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Isolation of New Bacterial Species from Drinking Water Biofilms and Proof of Their In Situ Dominance with Highly Specific 16S rRNA Probes

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A polyphasic approach involving cultivation, direct viable counts, rRNA-based phylogenetic classification, and in situ probing was applied for the characterization of the dominant microbial population in a municipal drinking water distribution system. A total of 234 bacterial strains cultivated on R2A medium were screened for bacteria affiliated with the in situ dominating beta subclass of *Proteobacteria*. The isolates were grouped according to common features of their cell and colony morphologies, and eight representative strains were used for 16S rRNA sequencing and the development of a suite of strain-specific oligonucleotide probes. Phylogenetic analysis indicated that all of the isolates were hitherto unknown bacteria. Three of them, strains B4, B6, and B8, formed a separate cluster of closely related organisms within the beta1 subclass of *Proteobacteria*. In situ probing revealed that (i) 67 to 72% of total bacteria, corresponding to more than 80% of beta-subclass bacteria, could be encompassed with the strain-specific probes and (ii) the dominating bacterial species were culturable on R2A medium. Additionally, two-thirds of the autochthonous drinking water population could be shown to be in a viable but nonculturable (VBNC) state by using a direct viable count approach. The comparison of isolation frequencies with the in situ abundances of the eight investigated strains revealed differences in their culturability, indicating variable ratios of culturable to VBNC cells among the strains. The further characterization of biofilms throughout the distribution network demonstrated strains B6 and B8 to be dominant bacterial strains in groundwater and distribution system biofilms. The other strains could be found at various frequencies in the different parts of the distribution system; several strains appeared exclusively in drinking water biofilms obtained from a house installation system.

Drinking water and the related microbial population in the distribution system, including biofilm bacteria, constitute one of the most extensively studied oligotrophic systems (12, 20, 23). Several studies have focused either on species causing infectious diseases, such as *Legionella pneumophila* (24), or on indicator organisms for fecal contamination, such as coliform bacteria (13, 31). Other investigations attempted to describe the whole spectrum of organisms by using culture-dependent techniques (7, 12, 32) or molecular approaches (9, 16) but did not identify the in situ dominating bacterial species.

By using conventional cultivation techniques, less than 1% of the bacterial population from oligotrophic systems can be cultivated, which has been described as "the great plate count anomaly" by Staley and Konopka (29). There are two possible explanations for this observation: first, the majority of the microbial population within these ecosystems is nonviable, and second, most of the cells are viable and active in their natural environment but cannot be cultivated. A number of studies clearly indicate that most of the bacteria present in oligotrophic aquatic systems are metabolically active (11, 21, 25, 29). The inability of these bacteria to form colonies on commonly used media, such as R2A (22), might be due to either (i) cells having transiently entered a viable but nonculturable (VBNC) state (19, 25) in response to oligotrophic conditions or (ii) the presence of bacterial species which are per se nonculturable on such media.

In situ hybridization with rRNA-targeted oligonucleotide probes, a method increasingly used to identify bacteria within their natural habitats (3, 4), provides the appropriate tool to evaluate these two hypotheses. In oligotrophic systems, the detection sensitivity of fluorescent oligonucleotides might be limited by low ribosome content or restricted cell accessibility (4). In several studies, however, oligonucleotide probes could be successfully applied for the phylogenetic characterization of microbial communities in low-nutrient habitats, such as drinking water and associated biofilms (9, 16) or oligotrophic lakes (1).

In the Berlin, Germany, drinking water distribution system, bacteria affiliated with the beta subclass of *Proteobacteria* constitute the dominant bacterial population, whereas bacteria belonging to the alpha and gamma subclasses are present only in low numbers (9). Just as in other oligotrophic systems, less than 1% of the bacteria can be recovered as CFU. Most cells, however, are able to utilize the nutrients present in the commonly used R2A medium (22) in a modified direct viable count (DVC) assay (9) and should thus, in principle, be culturable on this medium.

The aim of the present study was to characterize the dominant bacterial population of drinking water biofilms. The development of specific oligonucleotide probes for isolated bacterial strains and subsequent in situ probing were used to reveal the abundances of these bacteria in their natural habitat. Using this strategy, we were able to determine whether nonculturable species or potentially culturable species predominated in the drinking water biofilms.

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TABLE 1. Physical and chemical parameters for drinking water obtained from the Jungfernheide waterworks, Berlin

Parameter (unit)	Mean (minimal, maximal values) for 1996
Temp (°C)	12.5 (9.4, 15.6)
pH	7.4 (7.2, 7.7)
Total hardness (°dH)	20.2 (18.7, 22.1)
$KMnO_4$- O_2 (mg $liter^{-1}$)	2.3 (1.5, 3.0)
DOC[a] (mg $liter^{-1}$)	3.5 (3.2, 3.8)
Ca^{2+} (mg $liter^{-1}$)	124 (115, 135)
Mg^{2+} (mg $liter^{-1}$)	12.6 (11.4, 13.8)
Na^{+} (mg $liter^{-1}$)	50 (43, 57)
K^{+} (mg $liter^{-1}$)	8.2 (6.8, 9.4)
Total Fe (mg $liter^{-1}$)	0.03 (0.00, 0.10)
Total Mn (mg $liter^{-1}$)	0.02 (0.00, 0.05)
Cl^{-} (mg $liter^{-1}$)	81 (68, 94)
NO_3^{-} (mg $liter^{-1}$)	0.84 (0.37, 1.62)
NO_2^{-} (mg $liter^{-1}$)	0.00
SO_4^{2-} (mg $liter^{-1}$)	188 (170, 200)
F^{-} (mg $liter^{-1}$)	0.19 (0.16, 0.21)
Cl_2 (mg $liter^{-1}$)	0

[a] DOC, dissolved organic carbon.

MATERIALS AND METHODS

Sampling of biofilms. Modified Robbins devices (9) with polyethylene (PE) or glass slides as the substrata were installed at three locations of the Berlin drinking water distribution system: (i) in a well of the Jungfernheide waterworks located immediately after the pumping station, (ii) parallel to a main distribution pipe at a distance of about 5 km from the waterworks, and (iii) connected to a water tap in a house installation system at the Technical University Berlin. The anoxic groundwater which is used as the source for drinking water treatment is subjected to an aeration step, in which excess iron and manganese are precipitated, followed by a fast sand filtration without a final chlorination step. The physical and chemical parameters of the drinking water are summarized in Table 1.

