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Design and Application of rRNA-Targeted Oligonucleotide Probes for the Dissimilatory Iron- and Manganese-Reducing Bacterium *Shewanella putrefaciens*

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A 16S rRNA-targeted oligonucleotide probe specific for the iron (Fe^{3+})- and manganese (Mn^{4+})-reducing bacterium *Shewanella putrefaciens* was constructed and tested in both laboratory- and field-based hybridization experiments. The radioactively labeled probe was used to detect *S. putrefaciens* in field samples collected from the water column and sediments of Oneida Lake in New York and its major southern tributary, Chittenango Creek. *S. putrefaciens* was quantified by (i) hybridization of the probe to bulk RNA extracted from field samples and normalization of the *S. putrefaciens*-specific rRNA to total eubacterial rRNA, (ii) a colony-based probe hybridization assay, and (iii) a colony-based biochemical assay which detected the formation of iron sulfide precipitates on triple-sugar iron agar. The results of field applications indicated that the three detection methods were comparable in sensitivity for detecting *S. putrefaciens* in water column and sediment samples. *S. putrefaciens* rRNA was detected in the surficial layers of the lake and creek sediments, but the levels of *S. putrefaciens* rRNA were below the detection limits in the lake and creek water samples. The highest concentrations of *S. putrefaciens* rRNA, corresponding to approximately 2% of the total eubacterial rRNA, were detected in the surficial sediments of Chittenango Creek and at a midlake site where the Oneida Lake floor is covered by a high concentration of ferromanganese nodules. This finding supports the hypothesis that metal-reducing bacteria such as *S. putrefaciens* are important components in the overall biogeochemical cycling of iron, manganese and other elements in seasonally anoxic freshwater basins.

The biogeochemical cycling of metals in aquatic environments is thought to be critical to a variety of ecologically important processes, including primary productivity in oligotrophic surface waters (29, 47) and organic matter remineralization in nutrient-rich bottom sediments (24, 35). In many suboxic environments, microbial metal reduction is considered to be one of the principal driving forces in iron and manganese remobilization. Documenting the relative abundance and activity of metal-reducing bacteria is therefore central to understanding the principal factors that control overall metal cycling in natural water systems.

Only recently has the potential ecological importance of metal-reducing bacteria been recognized (17, 18, 24, 35). The recent interest in microbial Fe^{3+} and Mn^{4+} reduction can be attributed, at least in part, to the finding that some metal-reducing bacteria (e.g., *Shewanella putrefaciens*, *Geobacter metallireducans*) can obtain energy for anaerobic growth by coupling the oxidation of organic compounds to the reduction of either Fe^{3+} or Mn^{4+} (4, 25, 27, 34). The detection of high concentrations of reduced iron (Fe^{2+}) and manganese (Mn^{2+}) in the suboxic regimes of many aquatic environments has led to speculation that the metal-reducing bacteria may be important participants in the overall biogeochemical cycling of iron, manganese, and other elements (24, 35).

Although metal-reducing bacteria have been routinely isolated from a variety of freshwater and marine environments (21, 25, 27, 33, 36, 37), the relative abundance of these bacteria remains largely unknown. If the detection of high Fe^{2+} and Mn^{2+} chemical signals in suboxic aquatic environments is indeed indicative of the abundance or activity of metal-reducing bacteria, then these bacteria may be important members of the microbial communities that inhabit such environments. However, the use of Fe^{2+} and Mn^{2+} as indicators of in situ microbial metal reduction activity has limited potential because the oxidized and reduced forms of both Fe and Mn are known to participate in a large number of environmentally significant (chemical) redox reactions. For example, both Fe^{3+} oxides and Mn^{4+} oxides are readily reduced by various organic compounds (45, 46) and inorganic sulfides (S^{2-}) (7, 40), and Fe^{2+} is readily oxidized by Mn^{4+} oxides (26, 33), dissolved oxygen (O_2) (46), and possibly nitrite (NO_2^-) (15, 37). Chemical interactions such as these effectively mask the biological Fe^{3+} and Mn^{4+} reduction signals. Alternate methods are needed to differentiate the chemical and biological sources of Fe^{2+} and Mn^{2+} detected in natural water systems.

Molecular probes designed to detect specific microbial populations (or activities) provide a means for differentiating the sources of the chemical and biological signals detected in the environment. Specific rRNA-targeted oligonucleotide probes are particularly useful tools for characterizing targeted populations in complex microbial communities (1, 2, 39, 44). In this paper we describe the design of a 16S rRNA-targeted oligonucleotide probe and its use as an ecological tool for quantifying the relative abundance of a representative metal-reducing bacterium, *S. putrefaciens*, in the metal-rich waters and sediments of Oneida Lake in New York. In the field component of this study, we also compared the relative levels of *S. putrefaciens* as determined by nonspecific enrichment strategies and direct nucleic acid extraction techniques.

