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Molecular and Microscopic Identification of Sulfate-Reducing Bacteria in Multispecies Biofilms

R. I. AMANN,[1]† J. STROMLEY,[2] R. DEVEREUX,[1]‡ R. KEY,[1] AND D. A. STAHL[1,2]*

Departments of Veterinary Pathobiology[1] and Microbiology,[2] University of Illinois, 2001 South Lincoln Avenue, Urbana, Illinois 61801

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The population architecture of sulfidogenic biofilms established in anaerobic fixed-bed bioreactors was characterized by selective polymerase chain reaction amplification and fluorescence microscopy. A region of the 16S rRNA common to resident sulfate-reducing bacteria was selectively amplified by the polymerase chain reaction. Sequences of amplification products, with reference to a collection of 16S rRNA sequences representing most characterized sulfate-reducing bacteria, were used to design both general and specific hybridization probes. Fluorescent versions of these probes were used in combination with fluorescence microscopy to visualize specific sulfate-reducing bacterial populations within developing and established biofilms.

The greater part of microbial activity in nature is often associated with surfaces (17). Attached communities (biofilms) may be either beneficial or detrimental (8). Microbial aggregation or attachment is required in water treatment. However, in less controlled environmental settings, the sessile mode may contribute to extensive corrosion and biodeterioration (17). In medicine, attached communities are intimately related to host defense and disease (8). Understanding of the population ecology of surface communities is limited, irrespective of their pervasive and often conspicuous presence.

Characterization of biofilm populations has been hindered by available determinative techniques. Most notably, not all natural populations are amenable to pure-culture isolation. In addition, spatial heterogeneity and aggregation may compromise culture enumeration. Recent advances in analytical biochemistry (44), computer-assisted microscopy (6), and molecular biology have greatly expanded the array of tools that can be applied to the study of natural biofilms. More generally, nucleic acid hybridization and comparative sequence analyses are increasingly being applied to studies of environmental microbiology; these applications have been fostered by developments in both rapid sequencing techniques and comparative sequence analyses (18, 22, 33, 40). Notably, comparative sequencing of large rRNAs (16S-like and 23S-like) has served to cast both microbial classification and the design of nucleic acid hybridization probes within a phylogenetic framework (35, 45).

Oligonucleotide probes complementary to specific regions of rRNAs have been fabricated for the identification of both individual and phylogenetically coherent assemblages of microorganisms (15, 35, 37). Such probes are increasingly used to quantify natural microbial populations and to identify new isolates, serving to help alleviate reliance upon pure-culture isolation and phenotypic identification. In addition, natural populations have been characterized by sequencing of rRNAs (or their genes) directly isolated from environmental samples (14, 38, 39, 41). Isolation has been greatly facilitated by the use of the polymerase chain reaction (PCR). PCR primers complementary to conserved regions of rRNAs serve in general amplification (rRNA sequence from any species), whereas group-specific primers (complementary to less highly conserved tracts of a sequence) serve to retrieve rRNAs from selected microbial groups (26, 36, 42).

More recently, fluorescent dye-labeled oligonucleotide probes have been used for microscopic identification of single cells (3, 9, 36) and for the characterization of mixed populations by flow cytometry (2). Fluorescence is conferred by hybridization to the 16S or 23S rRNAs within fixed whole cells. The present study combines the techniques of selective PCR amplification, comparative sequencing, and whole-cell hybridization to selectively identify and visualize sulfate-reducing bacteria in both established and developing multispecies biofilms.

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MATERIALS AND METHODS

Bioreactors. Biofilms were developed in a completely mixed fixed-bed anaerobic bioreactor consisting of a glass column (2-cm diameter and 21.5-cm length) filled with 3-mm glass beads (Fig. 1). The bioreactor was established in August 1988 with inocula from a well-established bioreactor (B. Rittmann, Department of Civil Engineering, University of Illinois); the inocula for those were originally derived from groundwater and maintained under conditions comparable to those described here for approximately 1 year. Nutrient medium was composed of the following (milligrams per liter): glucose (30), NH_4Cl (58.2), KH_2PO_4 (85.5), K_2HPO_4 (64.2), $CaCl_2 \cdot 2H_2O$ (2), $MnCl_2 \cdot 2H_2O$ (0.2), $FeCl_2 \cdot 4H_2O$ (6.25×10^{-3}), $NaHCO_3$ (8.4), $MgSO_4 \cdot 7H_2O$ (2.5), and resazurin (0.9). A stock vitamin solution was added (1.25 ml/liter) following sterilization; the stock vitamin solution contained the following (milligrams per milliliter): biotin (2),

* Corresponding author.

† Present address: Lehrstuhl für Mikrobiologie, Technische Universität Munchen, D-8000 Munich 2, Germany.

‡ Present address: Environmental Research Laboratory, U.S. Environmental Protection Agency, Sabine Island, Gulf Breeze, FL.

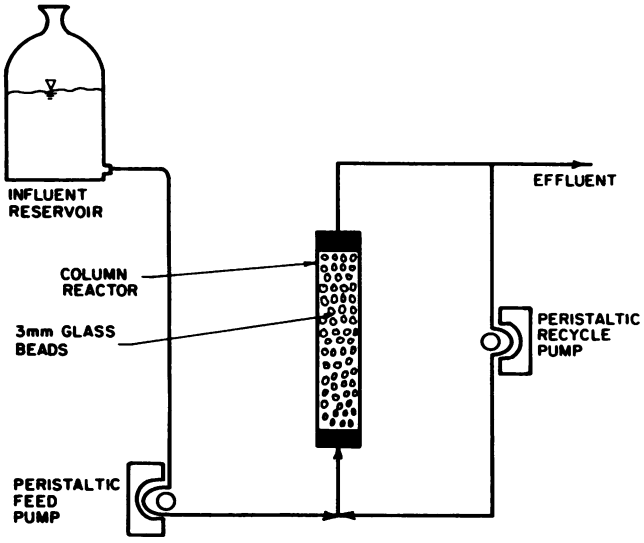

FIG. 1. Completely mixed fixed-bed bioreactor, modified from Rittmann et al. (32) with permission from the publisher.

folic acid (2), pyridoxine-HCl (10), riboflavin (5), thiamine (5), nicotinic acid (5), pantothenic acid (5), and vitamin B_{12} (0.1). The medium was sterilized by being autoclaved and reduced by the addition of $Na_2S \cdot 9H_2O$ (final concentration, 30 mg/liter). Flow rates through the reactor and recycle loop were 1 liter/day and 80 liters/day, respectively. Glass coverslips were placed into the top position of the bioreactor (parallel to the nutrient flow) for various times to allow biofilm development for microscopic observation. The bioreactor was placed in an anaerobic chamber during the removal of glass beads and coverslips.