Isolation of bacterial strains. PE and glass slides were removed after different exposure times from a modified Robbins device installed in a house installation system at the Technical University Berlin and immediately placed in sterile drinking water (filtered through a 0.2-μm-pore-size nitrocellulose membrane [Millipore, Eschborn, Germany]). Bacteria were detached from the slide surfaces with a sterile plastic scraper, pooled in a total volume of 2 ml of sterile drinking water, and vigorously vortexed. To determine total cell counts, 0.5-ml aliquots of the bacterial suspensions were filtered through a polycarbonate membrane (0.2-μm pore size; Millipore) placed on nitrocellulose support membranes (0.45-μm pore size; Millipore) by using a vacuum filtration unit (Schleicher and Schuell, Dassel, Germany). Bacteria attached on the polycarbonate membrane were stained with 4′,6-diamidino-2-phenylindole (DAPI) at a final concentration of 1 μg ml^{-1}. Serial dilutions of the bacterial suspension were plated on R2A agar (22) and incubated at 20°C for 10 days in the dark. Heterotrophic plate counts were determined on R2A agar, and randomly chosen colonies were transferred to liquid R2A medium, incubated at 20°C on a shaker, harvested after 1 to 7 days of incubation, and fixed as described previously (15).

Extraction of genomic DNA and amplification of 16S rRNA genes. Genomic DNA was isolated from bacterial species grown on R2A plates by using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and 16S rRNA sequences were amplified with the universal primers 616V (5′-AGA GTT TGA TC/TA/C TGG CTC AG-3′) and 1492 R (5′-CGG C/TTA CCT TGT TAC GAC-3′). PCR mixtures contained 200 μM each deoxynucleotide, 3 mM magnesium chloride, PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8), 50 pM each primer, 3 μg of genomic DNA, and 2.5 U of *Taq* polymerase (Boehringer, Mannheim, Germany). The PCR was performed in a Personal cycler (Biometra, Göttingen, Germany); the samples were subjected to an initial denaturing step of 2 min at 96°C, followed by addition of 2.5 U of *Taq* polymerase to each sample. The thermal profile consisted of 35 cycles of 45 s at 94°C, 2 min at 51°C, and 3 min at 72°C, with an increment of 5 s. The PCR products were purified with the QIAquick PCR purification kit (Qiagen).

16S ribosomal DNA sequencing and phylogenetic analysis. The purified DNA was sequenced with the fmol DNA cycle sequencing system (Promega, Madison, Wis.) according to the manufacturer's protocol, using the thermal profile recommended by Promega. The primer set included EUB338 and non-EUB338 (2), 606RII (5′-TA/GA CGG C/GCA/G GTG TGT ACA-3′), 610RII (5′-ACC GCG/T A/GCT GCT GGC AC-3′), 609RII (5′-ACT ACC/T A/C/GGG GTA TCT AAG/T CC-3′), 699RII (5′-A/GGG GTT GCG CTC GTT-3′), and 1492R. All primers were purchased 5′ labeled with 5(6)-carboxy-fluorescein-5-isothiocyanate from TIB MOLBIOL, Berlin, Germany. Sequences were generated with the Two Step direct blotter (Hoefer, San Francisco, Calif.) by using a 6% (wt/vol) Long Ranger gel (FMC, Rockland, Maine) and blotted on Biodyne A transfer membranes (Pall, Dreieich, Germany). The DNA was detected with the Southern-Light chemiluminescent detection system for fluorescein-labeled probes (Serva, Heidelberg, Germany). Phylogenetic analysis of the sequences was performed with the ARB software package (30). The 16S rRNA sequences were aligned by using the Aligner tool of the ARB software package and manually corrected according to primary and secondary structure similarities. Distance matrices were constructed from the aligned sequences and corrected for multiple base changes at single positions by the method of Jukes and Cantor (8). Phylogenetic trees were constructed by the neighbor-joining method of Saitou and Nei (26).

Oligonucleotides. The following oligonucleotides were used in this study: (i) EUB338, complementary to a region of the 16S rRNA conserved in the domain *Bacteria* (2), and non-EUB338, complementary to EUB338 and serving as a negative control for nonspecific binding; (ii) ALF1b, complementary to a region of the 16S rRNA characteristic for the alpha subclass of *Proteobacteria* (15); and (iii) BET42a and GAM42a, oligonucleotides complementary to regions of the 23S rRNAs of the beta (BET42a) and gamma (GAM42a) subclasses of *Proteobacteria* (15). The oligonucleotides were purchased 5′ labeled with the indocarbocyanine dye Cy3 from Biometra.

Strain-specific oligonucleotides for the isolated strains B1 to B8 were designed by using the Probe_Design tool of the ARB software package (30). Potential candidates for analytical in situ probes were compared to the available data set of 6,194 complete and partial 16S rRNA sequences to search for unrelated organisms that may be coincidentally homologous within the target sequences. The newly developed probes (termed beta1 to beta8b) were labeled with the indocarbocyanin dye Cy3; additionally, probes beta8a and beta8b were labeled with 5(6)-carboxy-fluorescein-*N*-hydroxysuccinimide-ester (FLUOS) and tetramethyl rhodamine-5-isothiocyanate (TRITC) as described previously (15).