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MATERIALS AND METHODS

Design and labeling of *S. putrefaciens*-specific oligonucleotide probes. An aligned data base of 16S rRNA sequences was obtained from the RNA Database Project (38) and the GenBank data bank (8). Sequences were aligned with the rRNA sequences of several *S. putrefaciens* strains (ATCC 8071, ATCC 8072, SC2A), as well as strains belonging to other species in the genus *Shewanella*, including *Shewanella hanedai* ATCC 35256 and ATCC 33224 and *Shewanella benthica* ATCC 43991, PT48 (48), PT99 (48), F1A (20), and OB54744 (12, 22). A comparison of the aligned sequences revealed a 30-nucleotide region that distinguished *S. putrefaciens* from other members of the genus *Shewanella* and all other eubacteria in the rRNA data base. The probe complementary to this region was subsequently prepared with an automated DNA synthesizer (Applied Biosystems, Foster City, Calif.). For fluorescent probe synthesis, the 5′ end was covalently linked to a hexyl amino-terminal group (Aminolink II; Applied Biosystems).

Approximately 500 ng of each probe was end labeled with [γ-^{32}P]ATP (ICN Biomedicals, Inc., Irvine, Calif.) by incubating the probe and radiolabeled nucleotide in the presence of T4 polynucleotide kinase (DuPont, Inc., Wilmington, Del.) (43). The ^{32}P-labeled probes were purified by using Nensorb columns (DuPont, Inc.), and their specific activities were determined by liquid scintillation counting. In addition, the *S. putrefaciens*-specific probe (designated SPN3) and the general eubacterial probe (designated EUB) (2) were fluorescently labeled with either sulfonyl chloride (Texas Red) or fluorescein isothiocyanate (FITC) (Molecular Probes, Inc., Eugene, Oreg.). Each probe was fluorescently labeled by using previously described procedures (13, 23).

Testing of SPN3 gene probe specificity. The specificity of the ^{32}P-labeled SPN3 gene probe was tested first in DNA-RNA hybridization experiments performed with total RNAs extracted from several known strains of *S. putrefaciens*, from other representatives of the genus *Shewanella* (*S. hanedai*, *S. benthica*), and from representatives of other phylogenetically related genera (see Fig. 2). Total RNA was extracted from pure cultures of each strain by using previously described procedures (41). Liquid cultures were grown in either Luria-Bertani medium (41) or marine 2216 broth (Difco Laboratories, Detroit, Mich.), harvested, washed, and resuspended in phosphate-buffered saline (PBS) (8.7 mM Na_2HPO_4, 1.5 mM $NaH_2PO_4 \cdot H_2O$, 0.15 M NaCl), and lysed by exposure to 2% (wt/vol) sodium dodecyl sulfate (SDS) for 10 min at room temperature. The resulting lysate was extracted sequentially with equal volumes of Tris-buffered (pH 5.1) phenol, phenol-chloroform-isoamyl alcohol (24:1:1), and chloroform-isoamyl alcohol (24:1). The aqueous phase was precipitated in 2 volumes of ice-cold 100% ethanol containing 0.1 volume of sodium acetate and incubated overnight at −20°C. The resulting precipitate was resuspended in 10 mM Tris buffer (pH 8), and the RNA was denatured for 5 min at room temperature in 3 volumes of 2% (vol/vol) gluteraldehyde. The final product was diluted 1:100 in an aqueous solution containing 1 μg of poly(A) (Sigma Chemical Co., St. Louis, Mo.) per ml, and samples were loaded onto nylon filters (Hybond N; Amersham Corp., Arlington Heights, Ill.) by using a slot blot apparatus (Millipore Corp., Bedford, Mass.). The filters were air dried, and the RNA was fixed to each filter by baking it at 80°C under a vacuum for 60 min. Hybridization experiments were conducted with the fixed RNA extracts and the ^{32}P-labeled gene probes as described below.

In a second test of SPN3 probe specificity, fluorescently labeled oligonucleotide probe SPN3 was used in situ to identify individual cells of *S. putrefaciens* in a mixed assemblage of bacteria viewed by epifluorescence microscopy (1, 2, 13, 23). Liquid cultures of *S. putrefaciens* 200 and *Aeromonas liquefaciens* ATCC 14715 were grown to mid-log phase, harvested, and resuspended in PBS. Mixed cell suspensions were fixed with 0.1 volume of 37% (vol/vol) formaldehyde for 2 h at room temperature and spotted onto subbed slides as described previously (13). After air drying, the slides were washed successively with 50% ethanol, 75% ethanol, and 90% ethanol, and 10 μl of hybridization buffer (5× SET buffer, 0.1% [wt/vol] SDS) was added to each well (1× SET buffer contained 0.15 M NaCl, 1.0 mM EDTA, and 20 mM Tris base [pH 7.8]). Approximately 50 ng of Texas Red-labeled probe SPN3 and 50 ng of FITC-labeled probe EUB were then added to each well, and the slides were incubated for 12 to 18 h at 45°C. Unhybridized and nonspecifically bound probes were removed by washing the slides in 0.2× SET buffer for 10 min at the appropriate wash temperatures (65 and 45°C for the SPN3 and EUB probes, respectively). The slides were stored in the dark before viewing by epifluorescence microscopy with a Zeiss Axiophot microscope equipped with an MC5 camera system.