Nucleic acid extraction. Nucleic acid was directly recovered from biofilms associated with bioreactor beads by mechanical disruption and phenol extraction (37). For each extraction, approximately 20 beads recovered from the bioreactor were placed in a 1.7-ml screw-top polypropylene tube and disrupted by bead beating as previously described but without the addition of the smaller glass beads to the tube (37).

Oligonucleotide probes and PCR primers. Oligonucleotide probes and PCR primers were designed in the context of an aligned collection of approximately 250 partial and complete 16S rRNA sequences. Probes were synthesized with an Applied Biosystems (Foster City, Calif.) DNA synthesizer (at the University of Illinois Biotechnology Center). The general probes for the bacterial domain (formerly eubacteria (47); EUB338; 5'-GCTGCCTCCCGTAGGAGT-3') and for the sulfate-reducing bacteria (SRB385; 5'-CGGCGTCGCT GCGTCAGG-3') were previously described (2). The remaining probes were specifically fabricated for this study. Oligonucleotides used for conjugation with fluorescent dye were synthesized with an aminohexylphosphate linker (Aminolink 2; Applied Biosystems). All oligonucleotides (with the exception of those derivatized with Aminolink 2) were purified by high-pressure liquid chromatography. Aminolink oligonucleotides were coupled with tetramethylrhodamine isothiocyanate and purified as previously described (3).

cDNA synthesis and amplification. Reaction mixture components were combined in a 0.5-ml polypropylene tube. The 20-μl mixture consisted of 1 μg of bulk nucleic acid and 10 pmol of a 3'-end primer (UNIV907-R; 5'-tctagaagcttC CCCGTCAATTCCTTTGAGTTT-3' [restriction site is in lowercase type]) in 50 mM KCl–10 mM Tris-HCl–5 mM dithiothreitol–0.5 mM each deoxynucleoside triphosphate. The mixture was overlaid with 50 μl of light mineral oil (Sigma Chemical Co., St. Louis, Mo.), heated to 100°C for 3 min, and immediately transferred to a 57°C water bath. Following 5 min of incubation, 20 U of avian myeloblastosis virus reverse transcriptase (Seikagaku America, St. Petersburg, Fla.) was added and incubation was continued for 40 min at 57°C. The PCR was initiated by the addition of 2.5 U of *Taq* polymerase (Perkin Elmer Cetus, Norwalk, Conn.)–10 pmol of a 5'-end primer (SRB385-F; 5'-cccgg gatCCTGACGCAGCIACGCCG-3' [I corresponds to inosine])–buffer–water (in a solution containing, in a 50-μl final volume, 50 mM KCl, 4 mM $MgCl_2$, 10 mM Tris-HCl, 2 mM dithiothreitol, and 60 ng of bovine serum albumin per μl [Life Technologies Inc., Gaithersburg, Md.]). A Perkin Elmer (Norwalk, Conn.) DNA thermal cycler was used for temperature cycling. Temperature cycles (20 total) were 3 min (4 min for the first cycle) of denaturation (95°C), 2 min of annealing (62°C), and 3 min of extension (72°C).

Cloning and sequencing. Amplified and plasmid [pGEM-3Zf(−); Promega Corp., Madison, Wis.] DNAs were digested with *Hin*dIII and *Bam*HI restriction endonucleases in accordance with the manufacturer's instructions (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The cut plasmid was dephosphorylated with alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Enzymes and salts were removed from DNA digests by phenol-chloroform extraction and ethanol precipitation (23). Amplified and plasmid DNAs were mixed in a molar ratio of 1:3 and incubated for 3 h at 12°C with 1 U of T4 DNA ligase in a reaction solution provided by the manufacturer (Bethesda Research Laboratories). *Escherichia coli* TB1 (3a) was transformed by the protocol of Cohen et al. (7). Recombinant clones were identified as white colonies on chromogenic indicator plates (tryptone, 10 g/liter; yeast extract, 5 g/liter; agar, 12g/liter; ampicillin, 100 mg/liter; 5-bromo-4-chloro-3-indolyl-β-galactopyranoside, 40 mg/liter). Isolated clones were grown overnight in 1.5 ml of broth medium containing 100 mg of ampicillin per liter. Recombinant plasmids were isolated by an alkaline lysis protocol (4) and analyzed by agarose gel electrophoresis. The nucleotide sequence was determined by plasmid template sequencing with [^{35}S]dATP (New England Nuclear Corp., Wilmington, Del.) and Sequenase (U.S. Biochemical Corp., Cleveland, Ohio) as recommended by the suppliers. Standard protocols were used for gel electrophoresis and autoradiography (23).

Phylogenetic tree inference. 16S rRNA sequences were aligned on the basis of conserved features of primary and secondary structures (46). Sequence similarities were calculated with only nucleotide positions of unambiguous alignment in all sequences compared. These were converted into evolutionary distances by the method of Jukes and Cantor (21). Phylogenies were determined from the evolutionary distance estimates by use of the algorithm of De Soete (10).