In situ hybridization with fluorescent oligonucleotide probes. Fixation of biofilm samples and pure cultures as well as in situ hybridizations with Cy3-labeled probes EUB338, ALF1b, BET42a, and GAM42a were performed as described by Manz et al. (16). The hybridization stringency was adjusted by raising the hybridization temperature to 46°C and adding formamide to a final concentration of 40%. In the washing solution, the stringency was maintained by lowering the sodium chloride concentration according to the formamide concentration used in the hybridization buffer. Hybridization stringencies for Cy3-labeled probes beta1 to beta8b were adjusted by stepwise addition of formamide in whole-cell hybridizations against selected reference strains displaying one or two mismatches within the target region. The formamide concentrations necessary to discriminate nontarget organisms were 35% for probe beta1; 40% for probes beta2, -3, -4, -5, -6, and -8a; and 50% for probes beta7 and -8b. The washing buffer for all probes contained 20 mM Tris-HCl (pH 8) and 0.01% sodium dodecyl sulfate. The sodium chloride concentration was adjusted to 88 mM for probe beta1; 62.4 mM for probes EUB338, ALF1b, BET42a, GAM42a, and beta2, -3, -4, -5, -6, and -8a; and 31.2 mM for probes beta7 and -8b. For the simultaneous application of probes beta8a and beta8b labeled with FLUOS and TRITC, respectively, hybridization stringencies were lowered to 35% formamide in the hybridization buffer and 88 mM NaCl in the washing solution, due to the lower sensitivity of these fluorochromes.

Modified DVC assay. The potential capacity of bacteria in drinking water to metabolize the nutrients present in R2A medium (22) was determined by using a modification of the DVC technique proposed by Kogure et al. (10). Biofilms were incubated for 8 and 16 h in R2A medium containing the gyrase inhibitor pipemidic acid to prevent cell division during incubation (9). Because of the prolonged incubation period of 16 h, the concentration of pipemidic acid was raised to 30 mg $liter^{-1}$. Total cell counts, cell lengths of 100 randomly chosen cells, and percentages of cells hybridizing with the *Bacteria*-specific oligonucleotide probe EUB338 were determined for the attached microbial population prior to incubation as well as 8 and 16 h after incubation.

Microscopy and documentation. Fluorescence was detected by epifluorescence microscopy with a Zeiss (Oberkochen, Germany) Axioskop fitted with a 50-W high-pressure bulb; Zeiss light filter sets no. 01 for DAPI (excitation, 365 nm; dichroic mirror, 395 nm; suppression, 397 nm), no. 09 for FLUOS (excitation, 450 to 490 nm; dichroic mirror, 510 nm; suppression, 520 nm), and no. 15 for TRITC (excitation, 546 nm; dichroic mirror, 580 nm; suppression, 590 nm); and HQ light filter 41007 (AF Analysentechnik, Tübingen, Germany) for Cy3-labeled probes (excitation, 535 to 550 nm; dichroic mirror, 565 nm; suppression, 610 to 675 nm). Black-and-white micrographs were taken on Ilford 400 ASA film; exposure times were 8 to 30 s. For statistical evaluation, at least 10 microscopic fields (100 by 100 μm) and a minimum of 1,000 cells were chosen randomly and enumerated. Statistical analysis (standard error) and Gaussian curve fits were done with SigmaPlot 2.0 (Jandel Scientific, Erkrath, Germany) software programs.

RESULTS

Isolation strategy. Biofilms grown on PE or glass slides were sampled after different exposure times (2, 7, 14, and 21 days) in January, May, August, and October 1996 to take possible seasonal and age-dependent variations of the population compo-

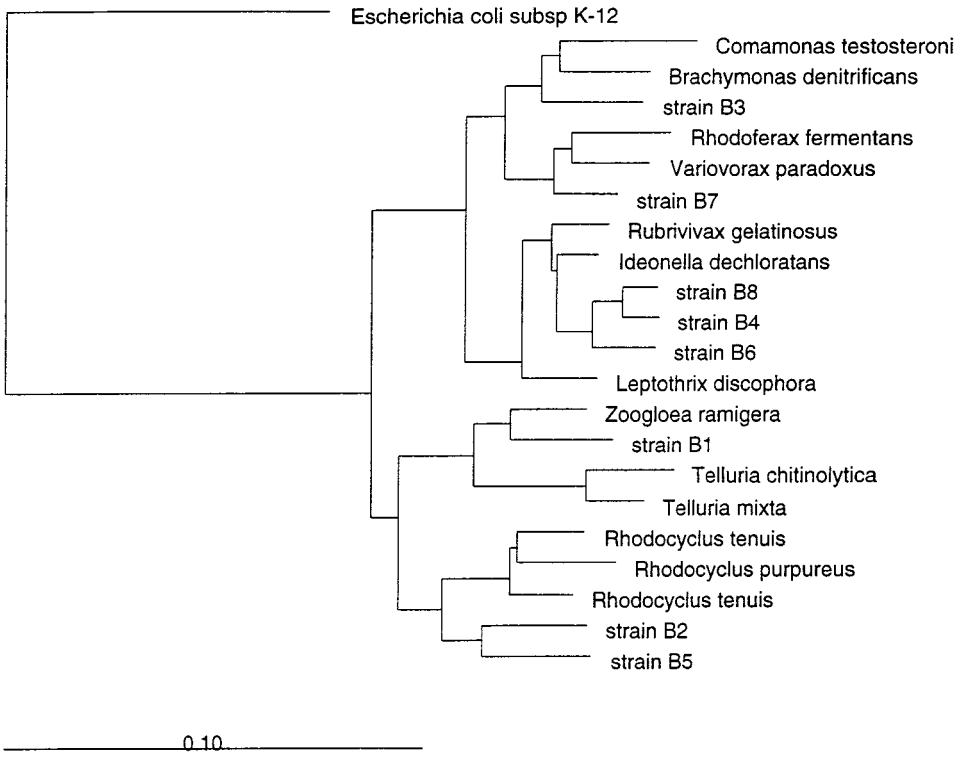

FIG. 1. Phylogenetic tree inferred from 16S rRNA sequence data, showing the relationships of the eight isolated strains to their closest known relatives. Distance matrices were constructed from the aligned sequences and corrected for multiple base changes at single positions by the method of Jukes and Cantor (8), and a phylogenetic tree was constructed by the neighbor-joining method of Saitou and Nei (26) by using the ARB software package (30). The scale bar represents 10 nucleotide substitutions per 100 nucleotides.

sition into account. In general, bacteria could be cultivated on R2A agar to between 0.1 and 1.5% of the total bacterial cell counts. A total of 234 colonies were analyzed by in situ hybridizations with oligonucleotide probes specific for the alpha, beta, and gamma subclasses of *Proteobacteria*. All of the colonies could be successfully assigned to one of these taxa. Strains belonging to the beta subclass were grouped according to their cell and colony morphologies, and one representative strain of each group was subsequently used for DNA isolation and 16S ribosomal DNA sequencing. After the successful design of a set of eight strain-specific probes, the remaining isolates from each group of morphologically similar strains were analyzed by using these oligonucleotides. In situ hybridizations revealed that all members of a distinct group could be detected with the corresponding strain-specific oligonucleotide probe.