Sampling locations. Water and sediment samples were collected from Oneida Lake in New York and its major southern tributary, Chittenango Creek, in October 1991. The sampling locations in Oneida Lake (Fig. 1) included two nearshore sites (depth, 10 m), designated sites OL-1 and OL-2, that flanked a shallower midlake site (depth, 3 m), designated site OL-3. Site OL-3 was located in Shackleton Shoals, a rocky outcropping known to contain large numbers of ferromanganese nodule concretions (see below). The Chittenango Creek sites (sites CC-1 and CC-2) were located approximately 5 miles (ca. 8 km) upstream from the mouth of the creek in regions that had high and low flow rates, respectively. At each field site, samples were collected along a vertical transect of the water column and underlying sediment. At lake sites OL-1 and OL-2, water column samples (1.5 liters) were collected at 3-m intervals with a Niskin bottle, and a sediment sample was obtained with a small gravity coring device. At Shackleton Shoal site OL-3 and Chittenango Creek sites CC-1 and CC-2 only a grab sample of each sediment was collected. All samples were immediately placed on ice and transported to a shore-based laboratory for processing within 1 to 2 h.

Sample processing and bulk RNA extraction procedures. An aliquot (100 μl) was removed from each sample, and serial dilutions were spread on nutrient agar (Difco Laboratories). After aerobic incubation for 7 to 10 days in the dark at room temperature, colony lifts of each sample were replicated on (i) triple-sugar iron agar (TSI agar; Difco Laboratories) and (ii) nylon filters placed directly on a second nutrient agar plate. The colony lifts were incubated aerobically in the dark for 7 to 10 days at room temperature, and the resulting colonies were scored for iron sulfide formation (TSI agar preparations) or processed further (nylon filter preparations). Colonies were lysed directly on the nylon filters by using previously described procedures (41). The filters were soaked in a 10% (wt/vol) SDS solution for 3 min and then in a 0.5 M NaOH–1.5 M NaCl solution for 5 min and neutralized in a 0.5 M Tris (pH 7.4)–1.5 M NaCl solution for 5 min and in 2× SET buffer for 5 min. The filters were air dried, and the nucleic acids were immobilized by baking the filters in a vacuum at 80°C for 1 h. Any remaining

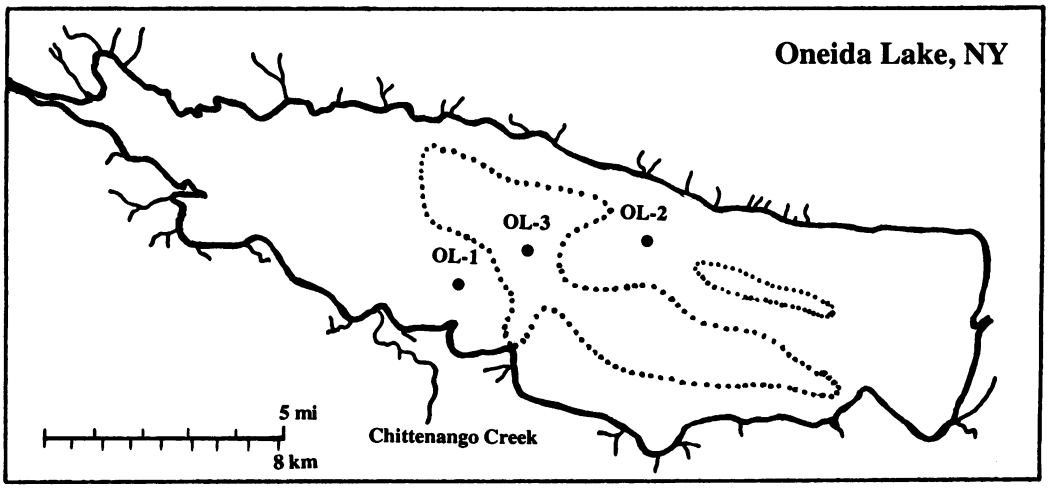

FIG. 1. Sampling locations OL-1, OL-2, and OL-3 in Oneida Lake. The dotted line indicates the approximate location of the midlake ferromanganese nodule region described by Dean and Greeson (10). Chittenango Creek empties into the lake from the south.

cell debris was washed off with 5× SET buffer before hybridization.

Water samples (1.5 liters) from each site were passed through 0.22-μm-pore-size nitrocellulose filters (Millipore Corp.), and the filters were stored at −20°C before processing. Total RNA was extracted directly from each filter by using a slight modification of previously described lysis procedures (42). Captured cells were washed off the filters in 1 ml of lysis buffer (1 mg of lysozyme [Sigma Chemical Co.] per ml, 40 mM EDTA, 50 mM Tris [pH 5.1], 75 mM sucrose) and incubated for 30 min at 37°C. Both cells and washed filters were incubated in solutions containing proteinase K (final concentration, 0.5 mg ml^{-1}; Sigma Chemical Co.) and SDS (final concentrations, 1% [wt/vol]) for 30 min at 55°C. The resulting lysates were extracted with phenol, chloroform, and isoamyl alcohol, precipitated overnight in ethanol, and dissolved in TE buffer (10 mM Tris base, 1 mM EDTA; pH 7.4) as described above. The samples were denatured, serially diluted, and loaded onto nylon filters (44), and the RNA was immobilized as described above.