Biofilm fixation and whole-cell hybridization. Biofilms on glass coverslips were fixed by immersion of pieces of coverslips in glass scintillation vials filled with phosphate-buffered saline (390 mM NaCl, 30 mM $NaPO_4$ [pH 7.2])–4% paraformaldehyde for 20 h at 4°C. The paraformaldehyde solution was prepared immediately before use as previously described (3). Fixative was removed by washing of the coverslips twice for 3 min each time in 0.1% nonionic detergent (Nonidet P-40; Sigma), dehydration, and postfix-

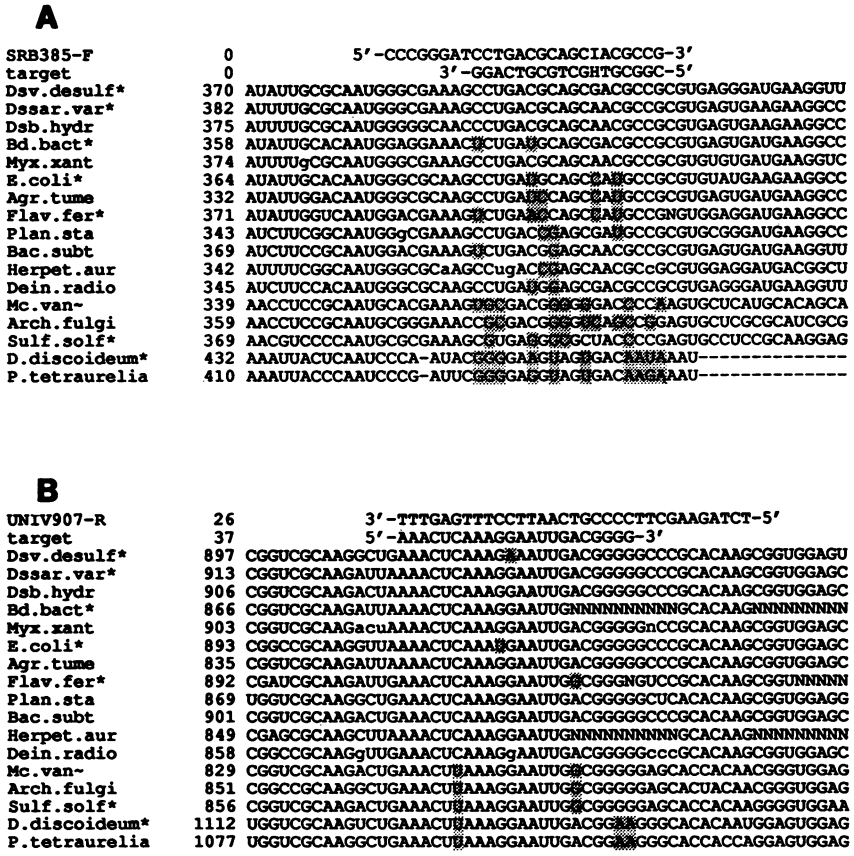

FIG. 2. PCR primers and priming sites. Shown are 16S rRNA sequence alignments near the target regions of PCR amplification primers SRB385-F and UNIV907-R. An asterisk marks the species used to demonstrate selective amplification with this PCR primer set (see Fig. 3). Positions that differ from the target sequence are indicated by shading. The 16S rRNAs shown (in order) are as follows (the GenBank accession number follows the reference citation): *Desulfovibrio desulfuricans* (31; M34113), *Desulfosarcina variabilis* (11; M34407), *Desulfobacter hydrogenophilus* (11; M34412), *Bdellovibrio bacteriovorus* (28), *Myxococcus xanthus* (31; M34114), *Escherichia coli* (5; J01695), *Agrobacterium tumefaciens* (48; M11223), *Flavobacterium ferrugineum* (28), *Planctomyces staleyi* (46b), *Bacillus subtilis* (16; X00007), *Herpetosiphon aurantiacus* (30; M34117), *Deinococcus radiodurans* (43; M21413), *Methanococcus vannielii* (20; M36507), *Archaeoglobus fulgidus* (1; X05567, Y00275), *Sulfolobus solfataricus* (29; X03235), *Dictyostelium discoideum* (25; X00134), and *Paramecium tetraurelia* (34; X03772). The sequence of *Leptonema illini* (not shown) through the target region indicated in panel A is UCUGACGCAGC GACGCCG (28; N34118), and the sequence of *D. acetoxidans* (not shown) is identical to that of the other sulfate-reducing bacteria through this region (13).

ation in 50, 80, and 100% ethanol for 3 min each. All manipulations were performed with a minimum of agitation to minimize biofilm loss. Fixed biofilms were stored in a dessicator at 4°C prior to hybridization as previously described (3). For each hybridization, 9 μl of hybridization solution (0.9 M NaCl, 20 mM Tris-HCl [pH 7.2], 100 μg of polyadenylic acid per ml, 0.1% sodium dodecyl sulfate [SDS]) was mixed with 1 μl of fluorescent probe (50 ng), and the mixture was applied to a fixed coverslip fragment placed in a glass window of a Teflon-coated microscope slide (Cell Line Associates, Inc., Newfield, N.J.), completely immersing the coverslip. Hybridization fluid was restricted to the immediate coverslip region by the surrounding hydrophobic Teflon coating. Formamide-amended solutions (30% [wt/vol]) were also used for hybridizations with the probes for population type 1 and population type 2. Following incubation at 45°C (37°C for the 30% formamide solutions) for 1 h in a moisture chamber, the coverslip was washed twice for 15 min each time in 0.9 M NaCl–0.1% SDS–0.02 M Tris-HCl (pH 7.2) at 48°C. Residual salt was removed by a brief wash with water. The coverslip was air dried prior to microscopic observation.

Photomicroscopy. Hybridized biofilms were viewed immediately with a model BH2 microscope fitted for epifluorescence (Olympus Optical Co., Tokyo, Japan) with the green filter set (BL0892). A hybridized coverslip fragment was mounted in distilled water beneath a coverslip and viewed under oil immersion with UV fluorite flat-field objectives (×40 and ×100). Biofilm thickness was estimated by use of the microscope fine adjustment. Black and white photomicrographs were made with Kodak Tri-X-Pan film (ASA 400). Exposure times were approximately 0.5 s for phase contrast and 15 to 40 s for epifluorescence.

