done as described by Manz et al. (25). Optimal hybridization stringency required the addition of formamide to a final concentration of 20% (probes ALF and EUB), 25% (probe HGC), or 35% (probes ACA, CF, BET, and GAM) in the hybridization buffer. In the washing buffer (containing no formamide), the sodium chloride concentration was adjusted to guarantee sufficient stringency (25). Since single-mismatch discrimination was required for probes BET and GAM, these probes were used with competitor oligonucleotides as described earlier (25). Slides were examined with an Axioplan microscope (Zeiss, Oberkochen, Germany) with filter sets 09 and 15. For each sample, more than 10,000 cells stained with probe EUB were enumerated. In the sludge samples, there was a nonhomogeneous distribution of the bacteria (aggregates). Therefore, a simple statistical evaluation was not possible. Black-and-white photomicrographs were taken with Kodak Tmax400 film. Exposure times were 0.06 s for phase-contrast micrographs and 15 to 30 s for epifluorescence micrographs.

Dot blot hybridization. The specificity of oligonucleotide probe ACA was evaluated by dot blot hybridization of reference nucleic acids extracted from 66 pure cultures of bacteria (representing a diverse collection of taxa) with a digoxigenin-labeled probe. The composition of the membrane is shown in Table 1. Gram-positive cells were mechanically disrupted with glass beads (0.17 mm in diameter) in combination with sonication (2 min). Reference nucleic acids were isolated as described before (25). The concentrations of nucleic acids were determined spectrophotometrically by measuring the A_{260}. Isolated reference nucleic acids were immobilized on nylon membranes (Quiagen; Quiabrane, Düsseldorf, Germany) by use of a dot blot apparatus (Schleicher and Schuell, Dassel, Germany). Probing with digoxigenin-labeled oligonucleotides was done as described before (25). Hybridization with probe ACA was performed with a hybridization buffer containing 35% formamide; that with probe EUB was performed with hybridization buffer containing 0% formamide.