Phylogenetic analysis. The phylogenetic tree shown in Fig. 1 reflects the phylogenetic relationships of the eight strains B1 to B8 to their next relatives. Three of them, namely, strains B4, B6, and B8, formed a distinct cluster within the beta1 subclass of *Proteobacteria*, whereas strains B2 and B5 constituted a distinct cluster in the beta2 sublineage. The rRNA sequence similarity between strains B4 and B8 was 98.4%, that between strains B6 and B8 was 97.2%, and that between strains B6 and B4 was 96.6%. *Ideonella dechloratans*, which is the closest relative of the B4-B6-B8 cluster, displayed 16S rRNA similarities of 96.7% to strain B6, 96.6% to strain B8, and 96.4% to strain B4. The other new isolates were more distantly related to their next neighbors, with similarity values ranging from 94 to 96%. Strains B2 and B5 had 94.4% sequence similarity.

Design of oligonucleotide probes. Specific probes for all isolated bacterial strains were designed by using the ARB software package (30). Computer-aided sequence comparison revealed that the probes beta2, -3, -4, -5, and -6 showed at least one mismatch with all accessible 16S rRNA sequences. Probe beta1 showed homology within the target regions of four partial sequences obtained from 16S rRNA libraries of beta *Proteobacteria* (GenBank accession no. U34035, X91274, X91526, and X74914), probes beta7 and beta8a displayed homology to one sequence (accession no. X91427 and X91174, respectively), and probe beta8b showed homology to partial sequences deposited under accession no. X84573, X84597, and X84617. By using the two probes for strain B8 labeled with different fluorochromes simultaneously, potential nontarget organisms could be excluded. The sequences and positions of the oligonucleotides are summarized in Table 2. All oligonucleotides (labeled with the fluorochrome Cy3) were tested against se-

TABLE 2. Sequences and target sites of 16S rRNA oligonucleotide probes specific for drinking water strains B1 to B8

Probe	Sequence (5′→3′)	Target site[a]
beta1	CTCTGCCAGATTCTAGCC	649–666
beta2	GGTACCGTCATCCACACAGGG	469–489
beta3	CGGTACCGTCATGGACC	474–490
beta4	GCCGTGCAGTCACAAGTGC	633–651
beta5	GGTACCGTCATCCACGCAGAG	469–488
beta6	CTAGCCTTGCAGTCACAAAGGC	633–654
beta7	GCCTTGCAGTCACAAGTGC	633–651
beta8a	GCCTTGCAGTCACAAATGC	633–654
beta8b	CCAGGTTGCCCCGCGTTAC	69–87

[a] *E. coli* numbering (5).

TABLE 3. Comparison of in situ abundances and frequencies of isolation of the newly isolated drinking water bacteria in 14-day-old biofilms obtained in October 1996

Oligonucleotide probe	Mean % of total bacteria (SE) on:		No. of colonies on R2A
	PE slides	Glass slides	
EUB338	87 (0.9)	84 (1.5)	100
ALF1b	1 (0.6)	2 (0.8)	17
BET42a	78 (3.6)	80 (1.2)	57
GAM42a	2 (0.7)	2 (0.8)	26
beta1	<0.1	0	5
beta2	15 (5)	2 (0.8)	25
beta3	1 (0.5)	0	0
beta4	14 (1.4)	12 (2.5)	2
beta5	0.5 (0.3)	0	1
beta6	21 (3.5)	19 (3.8)	20
beta7	0	<0.1	3
beta8b	16 (6)	34 (3.5)	1

lected reference strains displaying one or two mismatches within the target region. The hybridization stringency necessary to discriminate between target and nontarget organisms was adjusted by the addition of formamide.

In situ abundance and isolation frequencies. PE or glass slides were exposed as substrata for a period of 14 days in October 1996 in a house installation system located at the Technical University Berlin. One part of the biofilms grown on the slides was directly fixed in a 4% formaldehyde solution for subsequent in situ analysis of the bacterial population composition, and the other part was detached and used for isolation of bacteria by plating on R2A agar. In situ hybridization with the probes specific for the alpha, beta, and gamma subclasses of *Proteobacteria* demonstrated the predominance of bacteria belonging to the beta subclass, which formed 78% of the total bacterial population on PE slides and 80% on glass slides. Bacteria belonging to the alpha and gamma subclasses were present at significantly lower percentages, varying between 1 and 2% of total cell counts. Compared to the monitored in situ distribution, the analysis of 100 randomly picked bacterial colonies obtained from R2A agar demonstrated a population shift towards the apparently more easily culturable bacteria belonging to the alpha and gamma subclasses (Table 3). Ten days after incubation, heterotrophic plate counts on R2A agar reached 1.8% of the total bacterial counts.

The highly specific probes developed for strains B1 to B8 revealed that all of the previously isolated strains could be detected in the biofilms, although some strains were present in very low numbers and not on both materials (Table 3). On both substrata, strains B4, B6, and B8 could be detected in significant amounts, ranging between 12 and 34% of the total bacterial population. Up to 78% of the biofilm bacteria grown on PE slides which were detectable with the *Bacteria*-specific probe EUB338 could be affiliated with one of the eight newly isolated strains, corresponding to 87% of the beta *Proteobacteria* within the community. On glass slides, the isolated strains accounted for 80 and 84% of the bacteria detected with probes EUB338 and BET42a, respectively. All cells yielding hybridization signals with probe beta8a also hybridized with probe beta8b and vice versa, greatly reducing the probability of false-positive detection of target organisms.