Nucleotide sequence accession numbers. The GenBank accession numbers for population type 1 and population type 2

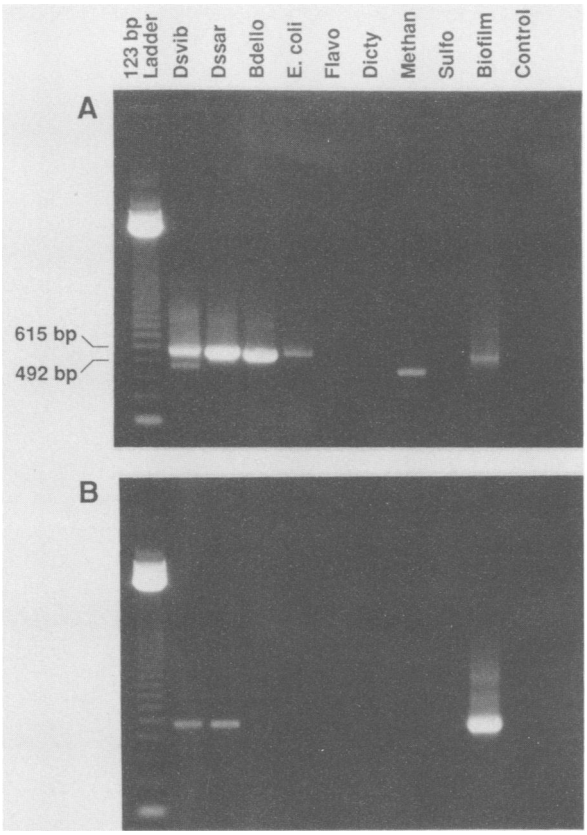

FIG. 3. Effect of temperature on selective PCR amplification of sulfate-reducing bacteria (see Materials and Methods for details). The rRNA template was derived from the organisms listed in Fig. 2. (A) Reverse transcription at 37°C and PCR primer annealing at 37°C. (B) Reverse transcription at 57°C and PCR primer annealing at 62°C.

partial 16S rRNA sequences are, respectively, M80617 and M80618.

RESULTS

Selective recovery of 16S rRNA sequences from sulfate-reducing bacteria. A PCR primer set (UNIV907-R and SRB385-F) was designed for the selective recovery of 16S rRNA sequences from sulfate-reducing bacteria. Each primer contained restriction enzyme recognition sites (*Bam*HI or *Hin*dIII) at the 5′ end of a sequence complementary to the 16S rRNA to facilitate cloning following amplification. The specificity of this primer set was defined by the SRB385-F primer (the UNIV907-R primer is complementary to most 16S-like rRNAs). The sequences of the two primers in relation to target and nontarget group sequences are shown in Fig. 2. The primer annealing temperature and magnesium concentrations were established by use of a reference collection of target and nontarget 16S rRNAs. Incubation with reverse transcriptase at 57°C (in contrast to 37°C) was shown to improve discrimination (Fig. 3). An appropriately sized fragment was amplified from RNA extracted from the sulfate-reducing bacteria examined. However, amplification was not entirely exclusive; certain others were weakly amplified by this primer set (Fig. 3). This result was anticipated given limited sequence variations within the SRB385-F target sequence among certain nontarget 16S rRNAs. In addition, more recent comparisons with an extensive 16S rRNA data base (28) revealed that several species of nontarget genera also share this target sequence (e.g., *Chlorobium*, *Campylobacter*, and *Clostridium*). A larger number differ at a single nucleotide position. Thus, this primer set is less selective than initially indicated. However, the intent of selective PCR amplification was to encompass the group targeted for sequence analysis. Since

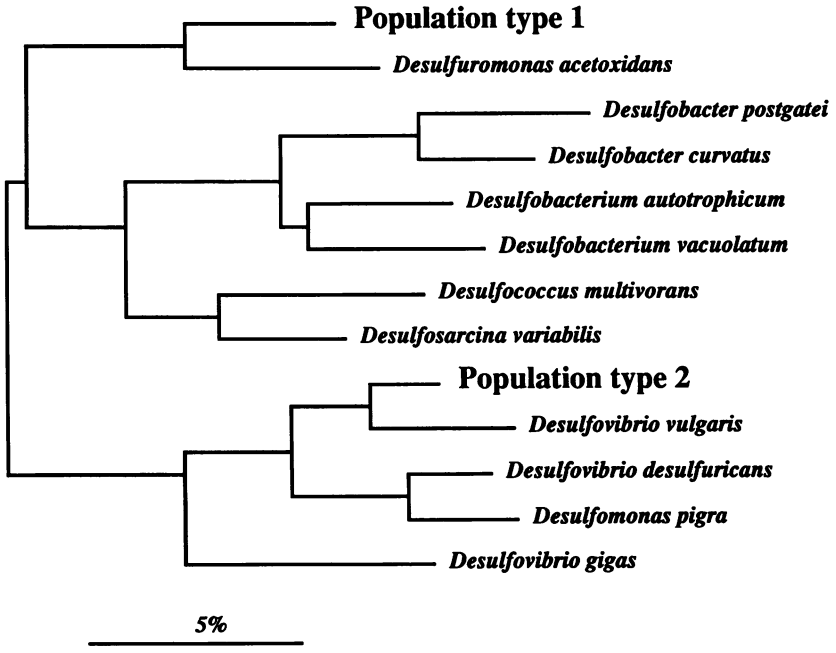

FIG. 4. Phylogenetic tree inferred from the 16S rRNA sequence divergence of characterized sulfate-reducing bacteria (11) and the bioreactor cloned amplification products. Approximately 500 nucleotides of sequence (flanked by positions 385 to 907, *E. coli* numbering) were used to infer relationships (see Materials and Methods for details). The bar represent 0.05 estimated nucleotide change per position.