Sediment subcores were kept at −20°C and processed immediately after thawing. Total RNA was extracted from each subcore by using a bead-beating technique (44). Sediment (1.0 g, wet weight) was transferred to a vial containing 0.5 ml of baked glass beads (Sigma Chemical Co.), 50 μl of 20% (wt/vol) SDS, 200 μl of lysis buffer (pH 5.1), 200 μl of Na_2HPO_4 (pH 6.0), and 400 μl of hot phenol (55°C) buffered with 50 mM sodium acetate and 10 mM EDTA (pH 5.1). The

TABLE 1. Aligned region of the 16S rRNA sequence targeted by *S. putrefaciens*-specific probe SPN3[a]

Strain or probe	Sequence
Pseudomonas testosteroni	GUC-AUGACGGUACCG-U-AAG-AAUAAGCACC-GGCU
Alcaligenes eutrophus	UCG-AUGACGGUACCG-G-AAG-AAUAAGCACC-GGCU
Pseudomonas aeruginosa	GUU-UUGACGUUACCA-A-CAG-AAUAAGCACC-GGCU
Pseudomonas mendocina	UGUUUUGACGUUACCr-A-CAG-AAUAAGCACC-GGCU
Oceanospirillum linum	GCU-GUGACGUUACUC-G-CAG-AAGAAGCACC-GGCU
Proteus vulgaris	CAA-UUGACGUUACCC-G-CAG-AAGAAGCACC-GGCU
Serratia marcescens	CAA-UUGACGUUACUC-G-CAG-AAGAAGCACC-GGCU
Vibrio parahaemolyticus	CrU-UUGACGUUAGCG-A-CAG-AAGAAGCACC-GGCU
Shewanella hanedai ATCC 35256[b]	ACU-GUGACGUUACUC-G-CAG-AAGAAGCACC-GGCU
Shewanella hanedai ATCC 33224[b]	ACU-GUGACGUUACUC-G-CNG-AAGAAGCACC-GGCU
Shewanella benthica ATCC 43991[b]	GCU-UUGACGUUACUC-G-CAG-AAGAAGCACC-GGCU
Shewanella benthica OB54744[b]	GCU-GUGACGUUACUC-G-CAG-AAGAAGCACC-GGCU
Shewanella benthica PT48[b,c]	GCU-GUGACGUUACUC-G-CAG-AAGAAGCACC-GGCU
Shewanella benthica PT99[b,c]	NCU-NUGACGUUACUC-G-CAG-AAGAAGCACC-GGCU
Shewanella benthica F1A[b,c]	GCU-NUGACGUUACUC-G-CAG-AAGAAGCACC-GGCU
Shewanella putrefaciens SC2A[b]	ACU-NUGACGUUACCU-A-CAG-AAGAAGGACC-GGCU
Shewanella putrefaciens 8072[b]	GCU-GUGACGUUACCU-A-CAG-AAGAAGGACC-GGCU
Shewanella putrefaciens 8071[b]	UCU-GUGACGUUACCU-A-CAG-AAGAAGGACC-GGCU
SPN3 probe target	CU-GUGACGUUACCU-A-CAG-AAGAAGGACC-GG

[a] The region corresponds to residues 477 to 506 in the *Escherichia coli* numbering system (9). The boldface type indicates mismatches. Aligned sequences were obtained from the RNA Database Project (38) or the GenBank data bank (8).

[b] With the exception of the previously reported (22) small-subunit rRNA sequence of *S. benthica* OB54744, all *Shewanella* small-subunit rRNA sequences were determined by DeLong and Franks (12).

[c] The sources of the *S. benthica* strains are as follows: PT48 and PT99, Yayanos deep-sea culture collection (48); and F1A, Jannasch-Wirsen deep-sea culture collection (20).

mixture was beaten three times (2 min per cycle) with a mini-bead beater (Bio Spec, Inc., Bartlesville, Okla.) with a 2-min period of incubation at 55°C between cycles. The beads and any residual solids were separated by centrifugation at 4,000 × *g* for 2 min, and the supernatant was extracted with phenol, chloroform, and isoamyl alcohol, precipitated overnight in ethanol, and resuspended in TE buffer (pH 7.4) as described above. Samples were loaded onto nylon filters and the RNA was immobilized as described above.

Nucleic acid hybridization experiments. Immobilized nucleic acid samples from either colony blot or slot blot preparations were incubated at 45°C in a prehybridization solution containing 0.9 M NaCl, 50 mM $NaPO_4$ (pH 7.0), 5 mM EDTA (pH 7.2), 0.5% (wt/vol) SDS, 0.5 mg of poly(A) per ml, 2 mg of Ficoll per ml, 2 mg of polyvinylpyrrolidone per ml, and 2 mg of bovine serum albumin (Sigma Chemical Co.) per ml. After prehybridization for 2 h, the appropriate ^{32}P-labeled probe was added to a final concentration of approximately 2×10^7 cpm per filter, and the hybridizations were carried out at 45°C for 18 to 24 h. Unhybridized probe was removed by washing each filter in a rinse solution (1× SET, 0.5% SDS) for 30 min at room temperature with gentle shaking. Nonspecifically bound probe was subsequently removed by washing each filter in 0.2× SET for 30 min at the appropriate temperature (65°C for SPN3 probe and 45°C for EUB and UNIV probes). The filters were air dried and exposed to preflashed (Kodak XRP-5 film) in the presence of an intensifying screen for 1 to 24 h at −80°C.