Hybridization of the 57 bacterial colonies affiliated with the beta *Proteobacteria* revealed that all of them could be assigned to one of the previously isolated strains B1 to B8. The comparison between the in situ abundances of strains B1 to B8 and their frequencies of isolation on R2A agar clearly demonstrated differences in culturability for the investigated strains (Table 3). Although strains B1 and B7 made up less than 0.1% of the total bacterial population in situ, 5 and 3% of the colonies hybridized with probes beta1 and beta7, respectively. In contrast, strains B4 and B8 made up between 12 and 34% of the bacteria in situ, but only 2 and 1% of the colonies hybridized with probes beta4 and beta8b, respectively. The isolation frequencies for strains B2, B5, and B6 reached values comparable with their in situ abundances (Table 3).

Population composition within the distribution system. To determine the occurrence and levels of abundance of the eight isolated strains in the distribution system, PE and glass slides were exposed for 2 weeks between October and November 1996 in Robbins devices installed in a groundwater well at the Jungfernheide waterworks, Berlin, and in a main pipe 5 km from the waterworks. Just as in the house installation system, bacteria of the beta subclass of *Proteobacteria* dominated the biofilm population of the main pipe. In contrast, the population composition in groundwater biofilms differed significantly: beta-subclass bacteria accounted for only about one-third of total cell counts, whereas alpha *Proteobacteria* made up between 39% (±5.2%) and 41% (±5.5%) of the bacterial community. Gamma *Proteobacteria* formed a minor part of the population, ranging from 5% (±2.3%) to 7% (±2.7%) of total cell counts (Table 4).

Cells hybridizing with probes beta3, -6, and -8b could be detected in significant numbers both in groundwater and in the main pipe. Together, they accounted for 66 to 82% of the beta-subclass bacteria in groundwater and for 81 to 84% in the main pipe. Strain B8 was by far the most abundant of the isolated strains in groundwater as well as in the distribution net (Table 4). Interestingly, strain B3 was quite numerous in the distribution net, accounting for up to 18% (±4.5%) of total cell counts, but was present in very low numbers within the house installation system and in groundwater. All other strains could not be detected in groundwater and appeared in very low numbers in the distribution net but reached up to 15% (±5%) for strain B2 and 14% (±1.4%) for strain B4 of total cell counts in the house installation system (Table 3).

DVCs. PE slides exposed for 14 days in the house installation system at the Technical University Berlin in January 1997 were subjected to a modified DVC assay. Cell densities remained constant, with 1.8×10^6 ($\pm 1.7 \times 10^5$) cells cm^{-2} before incu-

TABLE 4. Population compositions of 14-day-old biofilms obtained from groundwater and a main pipe of the drinking water distribution system between October and November 1996

Oligonucleotide probe	Mean % of total bacteria (SE) in:			
	Groundwater		Main pipe	
	PE slides	Glass slides	PE slides	Glass slides
EUB338	86 (1.2)	86 (1.7)	85 (2.3)	86 (2.3)
ALF1b	39 (5.2)	41 (5.5)	1 (0.5)	2 (0.6)
BET42a	33 (3.4)	35 (3.6)	80 (1.7)	79 (2.4)
GAM42a	7 (2.7)	5 (2.3)	1 (0.8)	2 (0.8)
beta1	0	0	0	0
beta2	0	0	1 (0.4)	0
beta3	1 (0.4)	1 (0.3)	17 (7.6)	18 (4.5)
beta4	0	0	0	0
beta5	0	0	1 (0.3)	0
beta6	5 (1.6)	5 (2)	4 (1)	6 (2.5)
beta7	0	0	0	0
beta8b	21 (5.1)	17 (3)	44 (4.5)	42 (4.9)

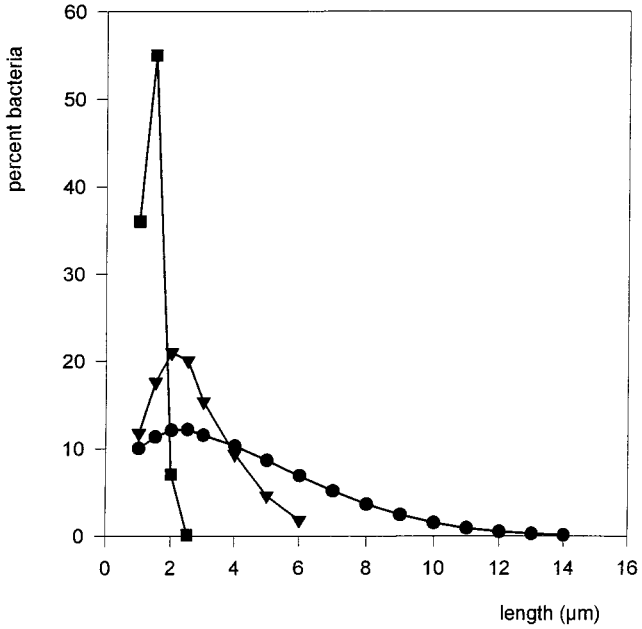

FIG. 2. Influence of incubation in pipemidic acid-amended R2A medium on the distribution of cell lengths within the bacterial biofilm community. Cell lengths of 100 randomly chosen bacteria were determined microscopically and subjected to a Gaussian curve fit by the least-squares method. The graphs show the distribution of bacterial cell lengths in the native biofilm (■) and after incubation for 8 h (▼) and 16 h (●).

bation and 1.5×10^6 ($\pm 1.5 \times 10^5$) cells cm^{-2} and 1.8×10^6 ($\pm 1.6 \times 10^5$) cells cm^{-2} after 8 and 16 h of incubation, respectively. The microscopically measured cell lengths of 100 bacteria were subjected to a Gaussian curve fit by the least-squares method, and the resulting graphs are given in Fig. 2. In the original biofilm, bacteria showed a very homogeneous cell size, ranging from 1 to 2 μm; only 2% of the cells had a length greater than 2 μm. After activation for 8 and 16 h, however, 49 and 68% of the bacteria, respectively, reached cell lengths of more than 2 μm, clearly indicating that the majority of the cells were able to utilize the nutrients present in R2A medium.