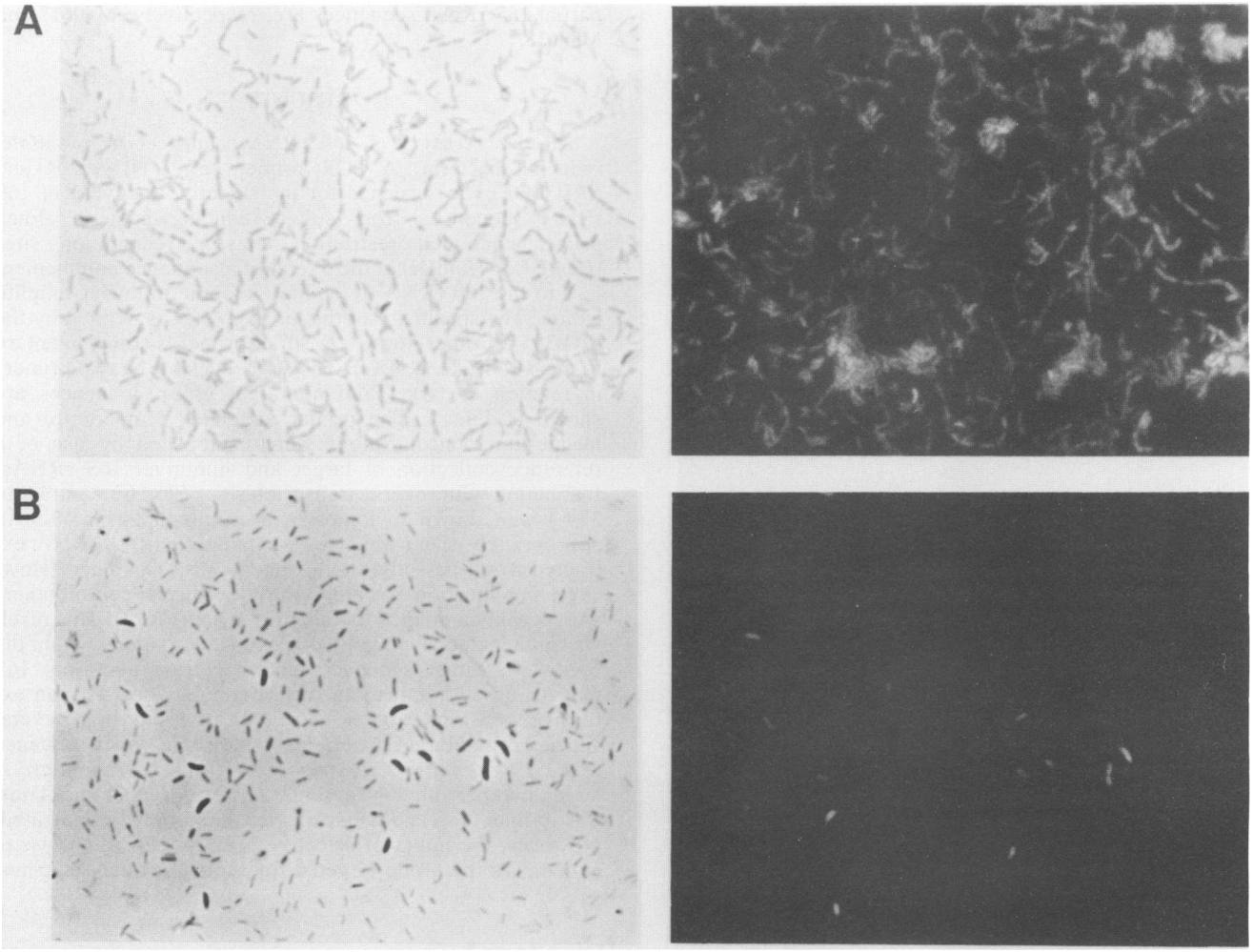

FIG. 5. Photomicrographs of biofilm colonization time series with the fluorescent probes for bacteria (A) and sulfate-reducing bacteria (B to D). Glass coverslips were placed in the bioreactor for 1 h (A), 24 h (B), 5 days (C), and 25 days (D) prior to fixation, hybridization, and microscopic observation. Phase-contrast (left) and fluorescence (right) photomicrographs (×100 objective) of the same field are shown for each time.

amplification is selective, not specific, it cannot be used quantitatively. The use of population-specific hybridization probes, such as the fluorescent probes described below, should provide better measures of natural abundance.

The products of amplification of bioreactor-derived nucleic acids were of the expected size (Fig. 3). Digestion with *Bam*HI and *Hin*dIII resulted in an internal cut in some of the amplified products (approximately 120 bp from the 3′ primer site). Following restriction endonuclease digestion, the amplified products were ligated in a plasmid vector and cloned in *E. coli*. Virtually all recombinant clones (white colonies) contained plasmids with inserts of the expected sizes. Three unique sequences were identified in 12 clones examined. Two of the sequences were closely related to the 16S rRNA sequences of characterized sulfate-reducing bacteria. Sequence (population type I) 1 was 96% similar (through the region compared) with the *Desulfuromonas acetoxidans* 16S rRNA sequence, and sequence 2 (population type II) was most closely related to the *Desulfovibrio vulgaris* 16S rRNA sequence (98% similar). A third sequence was also retrieved from the sulfate-reducing bacterium sequence-enriched recombinant library (data not shown). This was specifically, but distantly, related to available spirochete 16S rRNA sequences. The recovery of a spirochetelike sequence was not unexpected, in that spirochetes are conspicuous members of the biofilm community and the 16S rRNAs of characterized representatives share substantial similarities within the selective primer target sequence (see Fig. 2). Phylogenetic relationships between bioreactor-derived partial 16S rRNA sequences and those of characterized sulfate-reducing bacteria are shown in Fig. 4.

Visualization of biofilm populations by in situ whole-cell hybridization. For microscopic examination of biofilm populations, glass coverslips were placed in the bioreactor for various periods of time (1 h, 24 h, 5 days, or 25 days). As is evident in the phase-contrast photomicrographs (Fig. 5), biofilms rapidly developed on newly placed glass surfaces. Attachment was apparent within several hours. A monolayer was established after about 1 day and becoming multilayered (5- to 10-μm thickness) within 3 weeks. These biofilms were examined by both phase-contrast and epifluorescence microscopies following hybridization with population-specific flu-