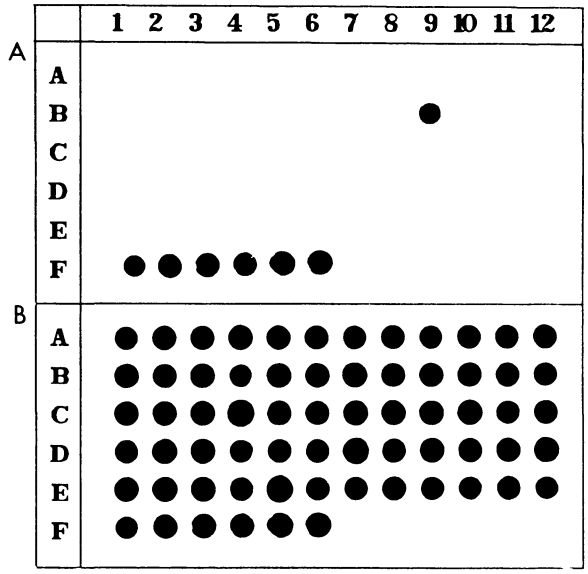


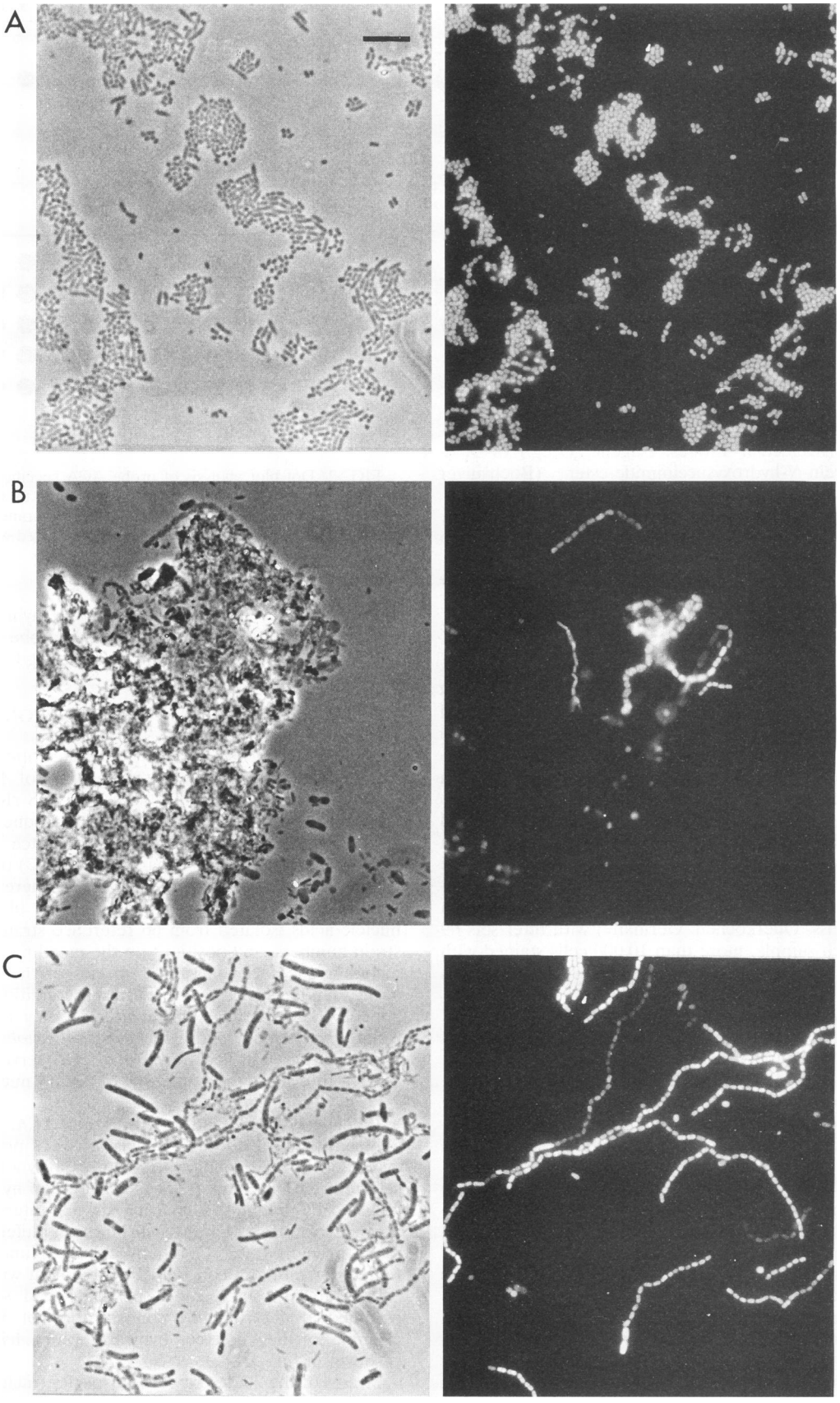
FIG. 2. Dot blot analysis of probe ACA specificity. Nucleic acids were isolated from 66 reference strains and immobilized on the membrane as indicated in Table 1. (A) Membrane hybridized with probe ACA. (B) Identical membrane rehybridized with probe EUB.

RESULTS AND DISCUSSION

Specificity of probe ACA. The oligonucleotide probe ACA is complementary to a sequence in helix 23a of *A. calcoaceticus*. A comparison with all other accessible 16S rRNA sequences (1,500 complete or almost complete sequences [22, 27]) showed that ACA has at least one mismatch. Currently, 16S rRNA sequences of only a part (about 25%) of the described bacterial species have been determined; therefore, the specificity of probe ACA was evaluated by dot blot analysis with nucleic acids isolated from 66 reference strains (for sources, strain numbers, and positions on the membrane, see Table 1), including the seven described species of the genus *Acinetobacter*. Under stringent hybridization conditions (35% formamide, 46°C), probe ACA hybridized only to nucleic acids isolated from members of the genus *Acinetobacter* (Fig. 2A). Hybridization with bacterial probe EUB served as a positive control to examine the amounts of bound nucleic acids (Fig. 2B).