In situ hybridizations of biofilm communities with probe EUB338 before and after incubation clearly demonstrated cell elongation of the majority of cells (Fig. 3). In the native biofilm, 89% of the biofilm-associated bacteria, displaying uniform cell lengths of 1 to 2 μm, could be detected with probe EUB338 (Fig. 3A). After incubation for 8 and 16 h in R2A medium amended with pipemidic acid, the percentage of hybridized cells increased slightly but not significantly, to 90 and 91% of total cell counts, respectively. After incubation for 8 h, an increase in cell length could be seen (Fig. 3B), resulting in a population of various morphotypes and sizes, ranging from 1 to 14 μm, after 16 h of incubation (Fig. 2 and 3C). Hybridizations performed with the strain-specific probes revealed that only strains B4, B6, and B8 were present in large numbers in the biofilms obtained in January: 48% (±5%) of total cell counts hybridized with probe beta8b, 14% (±5%) hybridized with probe beta4, and 10% (±3.4%) hybridized with probe beta6. With probe beta5, single bacterial cells, accounting for less than 0.1% of the total population, could be detected. All other strains were not present in the biofilm communities. Interestingly, the bacterial population structure of biofilms obtained in January differed markedly from that of biofilms obtained in October (Table 3), with a strong increase of the abundance of strain B8, from 16% (±6%) to 48% (±5%) of total cell counts.

Cell elongation, defined as an increase in cell length to more than 2 μm, was determined for more than 600 cells yielding

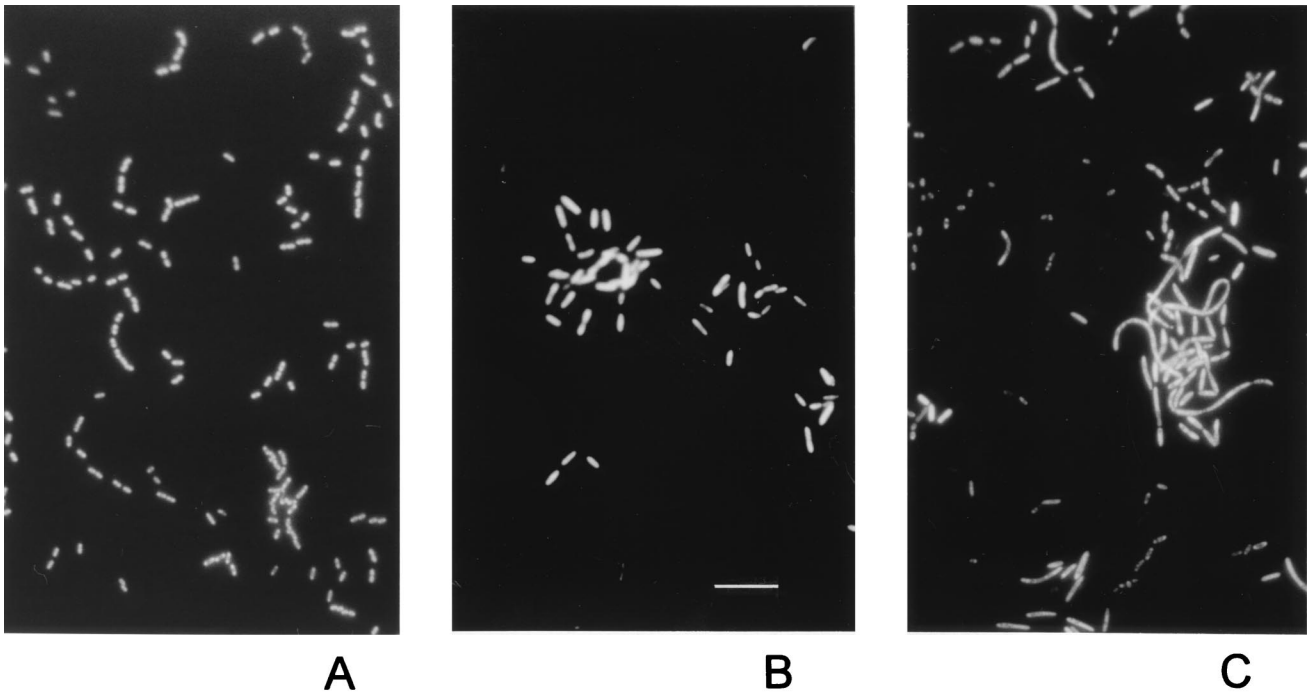

FIG. 3. Modified DVC method adapted for application of in situ probing. Epifluorescence photomicrographs of in situ hybridizations with Cy3-labeled probe EUB338 show bacteria in a native, 14-day-old biofilm on PE displaying a uniform cell length (A) and then an increase of cell length after 8 h (B) and various sizes after 16 h (C) of incubation in pipemidic acid-amended R2A medium. Original magnification, ×1,000; exposure time, 30 s; bar, 10 μm.

TABLE 5. Distribution of elongated and nonelongated cells in biofilm communities after incubation for 16 h in R2A medium amended with pipemidic acid[a]

Oligonucleotide probe	% of cells	
	Elongated (>2μm)	Nonelongated (≤2μm)
EUB338	68	32
beta4	92	8
beta6	96	4
beta8b	73	27

[a] PE slides were exposed for 14 days in January 1997.

hybridization signals with probes beta4, beta6, and beta8b. As shown in Table 5, the three drinking water isolates differed significantly in their ratios of elongated to nonelongated cells. The most abundant strain in the ecosystem, strain B8, displayed the highest percentage of nonelongated cells, with 27% of all cells yielding hybridization signals with probe beta8b.

DISCUSSION

Up to now, studies on the species composition of microbial populations in drinking water and the associated biofilms relied mainly on cultivation methods (12, 32, 34). Some bacterial species, such as *Pseudomonas* spp., have been frequently isolated from drinking water (12, 32, 34), but no information on the abundance of these bacteria within the ecosystem was obtained. In the present study, we developed a strategy to identify the in situ dominant bacterial species. This could be achieved by the design of specific oligonucleotide probes for bacterial strains isolated on R2A agar and subsequent in situ probing within the natural habitat.