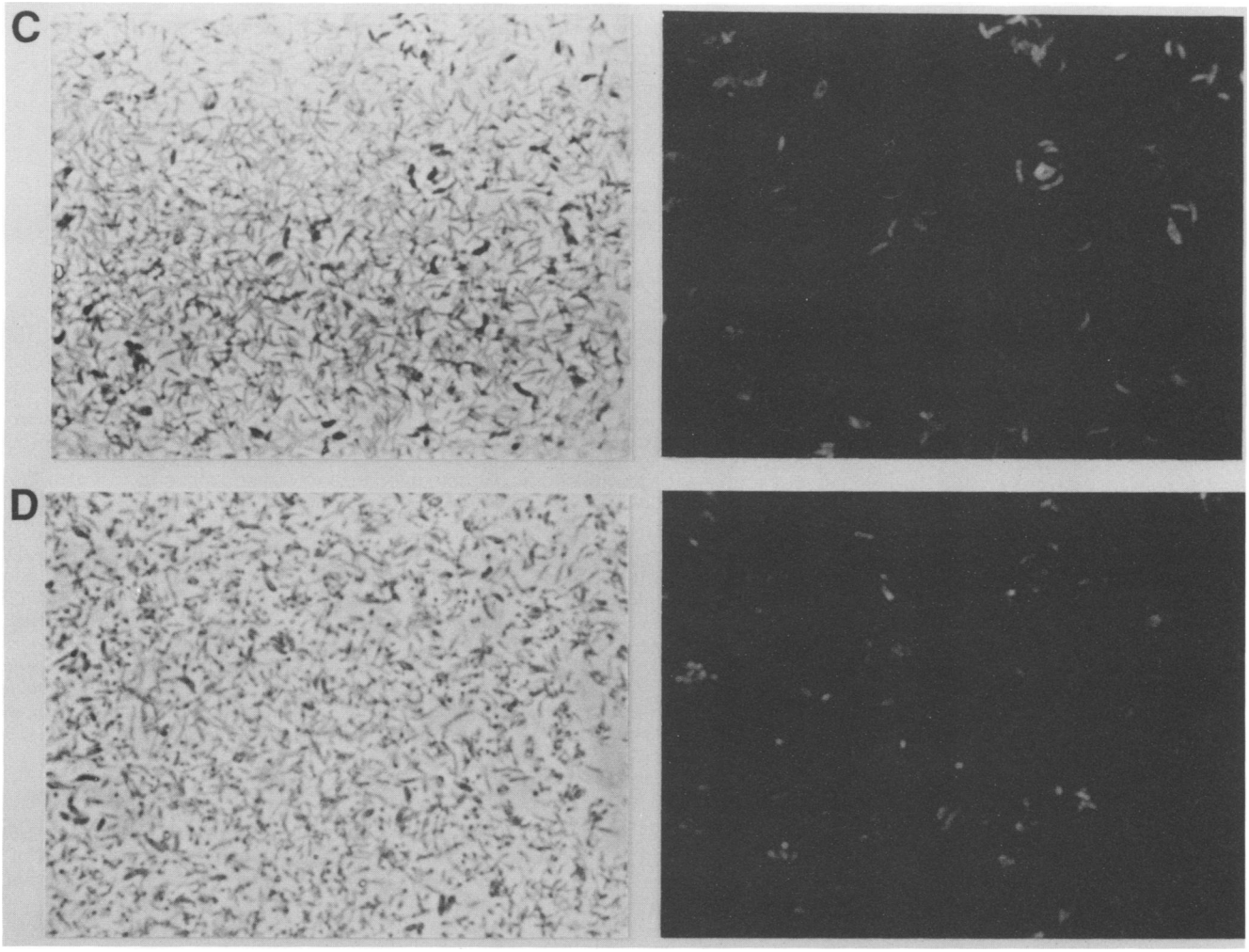

FIG. 5—*Continued.*

orescent probes. The specified conditions of fixation and hybridization rendered virtually all biofilm population members permeable to the fluorescent probes (see below and Fig. 5). Five fluorescent probes were used to visualize different populations of sulfate-reducing bacteria. The general probe (SRB385) was designed on the basis of available 16S rRNA sequences of sulfate-reducing bacteria as previously described (2, 11, 12). The PCR amplification primer (SRB385-F) is an extended version of the complement to this sequence (with an inosine substitution at one position). The remaining probes were specifically fabricated for this study and are complementary to variable regions of sequence within the two sulfate-reducing bacterium-like 16S rRNA sequences obtained by selective PCR amplification (Fig. 6).

A general observation in preliminary biofilm hybridization experiments was low specific (single-cell) and high background (nontarget) fluorescence. Both cells and noncellular biofilm matrix material appeared to contribute to the background. Background fluorescence, in general, increased with the age of the biofilm. Therefore, the influence of several different biofilm fixation and hybridization protocols on the background were examined. Variations included (i) fixation in different concentrations of formaldehyde (1, 3, or 4%) for various times (1 h, 16 h, or 14 days) with or without air drying or dehydration through an ethanol wash series (50, 80, and 100%), (ii) fixation in glutaraldehyde, and (iii) fixation in lysine-glutaraldehyde (19). The evaluation of nonspecific fluorescence was a relative assessment, comparing the fluorescence of all cells (and extracellular material) with the fluorescence of morphologically distinct populations identified by the specific probes (see below and Fig. 7). Of the various parameters examined, the use of freshly prepared solutions of paraformaldehyde and the inclusion of an ethanol dehydration series (following fixation) were most important for reducing nonspecific fluorescence. Also, the inclusion of formamide in the hybridization solution was found to enhance the hybridization of certain probes examined (see below). The colonization time series demonstrated both comparable probe penetration of different biofilm populations (Fig. 5A) and the applicability of fluorescent probes for visualizing specific populations within both newly established and more established biofilms (Fig. 5B and D).

Two probes were initially designed to specifically hybridize with the *D. vulgaris*-like and *D. acetoxidans*-like 16S rRNAs (sequences not shown). These probes were complementary to a relatively variable region of the 16S rRNAs in the vicinity of positions 453 to 470 (*E. coli* numbering). However, we failed to observe specific fluorescence of