Whole-cell hybridization with probe ACA. The specificity and sensitivity of fluorescent derivatives of probe ACA were evaluated by hybridization to mixtures of whole fixed cells of selected reference strains. The stringency of hybridization was adjusted by gradually increasing (in 5% intervals) the formamide concentration in the hybridization buffer while keeping ionic strength (0.9 M sodium chloride) and hybridization temperature (46°C) constant. In agreement with the dot blot hybridization results, good specificity and sensitivity were obtained at a formamide concentration of 35%. A typical example for the whole-cell hybridization of *Acinetobacter* spp. is shown in Fig. 3A.

Strains of the 20 described genospecies (including the seven described species) were tested by whole-cell hybridization


A
B
C

(Table 2). All of these strains showed a bright hybridization signal with probe ACA.

In situ hybridization of activated sludge. The composition of the microbial population in the anaerobic and aerobic stages of a wastewater treatment plant with EBPR (Hirblingen, Germany) was determined by fluorescent-oligonucleotide probing. In the sample from the anaerobic basin, 83% (±8%) of the cells visualized by DAPI were also detected by bacterial probe EUB. In the sample from the aeration basin, 78% (±10%) of all DAPI-stained cells hybridized with the bacterial probe. These findings demonstrated that the bulk of the fixed cells were members of the domain *Bacteria* which were accessible to oligonucleotide probes and contained sufficient rRNA for detection. The high EUB count/DAPI count ratio shows that whole-cell hybridization with rRNA-targeted oligonucleotide probes is well suited for the analysis of bacterial community structure in activated sludge. In more oligotrophic environments, this ratio is significantly lower (unpublished observations). Hybridization with probes for the alpha, beta, and gamma subclasses of the class *Proteobacteria* as well as with probes for the *Cytophaga-Flavobacterium* cluster (probe CF) and gram-positive bacteria with a high G+C DNA content (probe HGC) resulted in easily detectable staining of individual cells. Application of the probe specific for the genus *Acinetobacter* (ACA) resulted in the visualization of rods and cocci commonly occurring in clusters or chains (Fig. 3B). The bright staining of the cells even within and underneath the sludge flocs facilitated reliable enumeration without further dispersion and declustering of the bacteria in the activated sludge. Probe ALF, BET, GAM, CF, HGC, and ACA counts for the anaerobic and aerobic stages are presented in Fig. 4A (given as percentages of cells hybridizing with bacterial probe EUB). In the anaerobic basin, probe BET and HGC counts (24% each) dominated over probe ALF (11%), CF (9%), and GAM (5%) counts. The majority of cells showing a positive hybridization signal with probe GAM (60%) could simultaneously be detected with probe ACA. In the aeration basin, the structure of the microbial consortium was similar. Cells hybridizing with probes BET (26%) and HGC (19%) were more abundant than those hybridizing with probe ALF (9%), GAM (10%), or CF (8%). Once again, cells hybridizing with probe ACA (7%) represented the bulk of the cells assigned to the gamma subclass of the class *Proteobacteria*.

Repeated sampling at the Hirblingen plant between February and May 1993 revealed no significant change in group-specific cell counts (data not shown). Gram-positive bacteria with a high DNA G+C content accumulated in all stages in comparison with levels in municipal wastewater treatment plants without EBPR. In the aeration basins of two large plants (München I, Grosslappen, Germany, and München II, Gut Marienhof, Germany) with chemical phosphate removal instead of EBPR, only 7% (München I, high load, 0.8 biological oxygen demand [BOD]/kg/day) or 1% (München II, stage 1, high load, 1.0 BOD/kg/day; stage 2, low load, 0.07 BOD/kg/day) of the cells hybridized with probe HGC, while the number of cells hybridizing with probe ACA was in the same range as in the anaerobic-aerobic activated sludge system (3% for München I; 8% for München II, stage 1; and 2% for München II, stage 2). These results indicate that bacteria other than *Acinetobacter* spp. must be involved in EBPR in the Hirblingen plant. Preliminary community structure analysis in the anaerobic and aerobic reactors of another plant showing highly efficient biological phosphate removal (Berlin-Ruhleben, Germany) revealed that in both compartments, the number of cells hybridizing with probe ACA was below 2%. Consequently, members of the genus *Acinetobacter* can only be of secondary importance in the EBPR process.