In quantitative slot blot experiments the signals obtained from serial dilutions of field sample bulk RNA were compared to the signals obtained from known rRNA standards fixed to the same filter. Autoradiographic signals were quantified from video images by using a gas proportional radioisotope detection system (Ambis, Inc., San Diego, Calif.). The autoradiographic signals were linear within the range from 0.5 to 50 ng for rRNA standards. The percentage of SPN3-specific rRNA was estimated from the slope of the amount of SPN3-specific probe bound per unit of rRNA divided by the slope of the amount of EUB-specific probe bound per unit of rRNA. The values were corrected for background hybridization values and were normalized to the slope of the amount of SPN3-specific probe bound per unit of rRNA with appropriate rRNA standards. RNA extracts from the following organisms were used as rRNA standards in each hybridization experiment: *S. putrefaciens* ATCC 8072 (SPN3- and EUB-positive control) and *Methanobacterium thermoautotrophicum* (an archeabacterium [20]; SPN3- and EUB-negative control).

RESULTS

Design of *S. putrefaciens* rRNA-targeted oligonucleotide probes. Members of the genus *Shewanella*, including *S. putrefaciens*, *S. hanedai*, and *S. benthica*, form a phylogenetically coherent lineage, as shown by the results of phylogenetic analyses of both 5S (14, 28) and 16S (12) rRNA sequences. The three strains of *S. putrefaciens* used to design *S. putrefaciens*-specific probe SPN3 were phylogenetically very closely related (level of unrestricted 16S rRNA sequence similarity, >98% [12]). A comparison of 16S rRNA sequences revealed a conserved tract of 30 nucleotides diagnostic for the *S. putrefaciens* strains examined in this study (ATCC 8071, ATCC 8072, and SC2A). The conserved tract differentiated *S. putrefaciens* from other

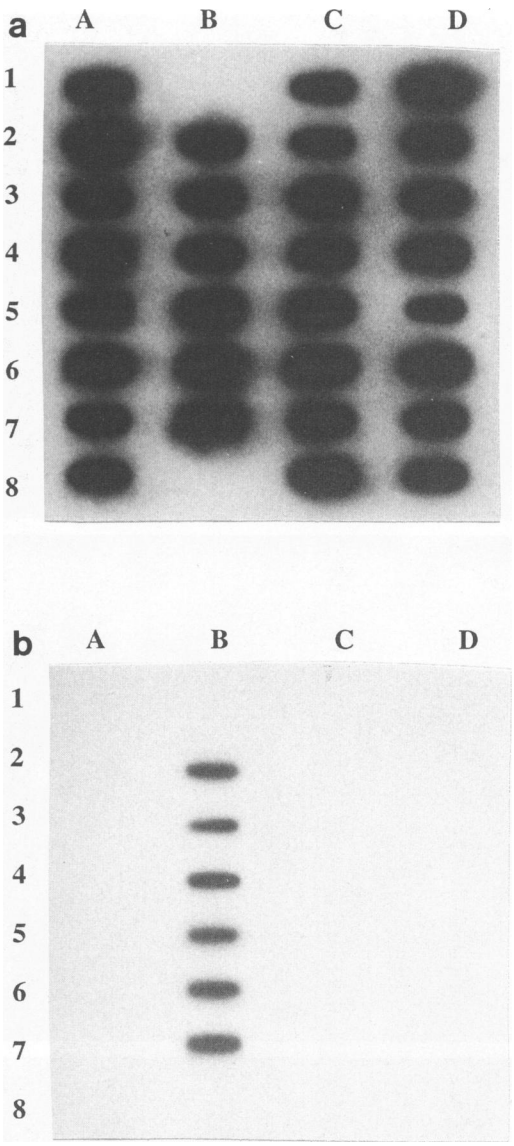

FIG. 2. Autoradiographs showing the degrees of hybridization of 16S rRNA-targeted universal probe UNIV (19) (a) and *S. putrefaciens* rRNA-targeted probe SPN3 (b) to total RNAs extracted from 30 bacterial strains. The RNA extracts from the bacterial strains were placed in slots as follows (the numbers in parentheses are references): slot A-1, *Vibrio fisheri* B399 (6); slot A-2, *Vibrio splendida* B714 (6); slot A-3, *Vibrio angustum* B559 (6); slot A-4, *Vibrio alginolyticus* B90 (6); slot A-5, *Vibrio orientalis* B719 (6); slot A-6, *Photobacterium leigonathii* B446 (6); slot A-7, *Photobacterium phosphoreum* B461 (6); slot A-8, strain SS9 (6); slot B-1, empty; slot B-2, *Shewanella putrefaciens* ATCC 8071; slot B-3, *Shewanella putrefaciens* ATCC 8072; slot B-4, *Shewanella putrefaciens* ATCC 8073; slot B-5, *Shewanella putrefaciens* ATCC 12099; slot B-6, *Shewanella putrefaciens* 200 (37); slot B-7, *Shewanella putrefaciens* SC2A (12); slot B-8, empty; slot C-1, *Escherichia coli* HB101; slot C-2, *Pseudomonas putida* ATCC 12633; slot C-3, *Pseudomonas mendocina* ATCC 25411; slot C-4, *Oceanospirillum linum* (6); slot C-5, *Alteromonas haloplanktis* ATCC 14393; slot C-6, *Alteromonas macleodii* NCMB 1963; slot C-7, *Aeromonas liquefaciens* ATCC 14715; slot C-8, *Aeromonas hydrophila* ATCC 15467; slot D-1, *Shewanella benthica* F1A (20); slot D-2, *Shewanella benthica* PT99 (48); slot D-3, *Shewanella benthica* PT48 (48); slot D-4, *Shewanella benthica* ATCC 43991; slot D-5, *Shewanella hanedai* ATCC 35256; slot D-6, *Vibrio marinus* (6); slot D-7, *Deleya cupida* A79 (6); and slot D-8, *Deleya venusta* A84 (6).