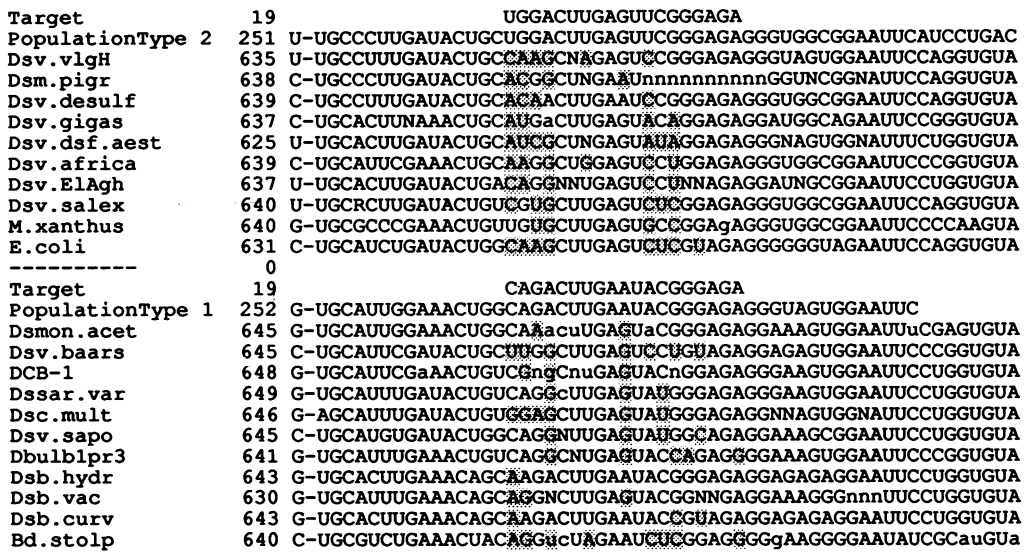

FIG. 6. Oligonucleotide probes for bioreactor populations 1 and 2. 16S rRNA sequence alignments are shown for the target regions of the two probes complementary to the 16S rRNA sequence (population type 1 or population type 2) recovered by selective PCR amplification. The probe sequence is the complement to the indicated target region. Sequences represented are from the following (the GenBank accession number follows the reference number): *Desulfovibrio vulgaris* Hildenborough (M34399) (Dsv.vlgh), *D. desulfuricans* (ATCC 27774) (28; M34113) (Dsv.desulf), *D. gigas* (M34400) (Dsv.gigas), *D. desulfuricans* subsp. *aestuarii* (Dsv.dsf.aest), *D. africanus* (M37315) (Dsv.africa), *D. desulfuricans* El Agheila (M37316) (Dsv.ElAgh), *D. salexigens* (M34401) (Dsv.salex), *D. sapovorans* (M34402) (Dsv.sapo), *D. baarsii* (M34403) (Dsv.baars), *Desulfomonas pigra* (M34404) (Dsm.pigr), *Desulfuromonas acetoxidans* (13) (Dsmon.acet), *Desulfomonile tiedjei* (strain DCB-1) (13), *Desulfosarcina variabilis* (M34407) (Dssar. var), *Desulfococcus multivorans* (M34405) (Dsc. mult), *Desulfobacter hydrogenophilus* (M34412) (Dsb.hydr), *D. curvatus* (M34413) (Dsb.curv), *Desulfobacterium vacuolatum* (M34408) (11, 12) (Dsb.vac), and *Bdellovibrio stolpii* (46c) (Bd.stolp), *Desulfobulbus propionicus* (M34411) (Dbulb1pr3), *E. coli* (J01695) (5), and *Myxococcus xanthus* (M34114) (31).

individual biofilm populations with either of these probes. Inclusion or omission of formamide in the hybridization solution did not alter this result. Failure to hybridize either could reflect the inaccessibility of the probe target site or, alternatively, could indicate that the sequences recovered by amplification and cloning did not represent major biofilm populations. To address these alternatives, we fabricated a second set of fluorescent oligonucleotide probes (Fig. 6) complementary to a different region of the 16S rRNAs. These probes identified two distinct biofilm populations (Fig. 6) that were morphologically consistent with their inferred phylogenetic placements: thin rods and vibrios for the population type 1 sequence (*Desulfuromonas* sp.-like) and the population type 2 sequence (*D. vulgaris*-like), respectively. Thus, the first set of probes presumably failed to hybridize because of the inaccessibility of the target region. Inclusion of formamide in the hybridization solution was essential for the observation of population-specific fluorescence with the second set of probes.

The sulfate-reducing bacterium general probe (SRB385) hybridized to two dominant cell morphotypes representing approximately 1 to 10% of the attached cells (Fig. 5). These were thick vibrios approximately 2.5 to 3 μm in length and 1 to 1.5 μm in diameter and thin vibrios 2 to 3 μm in length and 0.5 to 0.8 μm in diameter. Population-specific fluorescence did not appear to reflect variable probe penetration or different ribosome contents, since virtually all the cells visible by phase-contrast microscopy hybridized with a bacterium-specific probe (Fig. 5 and unpublished observations). The specificity of hybridization was confirmed by including an unlabeled homologous or heterologous oligonucleotide in the hybridization mixture. Hybridization to specific morphological types (as assessed by fluorescence) was quenched by the addition of a 40-fold excess of homologous probe but was not appreciably reduced by the addition of a comparable amount of heterologous probe (data not shown).

DISCUSSION

The direct molecular characterization of natural microbial populations was demonstrated earlier by sequence analyses of 5S rRNAs recovered from available biomass (38, 39). Those studies and more recent comparative 16S rRNA sequencing studies by Giovannoni et al. (14) and Ward et al. (41) pointedly illustrated the limitations of pure-culture isolation in surveys of natural microbial diversity; community members directly identified by molecular criteria were absent from pure-culture representations of the communities. Also, classification adds an additional confounding layer to studies of natural communities. There are many examples of standard phenotypic characterizations obscuring genetic diversity (24, 27). Thus, less biased molecular measures are increasingly used in studies of microbial systematics and ecology. Comparative rRNA sequencing has added a phylogenetic framework to molecular characterization.

Within the phylogenetic framework offered by rRNA sequence divergence, both general and specific hybridization probes have been fabricated to identify phylogenetically coherent assemblages as well as individual species or subspecies of microorganisms (3, 15, 37). In certain instances, this framework also serves in more directed studies of the relationship between community structure and community activity as a consequence of the observation that many physiologically specialized microbial groups are phylogenetically coherent or restricted to a limited number of assemblages (45, 47). Examples include the sulfate-reducing bac-