Neisser staining could not be combined with whole-cell hybridization but revealed metachromatic granules indicative of polyphosphate inclusions in cell morphotypes that bound probe HGC. There is much confusion concerning the quality of the Neisser staining procedure for the differentiation of polyphosphate and poly-β-hydroxybutyrate granules (36). Rees et al. (31) developed a double-staining procedure with Nile blue A and Neisser's methylene blue to show the two types of granules separately within individual cells. With this technique, we could demonstrate the presence of polyphosphate-accumulating gram-positive bacteria with a high G+C DNA content. For the morphotypes detected by probe BET, we found no polyphosphate inclusions, whereas poly-β-hydroxybutyrate granules were quite abundant (data not shown).

Microthrix parvicella, a filamentous, gram-positive bacterium recognized by its peculiar morphology (15), was also found in the Hirblingen plant. This organism was impermeable to fluorescent-oligonucleotide probes without further pretreatment and therefore was not included in our enumerations. Limited probe penetration into *M. parvicella* is probably caused by its extremely hydrophobic cell surface (33). Heavily stained volutin granules after the staining procedure of Rees et al. (31) indicated that it accumulated large amounts of phosphate. Pretreatment of *M. parvicella* filaments with diethyl ether for 15 min facilitated oligonucleotide probe penetration into this organism but deteriorated hybridization signals for gram-negative bacteria. *M. parvicella* hybridized with probe HGC, indicating an affiliation with gram-positive bacteria with a high G+C DNA content. This result must be confirmed by sequencing data.

Changes in community structure and function upon the addition of iron salts and acetic acid. In wastewater treatment plants with EBPR, additional chemical phosphate precipitation with iron salts is a common strategy (3) used to attain effluent concentrations of less than 2 mg of phosphorus/liter. The continuous addition of 10 kg of Fe^{3+} as $FeClSO_4$ per day for 60 days did not strongly affect the composition of the microbial consortia. In the anaerobic basin, 78% (±15%) of the microbial cells stained with DAPI also emitted bacterial probe EUB-conferred fluorescence. In the sludge from the anoxic zone and the aeration basin, 82% (±11%) and 79% (±11%) of all cells, respectively, reacted with the bacterial probe. Probe ALF, BET, GAM, CF, HGC, and ACA counts for the anaerobic, anoxic, and aerobic stages are presented in Fig. 4B (given as percentages of cells hybridizing with bacterial probe EUB). In the anaerobic basin, probe BET (26%) and HGC (17%) counts codominated over probe ALF (13%), CF (11%), and GAM (9%) counts. Simultaneous hybridization with probes ACA and GAM labeled with different fluors

FIG. 3. Whole-cell identification of *Acinetobacter* spp. For each panel, identical fields were viewed by phase-contrast microscopy (left) and epifluorescence microscopy (right). Bar, 10 μm (all photomicrographs). (A) Artificial mixture of fixed cells of *Pseudomonas fluorescens* and *Acinetobacter haemolyticus* hybridized with fluorescein-labeled probe ACA. (B) In situ hybridization of an activated sludge sample obtained from the aerobic stage of the Hirblingen plant with fluorescein-labeled probe ACA. (C) In situ hybridization of activated sludge from stage 1 of the München II plant with fluorescein-labeled probe ACA. Filamentous bacteria of Eikelboom type 1863 show bright signals.