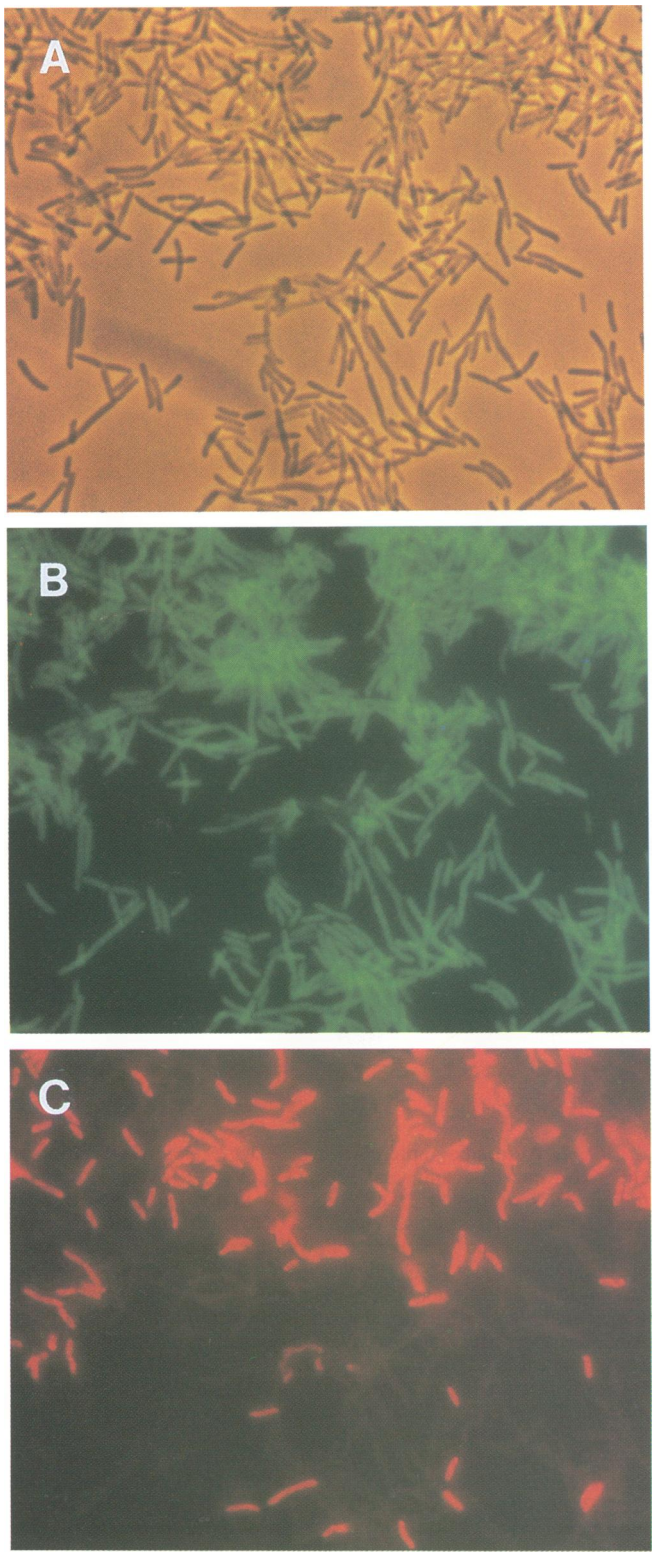

FIG. 3. (A) Phase-contrast micrograph (magnification, ×667) of a mixed bacterial assemblage containing laboratory-grown cells of *S. putrefaciens* 200 and *Aeromonas liquefaciens* ATCC 14715 in the mid-exponential growth phase. (B) and (C) Epifluorescence micrographs of the microscopic field shown in panel A after in situ hybridization with either FITC-labeled probe EUB (B) or Texas Red-labeled probe SPN3 (C). All exposure times were kept constant (120 s) to allow direct comparisons of probe-binding intensity to be made. Negative control epifluorescence experiments (data not shown) indicated that nonspecific probe binding and individual cell autofluorescence signals were below the limits of detection.

Shewanella species and from all other eubacteria available in the rRNA data base (8, 38) (Table 1).