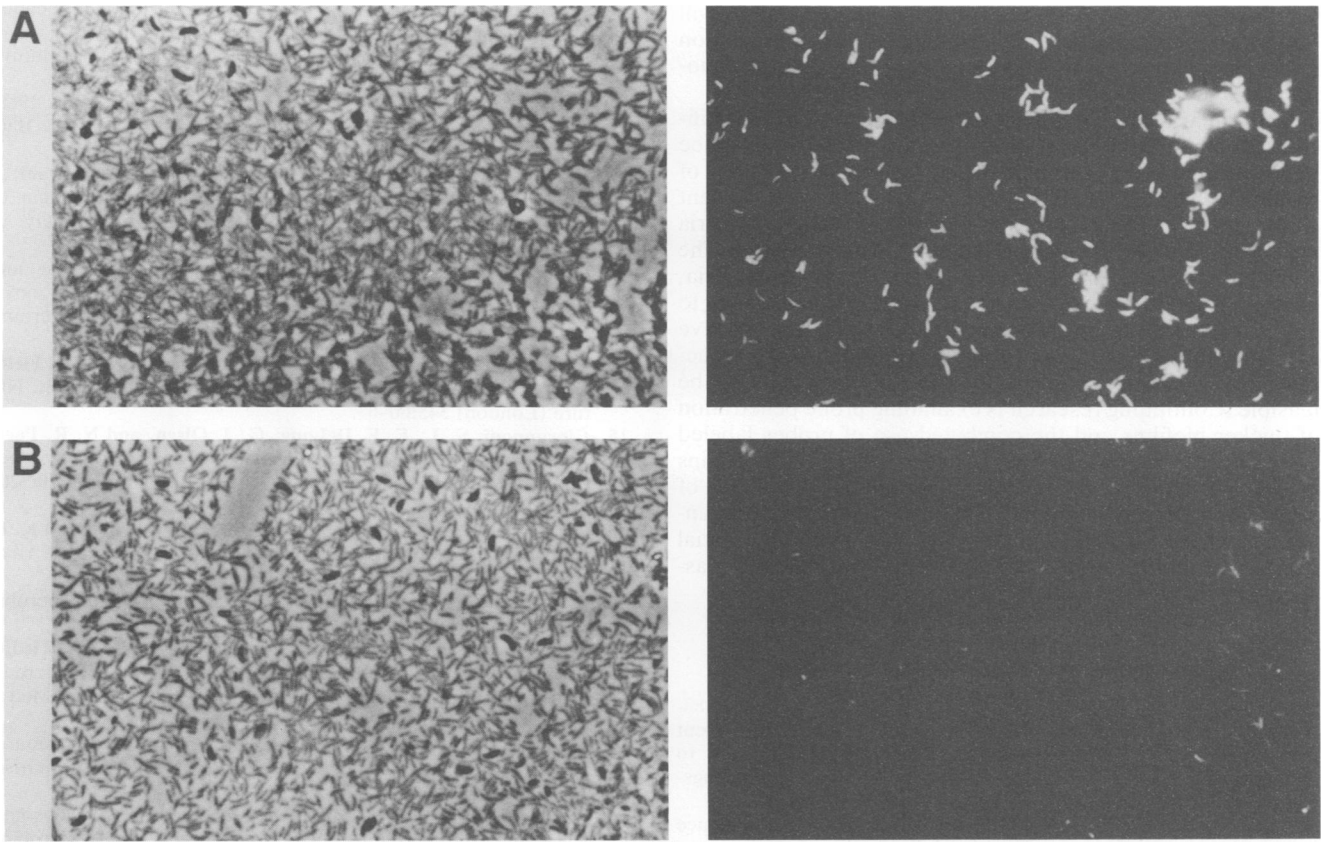

FIG. 7. Hybridization of biofilms to the bioreactor-derived specific probes (see Fig. 6). (A) *D. vulgaris*-like organisms (population type 2 probe). (B) *Desulfuromonas* sp.-like organisms (population type 1 probe). The phase-contrast (left) and fluorescence (right) micrographs do not correspond to the same field.

teria and methanogens. Thus, the use of explicit molecular criteria should serve to more directly establish the relationship between populations representing such groups and their activities under various environmental conditions. In the present study, the evolutionary coherence among mesophilic gram-negative sulfate-reducing bacteria served in the design of both selective PCR primers and hybridization probes.

Since rRNA constitutes a relatively large fraction of microbial biomass, 16S rRNA was used as a template for cDNA synthesis prior to PCR amplification. Also, the use of mechanical disruption (offering the most unbiased release of nucleic acid from a diverse community) does not favor the recovery of high-molecular-weight DNA. However, higher-order structure and base modification of rRNA may interfere with transcription. A related concern is incomplete transcription. Incomplete transcripts could (in principle) serve as primers in subsequent rounds of PCR amplification, resulting in chimeric 16S sequences. Although the sequences reported here show no marked deviation from evolutionary structural constraints (45, 46), we are nonetheless comparing amplification products derived from both rRNA and DNA primary templates. The combined use of both rRNA and DNA templates for PCR amplification may be necessary to provide the most complete community description.

A higher-resolution view of biofilm population architecture was provided by in situ hybridization with fluorescent oligonucleotide probes. Individual cells were visualized, providing information on their spatial distribution. The distribution of individual cells was markedly different for each of the bioreactor-specific populations identified by selective PCR amplification. On the basis of a qualitative visual assessment of numerous biofilm samples, population members represented by the *D. vulgaris*-like sequence appeared somewhat patchy in biofilm distribution, whereas the *Desulfuromonas* sp.-like population members were more uniformly distributed (Fig. 7 and data not shown). Although this observation has yet to be quantitatively evaluated, it could reflect such factors as local substrate limitation or differential motility of each member within the biofilm matrix. From a more practical standpoint, the spatial heterogeneity of biofilm populations has been suggested to contribute to microbially enhanced corrosion (17).

Since the sulfate-reducing bacterium general probe sequence was used both to visualize individual cells and to selectively recover 16S-like rRNA sequences, it was anticipated that the morphological types microscopically identified with the specific probes would also be revealed by the general probe. Although at least two distinct morphological types were apparent following hybridization with the general probe, the direct correspondence between these and population type 1 and population type 2 has yet to be demonstrated. In fact, the large phase-dense vibrios hybridizing with the general probe for the sulfate-reducing bacteria did not appear to correspond to the *D. vulgaris*-like population identified by the cloned-sequence-specific probe. They could have corresponded to a third population of sulfate-reducing

bacteria that has yet to be recovered. Continued studies will establish correspondence by simultaneous hybridization with general and specific probes labeled with different fluorescent dyes.

In summary, the combined use of selective PCR amplification, comparative sequencing, and oligonucleotide probe hybridization offers the basis for a systematic dissection of biofilm microbial community architecture. In the present study, mesophilic gram-negative sulfate-reducing bacteria were circumscribed by comparative molecular criteria. The contribution of the gram-positive sulfate-reducing bacteria, now represented by the genus *Desulfotomaculum*, has yet to be systematically explored. However, if the gram-positive members also demonstrate phylogenetic coherence, selective molecular recovery and identification should also be feasible. Continuing research is examining probe penetration of thicker biofilms and the combined use of probes labeled with different fluorescent dyes to define spatial relationships among populations. Given the consortiumlike character of biofilms and the dependence of many biofilm microorganisms upon the metabolism of associated organisms, spatial contiguity might reflect preferred or obligate metabolic associations.

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