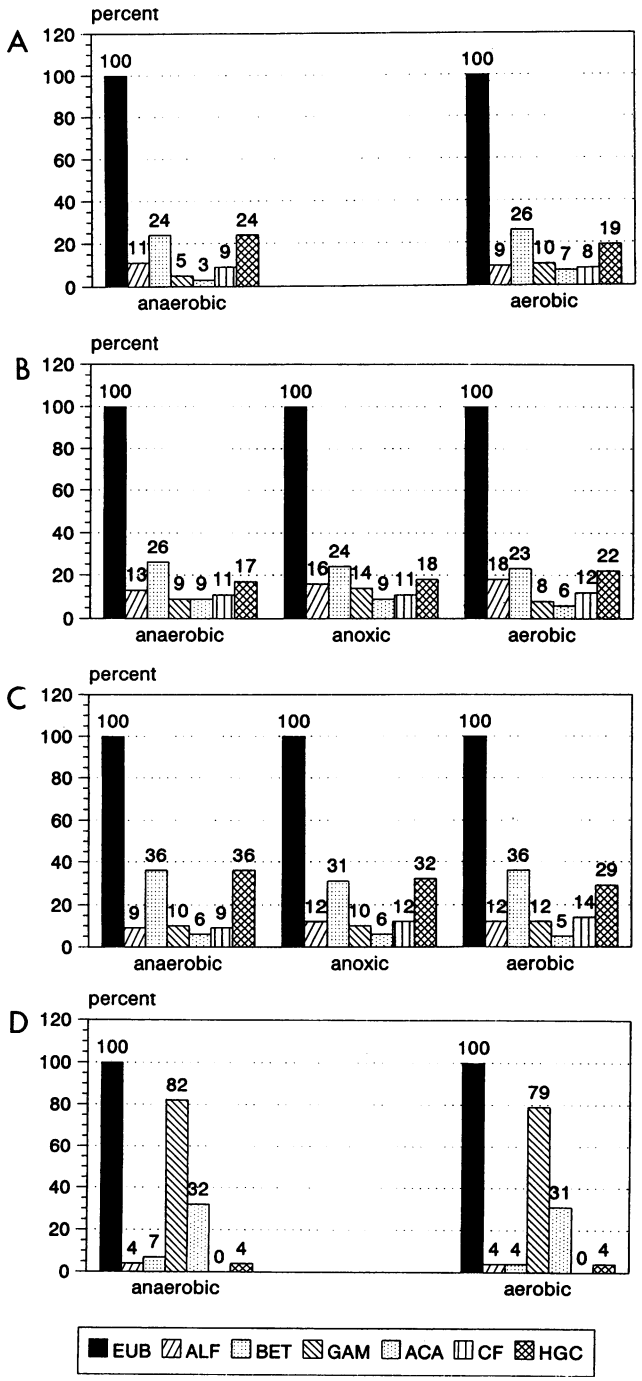


FIG. 4. Comparison of community compositions in activated sludge samples from the Hirblingen plant. (A) Microbial community structure during the EBPR mode of the plant, as determined by in situ hybridization (3 February 1993). (B) Microbial community structure in the EBPR and chemical phosphate precipitation mode of the plant, as determined by in situ hybridization (21 May 1993). (C) Microbial community structure in the EBPR and chemical phosphate precipitation mode of the plant following the addition of acetic acid, as determined by in situ hybridization (25 May 1993). (D) Heterotrophic saprophytes, as determined by whole-cell hybridization of isolates obtained on TSA from the sample shown in panel C.

showed that all cells hybridizing with probe GAM also hybridized with probe ACA (9%). In the anoxic zone and in the aeration basin, the population structure did not dramatically change. Cells hybridizing with probe BET (24% in anoxic zone and 23% in aeration basin) and HGC (18% in anoxic zone and 22% in aeration basin) were more frequent than cells hybridizing with probe ALF (16% in anoxic zone and 18% in aeration basin), GAM (14% in anoxic zone and 8% in aeration basin), or CF (11% in anoxic zone and 12% in aeration basin). Again, cells hybridizing with probe ACA (9% in anoxic zone and 6% in aeration basin) represented the major part of the cells assigned to the gamma subclass.