The phylogenetic analysis based on the 16S rRNAs of the eight representative bacterial strains revealed them to be new bacterial lineages, displaying less than 96.7% sequence similarity to their closest known relatives. According to the current concept that 97% rRNA sequence similarity corresponds to about 70% DNA-DNA relatedness (28), we conclude that five isolated strains (B1, B2, B3, B5, and B7) represent new bacterial species. Whether the closely related strains B4, B6, and B8 could be defined as separate bacterial species or strains of one species may be decided once additional taxonomic data are available.

The comprehensive set of newly developed strain-specific probes was subsequently used to characterize the in situ abundance of each strain in the drinking water habitat, revealing that the overwhelming majority of the cells could be assigned by in situ hybridization to one of the isolated strains. We therefore conclude that most of the bacteria from this strictly oligotrophic system are in principle culturable on R2A agar.

Variations in the species compositions of the biofilm communities obtained at the same sampling site but at different times of the year might be due to seasonal fluctuations of organic substrates in the drinking water. However, the question remains whether the population changes in the distribution net are primarily due to regrowth phenomena, as described by LeChevalier (13), or whether these changes reflect population shifts within the microbial community of the raw water source or in the treatment process.

The population composition of biofilms in the house installation system was also compared to biofilm communities originating from a groundwater well and a drinking water main pipe of the same distribution system (Tables 3 and 4). Although growth conditions certainly varied among the three sampling points, strains B3, B6, and B8 could be successfully detected in significant amounts at all three sampling points, indicating a high potential for adaptations and high competitiveness of these strains. The increased bacterial diversity in biofilms obtained from the house installation system is in good accordance with findings of LeChevalier et al. (12), who reported an increase in heterotrophic plate count diversity as water flowed through the drinking water distribution system. Since the retention time, the average temperature of the water, and the variety of installation materials in house installation systems are obviously higher than those in a distribution pipe, this might lead to an accumulation of assimilable organic substances originating from tubing materials, rubber fittings, and sealants (27). Some bacterial strains, such as strains B2 and B4, grew preferentially and to high cell numbers in the house installation system and could therefore be indicators for the availability of certain plasticizers, solvents, or other material-borne substances.

Oligonucleotide probes for the alpha, beta, and gamma subclasses of *Proteobacteria* as well as the newly designed strain-specific probes were used in a nested approach to compare the in situ abundances with the respective frequencies of isolation on R2A medium. Cultivation on R2A medium preferably supported growth of bacteria belonging to the alpha and gamma *Proteobacteria*. A culture-induced enrichment of bacteria belonging to the gamma subclass and simultaneous suppression of beta *Proteobacteria* has been described previously by Wagner et al. (33). Those authors, however, used nutrient-rich Luria-Bertani medium to isolate bacteria from activated sludge. In our study even bacteria hybridizing with the alpha-subclass probe were selectively enriched on the low-nutrient medium R2A. According to Zavarzin et al. (35), members of the alpha subclass are regarded as mostly oligotrophic species. But even typical oligotrophs such as *Caulobacter* spp. can be readily isolated from nutrient-rich habitats, such as activated sludge (14). The term oligotrophic should thus be used with caution; possibly this terminology should not be applied to organisms but should be restricted to the characterization of the nutrient contents of ecosystems (17, 18). Using specific oligonucleotide probes, we could show that not all of the eight strains belonging to the beta subclass were equally disfavored by cultivation and that culturability was not equally distributed among the investigated bacterial isolates (Table 3). Based on these results, we have to conclude that the ratio of culturable to nonculturable cells within one species differs considerably among the investigated strains, although they were all exposed to the same environmental conditions.

The capacity of single bacterial cells to respond to nutrient addition by cell elongation was determined by using a modified DVC method (10) adapted for application of in situ hybridization (9). Two-thirds of the biofilm-associated cells were elongated 16 h after incubation, clearly demonstrating the potential of these bacteria to utilize the nutrients present in R2A medium. The difference between heterotrophic plate counts and elongated cells determined by the DVC assay is commonly considered the portion of the bacterial population in a VBNC state (6, 19, 25). Accordingly, VBNC bacteria have been defined as cells demonstrating metabolic activity but incapable of undergoing the sustained cellular division required for growth in or on an artificial medium normally supporting growth of these cells (19). Applying this definition to the system investigated in this study, more than 65% of the bacterial population should be considered VBNC cells.

It appears to be more difficult to define the physiological state of the bacteria not elongating in the DVC assay. Appar-

ently, members of the investigated bacterial strains were concommitantly present in three distinct physiological states: (i) culturable, (ii) nonculturable but able to elongate in the DVC assay and thus considered VBNC, and (iii) nonculturable and unable to elongate in the DVC assay. Whether the nonelongating cells are dead or can recover and resume growth under certain environmental conditions remains to be determined. The closely related strains B4, B6, and B8 displayed significant differences in the ratio of elongated to nonelongated cells. Surprisingly, the most abundant strain within the biofilm community, strain B8, displayed the largest amount of nonelongated cells, raising the question whether this physiological state might confer competitive advantages in a low-nutrient environment.

To our knowledge, the biofilm community from this house installation system represents the first habitat from which the in situ dominant bacterial species have been successfully isolated, phylogenetically characterized, and detected by in situ probing in their natural environment. The strategy used in this study will provide the possibility to correlate information about the phylogenetic identity and the in situ distribution with physiological parameters obtained from pure cultures of the isolated bacteria.