Radioactively labeled probe SPN3 was tested for specificity by using total RNAs extracted from 30 bacterial strains (Fig. 2). These strains included representatives of each of three species of the genus *Shewanella* (*S. putrefaciens*, *S. hanedai*, *S. benthica*), as well as strains belonging to phylogenetically related genera. Autoradiographs of hybridization experiments performed with each RNA extract and ^{32}P-labeled probe SPN3 (Fig. 2) revealed that probe SPN3 hybridized strongly to the rRNAs of *S. putrefaciens* strains but not to the rRNA of any other strain (including *S. hanedai* and *S. benthica* strains).

The ability of fluorescently labeled probe SPN3 to specifically identify individual cells of *S. putrefaciens* in a mixed bacterial assemblage was evaluated by epifluorescence microscopy. A mixed cell suspension of *S. putrefaciens* 200 and *Aeromonas liquefaciens* ATCC 14715 was incubated in the presence of Texas Red-labeled probe SPN3 and FITC labeled probe EUB (1, 3, 44). Both the *S. putrefaciens* cells and the *Aeromonas liquefaciens* cells bound FITC-labeled probe EUB (FITC filter set) (Fig. 3B); however, only the *S. putrefaciens* cells bound Texas Red-labeled probe SPN3 (Texas Red filter set) (Fig. 3C).

Detection of *S. putrefaciens*-targeted rRNA signals in the water column and sediments of Oneida Lake and Chittenango Creek. Radioactively labeled oligonucleotide probe SPN3 was used in hybridization experiments to detect *S. putrefaciens* in water and sediment samples collected from Oneida Lake and its major southern tributary, Chittenango Creek (Fig. 1). Two probe hybridization techniques were used to determine the relative levels of *S. putrefaciens* in the field samples. In the first method, each sample was plated on nutrient agar, and the *S. putrefaciens* colonies were subsequently identified by the colony hybridization procedure. In the second method, *S. putrefaciens* rRNA signals were quantified by measuring the binding of probe SPN3 to bulk RNA extracted directly from the field samples and normalizing the SPN3 binding signal to the binding signal obtained with eubacterial probe EUB (1, 3, 44).

The relative levels of *S. putrefaciens* in the water and sediment samples from Oneida Lake and Chittenango Creek are shown in Table 2. Samples were collected at three lake sites and two creek sites. The colony hybridization method was used for all water and sediment samples collected at these five sites, while the direct RNA extraction method was used for only some samples. At lake site OL-1 (a nearshore site with a water depth of approximately 10 m [Fig. 1]), SPN3-positive RNA signals were not detected in any water or sediment sample. At lake site OL-2 (a farshore site with a water depth of approximately 10 m [Fig. 1]), only the colony hybridization technique was used to determine the relative amount of *S. putrefaciens*. SPN3-positive colonies (corresponding to approximately 0.1% of the total colony count on nutrient agar) were detected only in the surficial sediment layer at this site. SPN3-positive colonies were not detected in either the water column or deeper sediments at site OL-2. At lake site OL-3 (a site that was located approximately halfway between sites OL-1 and OL-2 in a relatively shallow

TABLE 2. Relative levels of *S. putrefaciens* in water and sediment samples obtained from Oneida Lake and Chittenango Creek

Site	Sample[a]	Depth (m)	% *S. putrefaciens* as determined by:	
			CFU method[b]	rRNA method[c]
OL-1	W1	0	ND (230)[d]	ND
	W2	3	ND (300)	ND
	W3	6	ND (310)	ND
	W4	9	ND (350)	NP[e]
	S1	Surficial	ND (105)	NP
	S2	0.6	ND (190)	NP
OL-2	W1	0	ND (630)	NP
	W2	3	ND (250)	NP
	W3	6	ND (250)	NP
	W4	9	ND (360)	NP
	S1	Surficial	0.1 (1,000)	NP
	S2	0.6	ND (1,000)	NP
OL-3	S1	Surficial	1.6 (980)	NP
CC-1	W1	0	ND (230)	ND
	S1	Surficial	1.0 (1,300)	2.2
CC-2	S1	Surficial	3.0 (750)	1.9

[a] W1 through W4, water samples; S1 and S2, sediment samples.
[b] Levels were calculated as follows: number of *S. putrefaciens*-positive CFU [as determined by colony hybridization experiments]/(total number of CFU on nutrient agar) × 100. The numbers in parentheses are the total numbers of CFU screened.
[c] Levels were calculated as described in Materials and Methods for the direct RNA extraction technique.
[d] ND, not detected or less than the background level.
[e] NP, experiment not performed.

region of the lake known as Shackleton Shoals; maximum depth, 3 m [Fig. 1]), an SPN3-positive signal was detected in the surficial sediment layer. Data obtained with the colony hybridization technique indicated that SPN3-positive colonies accounted for approximately 1.6% of the total colony count in samples collected from the surficial sediments at site OL-3.