To obtain effective biological phosphate removal, the influent must contain sufficient and appropriate substrates for the growth of phosphate-accumulating bacteria (32). To overcome the lack of substrates and to enhance the anaerobic phosphate release from phosphate-accumulating bacteria, some authors have suggested the addition of acetic acid to the anaerobic zone (32). We have analyzed the influence of this supplementary substrate on the composition of the microbial consortia and biological phosphate removal by adding acetic acid (20 mg/liter) to the return sludge for 72 h. After this period, 75% ± 9% (anaerobic basin), 83% ± 9% (anoxic zone), and 81% ± 10% (aeration basin) of the microbial cells stained with DAPI also emitted bacterial probe EUB-conferred fluorescence. These findings are in good agreement with those of control studies in which no additional acetic acid was used. Probe ALF, BET, GAM, CF, HGC, and ACA counts for the anaerobic, anoxic, and aerobic stages after the addition of acetic acid are summarized in Fig. 4C (again given as percentages of cells hybridizing with bacterial probe EUB). In the anaerobic basin, probe BET and HGC counts (36% each) dominated over probe GAM (10%), ALF (9%), and CF (9%) counts. Six percent of the cells hybridized with probe ACA. Similar findings were obtained in the anoxic zone and the aeration basin. Cells hybridizing with probe BET (31% in anoxic zone and 36% in aeration basin) and probe HGC (32% in anoxic zone and 29% in aeration basin) were more abundant than cells hybridizing with probe ALF (12% in anoxic zone and aeration basin), CF (12% in anoxic zone and 14% in aeration basin), or GAM (10% in anoxic zone and 12% in aeration basin). Probe ACA counts were in the same range as without acetic acid (6% in anoxic zone and 5% in aeration basin). The addition of acetic acid to the anaerobic basin enhanced the growth of gram-positive bacteria with a high G+C DNA content and of bacteria belonging to the beta subclass of the class *Proteobacteria*. It did not significantly change the number of *Acinetobacter* spp. Furthermore, one can suppose from these results that the formation of lower fatty acids, such as acetic acid, in the anaerobic stage of the plant may be the cause for the large numbers of gram-positive bacteria with a high G+C DNA content in the examined plant.

On-line phosphate determination. During the whole examination period, full-scale data for the Hirblingen plant (Fig. 5) showed a constant effective phosphate influx concentration (2.7 to 3 mg/liter, consisting of phosphate sewage diluted by phosphate return) and a low level of phosphate release (between 2.5 and 5.4 mg/liter) in the anaerobic reactor. In this part of the plant, only 4 to 10% of the total stored phosphate in the activated sludge was released into the bulk liquid (40). EBPR and EBPR with simultaneous chemical phosphate precipitation resulted in effluent phosphate concentrations of 0.2 to 0.7 mg/liter, significantly below the demanded threshold value of 2 mg of total phosphorus per liter. The addition of acetic acid to the return sludge slightly enhanced the release of stored phosphate into the anaerobic compartment (from approxi-

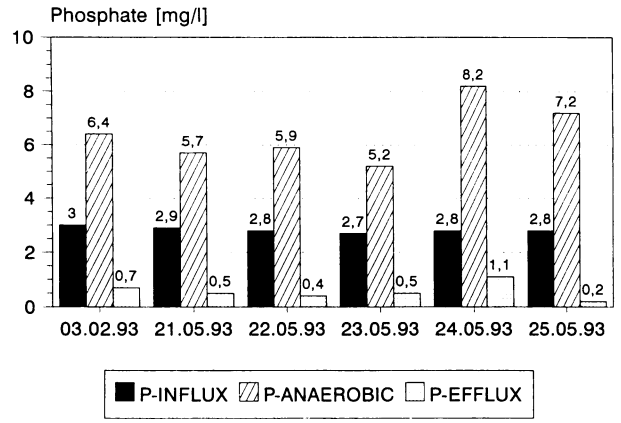


FIG. 5. On-line phosphate (P) determination in the Hirblingen plant for the sampling dates shown (day.month.year). The plant was in the EBPR mode without chemical phosphate precipitation on 3 February 1993 and in the EBPR and chemical phosphate precipitation mode on 21 May 1993. Acetic acid was added from 22 to 25 May 1993.

mately 3 to 5.4 mg/liter at the end of the 3-day period). Phosphate efflux from the aerobic reactor increased from approximately 0.5 to 1.1 mg/liter on the last day of acetic acid addition but decreased to 0.2 mg/liter on the next day. The increased value of 1.1 mg of phosphate per liter is within the regular variation of phosphate efflux concentrations. These data indicate that the microbial consortium in the Hirblingen plant is able to remove phosphate efficiently from phosphate sewage containing approximately 8 mg/liter to a phosphate efflux concentration below 1 mg/liter. The low level of phosphate release (below 10%) into the anaerobic compartment indicates that bacteria other than *Acinetobacter* spp., for which excessive phosphate release under anaerobic conditions was shown (i.e., 29), are responsible for phosphate removal.