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REFERENCES

1. **Alfreider, J. P., R. Amann, B. Sattler, F.-O. Glöckner, A. Wille, and R. Psenner.** 1996. Community analysis of the bacterial assemblages in the winter cover and pelagic layers of a high mountain lake by in situ hybridization. Appl. Environ. Microbiol. **62:**2138–2144.
2. **Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919–1925.
3. **Amann, R. I., W. Ludwig, and K.-H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.
4. **Amann, R. I.** 1995. Fluorescently labelled, rRNA-targeted oligonucleotide probes in the study of microbial ecology. Mol. Ecol. **4:**543–554.
5. **Brosius, J., T. L. Dull, D. D. Sleeter, and H. F. Noller.** 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. J. Mol. Biol. **148:**107–127.
6. **Byrd, J. J., H.-S. Xu, and R. R. Colwell.** 1991. Viable but nonculturable bacteria in drinking water. Appl. Environ. Microbiol. **57:**875–878.
7. **Costerton, J. W., J. C. Nickel, and T. I. Ladd.** 1986. Suitable methods for the comparative study of free-living and surface-associated bacterial populations, p. 49–84. *In* J. S. Poindexter and E. R. Leadbetter (ed.), Bacteria in nature—methods and special applications in bacterial ecology, vol. 2. Plenum Press, New York, N.Y.
8. **Jukes, T. H., and C. R. Cantor.** 1969. Evolution of protein molecules, p. 21–132. *In* H. N. Munro (ed.), Mammalian protein metabolism. Academic Press, New York, N.Y.
9. **Kalmbach, S., W. Manz, and U. Szewzyk.** 1997. Dynamics of biofilm formation in drinking water: phylogenetic affiliation and metabolic potential of single cells assessed by formazan reduction and *in situ* hybridization. FEMS Microbiol. Ecol. **22:**265–280.
10. **Kogure, K., U. Simidu, and N. Taga.** 1984. An improved direct viable count method for aquatic bacteria. Arch. Hydrobiol. **102:**117–122.
11. **Kuznetsov, S. I., G. A. Dubinina, and N. A. Lapteva.** 1979. Biology of oligotrophic bacteria. Annu. Rev. Microbiol. **33:**377–387.
12. **LeChevallier, M. W., T. M. Babcook, and R. G. Lee.** 1987. Examination and characterization of distribution system biofilms. Appl. Environ. Microbiol. **53:**2714–2724.
13. **LeChevallier, M. W.** 1990. Coliform regrowth in drinking water: a review. Res. Technol. J. Am. Water Works Assoc. **82:**74–86.
14. **MacRae, J. D., and J. Smith.** 1991. Characterization of caulobacters isolated from wastewater treatment systems. Appl. Environ. Microbiol. **57:**751–758.
15. **Manz, W., R. I. Amann, W. Ludwig, M. Wagner, and K.-H. Schleifer.** 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. Syst. Appl. Microbiol. **15:**593–600.
16. **Manz, W., U. Szewzyk, P. Ericsson, R. Amann, K.-H. Schleifer, and T.-A. Stenström.** 1993. In situ identification of bacteria in drinking water and adjoining biofilms by hybridization with 16S and 23S rRNA-directed fluorescent oligonucleotide probes. Appl. Environ. Microbiol. **59:**2293–2298.
17. **Martin, P., and R. A. MacLeod.** 1984. Observations on the distinction between oligotrophic and eutrophic marine bacteria. Appl. Environ. Microbiol. **47:**1017–1022.
18. **Morgan, P., and C. S. Dow.** 1986. Bacterial adaptions for growth in low nutrient environments. Spec. Publ. Soc. Gen. Microbiol. **17:**187–214.
19. **Oliver, J. D.** 1993. Formation of viable but nonculturable cells, p. 239–272. *In* S. Kjelleberg (ed.), Starvation in bacteria. Plenum Press, New York, N.Y.
20. **Pedersen, K.** 1990. Biofilm development on stainless steel and PVC surfaces in drinking water. Water Res. **24:**239–243.
21. **Poindexter, J. S.** 1981. Oligotrophy. Fast and famine existence. Adv. Microb. Ecol. **5:**63–89.
22. **Reasoner, D. J., and E. E. Geldreich.** 1985. A new medium for the enumeration and subculture of bacteria from potable water. Appl. Environ. Microbiol. **49:**1–7.
23. **Ridgway, H. F., and B. H. Olson.** 1981. Scanning electron microscope evidence for bacterial colonization of a drinking water distribution system. Appl. Environ. Microbiol. **41:**274–287.
24. **Rogers, J., and C. W. Keevil.** 1992. Immunogold and fluorescein immunolabelling of *Legionella pneumophila* within an aquatic biofilm visualized by using episcopic differential interference contrast microscopy. Appl. Environ. Microbiol. **58:**2326–2330.
25. **Roszak, D. B., and R. R. Colwell.** 1987. Survival strategies of bacteria in the natural environment. Microbiol. Rev. **51:**365–379.
26. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
27. **Schoenen, D.** 1986. Microbial growth due to material used in drinking water systems. Biotechnology **8:**627–642.
28. **Stackebrandt, E., and B. M. Goebel.** 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **44:**846–849.
29. **Staley, J. T., and A. Konopka.** 1985. Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Annu. Rev. Microbiol. **39:**321–346.
30. **Strunk, O., O. Gross, B. Reichel, M. May, S. Hermann, N. Stuckmann, B. Nonhoff, M. Lenke, A. Vilbig, T. Ludwig, A. Bode, K. H. Schleifer, and W. Ludwig.** ARB: a software environment for sequence data. Unpublished data.
31. **Szewzyk, U., W. Manz, R. Amann, K.-H. Schleifer, and T.-A. Stenström.** 1994. Growth and in situ detection of a pathogenic *Escherichia coli* in biofilms of a heterotrophic water-bacterium by use of 16S- and 23S-rRNA-directed fluorescent oligonucleotide probes. FEMS Microbiol. Ecol. **13:**169–176.
32. **Tall, B. D., H. N. Williams, K. S. George, R. T. Gray, and M. Walch.** 1995. Bacterial succession within a biofilm in water supply lines of dental air-water syringes. Can. J. Microbiol. **41:**647–654.
33. **Wagner, M., R. Amann, H. Lemmer, and K. H. Schleifer.** 1993. Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. Appl. Environ. Microbiol. **59:**1520–1525.
34. **Ward, N. R., R. L. Wolfe, C. A. Justice, and B. H. Olson.** 1986. The identification of gram-negative, nonfermentative bacteria from water: problems and alternative approaches to identification. Adv. Appl. Microbiol. **31:**293–365.
35. **Zavarzin, G. A., E. Stackebrandt, and R. G. E. Murray.** 1990. A correlation of phylogenetic diversity in the Proteobacteria with the influences of ecological forces. Can. J. Microbiol. **37:**1–6.