Cultivation and identification of isolates. Samples from the anaerobic basin and the aeration basin supplemented with acetic acid were plated on TSA. The nutrient-rich culture medium used in this study had been compared with several other media (23) and yielded a high plating efficiency. In the sample from the anaerobic basin, there were 1.3×10^7 CFU/ml (only 1.7% of the total microscopic counts of $7.6 \times 10^8 \pm 3.2 \times 10^8$); in the sample from the aeration basin, this number was 1.9×10^7 (in comparison, $4.7 \times 10^8 \pm 1.1 \times 10^8$ cells were visualized by DAPI [4%]). Randomly chosen colonies (80 from each sample) were classified by whole-cell hybridization (Fig. 4D). The majority of colonies retrieved from the anaerobic basin hybridized with probe GAM (82%), only 7% hybridized with probe BET, and 4% each hybridized with probes ALF and HGC. Thirty-two percent of the colonies could be determined to be members of the genus *Acinetobacter* by use of probe ACA. None of the colonies hybridized with probe CF. Colonies obtained from the aeration basin showed similar hybridization patterns. Seventy-nine percent of the colonies hybridized with probe GAM, and only a minority hybridized with probes ALF, BET, and HGC (4% for each probe). Thirty-one percent of the colonies hybridized with probe ACA. Once again, none of the colonies showed a positive hybridization reaction with probe CF. All colonies identified by fluorescent-oligonucleotide probing as *Acinetobacter* spp. were independently classified by fatty acid analysis. Over 90% of the colonies were identified as *Acinetobacter* spp. However, the match of the fatty acid profiles of the isolates with the profiles in the data base was not satisfactory (often below 0.5). Very likely, the environmental strains of *Acinetobacter* spp. are rather different from the clinical isolates of *Acinetobacter* spp. (34), which served as the source for the construction of the data base.

As already shown in an earlier study (39), the community compositions determined by cultivation were dramatically different from the in situ compositions. The nutrient-rich medium strongly favored the growth of bacteria belonging to the gamma subclass of the class *Proteobacteria*, most notably of *Acinetobacter* spp. Because of the strong selectivity of the presumably "nonselective media" used in most studies, the importance of acinetobacters in biological phosphate elimination was overestimated.

Filamentous bacterium of Eikelboom type 1863. In his key to the microscopic identification of filamentous bacteria in activated sludge, Eikelboom (15) described *Streptococcus*-like chains of gram-negative cells as type 1863. These hitherto-uncultured filaments are generally free floating between the flocs in the mixed liquor, showing a positive hybridization reaction with probe GAM (39) and with the *Acinetobacter*-specific probe (Fig. 3C). Gram staining of the filaments present in activated sludge samples showed a majority of gram-negative cells, with a few gram-positive cells. Members of the genus *Acinetobacter* are able to form chains of cells and are sometimes gram variable (20). These results strongly support a close affiliation of the filamentous bacterium of Eikelboom type 1863 with the genus *Acinetobacter*.

The in situ data have clearly demonstrated that members of the genus *Acinetobacter* do not play a major role in the EBPR process in the Hirblingen plant. The dominance of gram-positive bacteria with a high G+C DNA content and the presence of polyphosphate inclusions in these bacteria indicate that they may play an important role in the EBPR process. Nakamura et al. (26) have isolated a number of gram-positive bacteria from sludge accumulating large amounts of phosphate. Phosphate accumulation was also described for the genera *Nocardia*, *Rhodococcus* (24), and *Arthrobacter* (28), all gram-positive bacteria with a high G+C DNA content. A recent study on the occurrence of polyphosphate-dependent enzymes in coryneform bacteria isolated from sewage sludge (5) is an additional hint for the importance of gram-positive bacteria with a high G+C DNA content as phosphate-removing agents. Preliminary results on the microbial population structure of the sewage treatment plant at Berlin-Ruhleben using an EBPR process support this view.

Keeping in mind the overestimation of the number of *Acinetobacter* spp. caused by cultivation-dependent population shifts, investigations of the EBPR process should also include in situ population studies.

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