When the direct RNA extraction method was used, *S. putrefaciens* rRNA was not detected in water samples collected at Chittenango Creek site CC-1. However, *S. putrefaciens* rRNA was detected in the creek sediments at site CC-1. Data obtained with the direct RNA extraction technique indicated that *S. putrefaciens* rRNA accounted for approximately 2.2% of the total eubacterium-targeted rRNA in the surficial creek sediments at site CC-1, while data obtained with the colony hybridization technique indicated that SPN3-positive colonies accounted for approximately 1.0% of the total colony count on nutrient agar. In the surficial creek sediments at site CC-2, the relative amount of *S. putrefaciens* rRNA was similar to the relative amount found in the creek sediments at site CC-1. Data obtained with the direct RNA extraction and colony hybridization techniques indicated that *S. putrefaciens* accounted for approximately 3.0 and 1.9%, respectively, of the total eubacterial signal detected in the creek sediments at site CC-2.

Colony replicates of each nutrient agar enrichment culture were also grown on TSI agar. A colony replicate of the nutrient agar enrichment culture obtained with sediment samples collected at site OL-3 (Shackleton Shoals) is shown in Fig. 4. The 16 colonies that were SPN3 positive (Fig. 4C) also produced a black iron sulfide precipitate when they were grown aerobically on TSI agar. Because of profuse colony growth on TSI agar, several of the *S. putrefaciens*

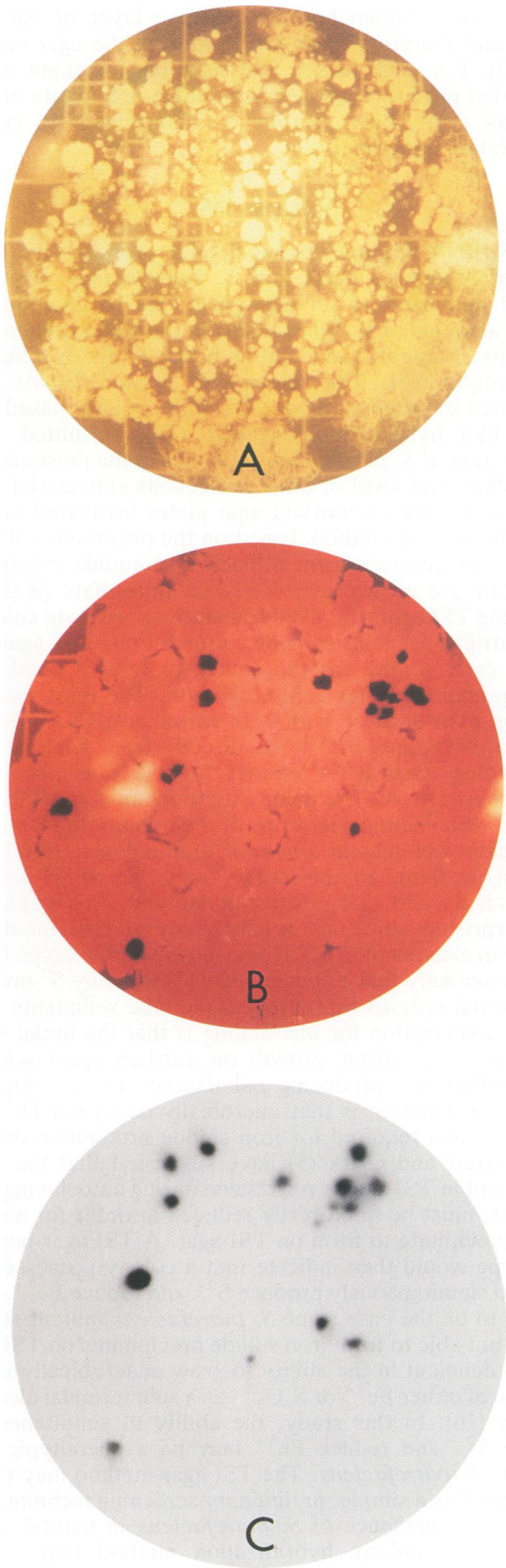

FIG. 4. Comparison of TSI agar and colony hybridization methods for detecting *S. putrefaciens* in surficial sediment samples collected from Oneida Lake site OL-3 (ferromanganese nodule site). (A) Nutrient agar enrichment master plate. (B) TSI agar replica plate. (C) Colony hybridization autoradiograph.

colonies were hidden beneath a dense layer of bacterial growth and therefore were not visible on the agar surface (Fig. 4B). For each of the five sampling locations, every colony that produced a black iron sulfide precipitate on TSI agar was also SPN3 positive as determined by colony hybridization experiments.

DISCUSSION

A 16S rRNA-targeted oligonucleotide probe for the Fe^{3+}- and Mn^{4+}-reducing bacterium *S. putrefaciens* was constructed and used as an ecological tool to examine the relative amounts of *S. putrefaciens* in the water column and sediments of Oneida Lake and its major southern tributary, Chittenango Creek. The amounts of *S. putrefaciens* were determined by three methods. The first method, based on a colony blot hybridization strategy (41), permitted rapid identification of *S. putrefaciens* colonies in the presence of a high background level of other indigenous eubacterial colonies, which arose on nutrient agar plates incubated aerobically. The second method, based on the observation that *S. putrefaciens* colonies form a black iron sulfide precipitate when they are grown aerobically on thiosulfate ($S_2O_3^{2-}$)-containing TSI agar, involved transferring replicate colonies from nutrient agar enrichment cultures onto TSI agar and scoring each colony for the ability to form a black iron sulfide precipitate after aerobic growth. The third method, based on hybridization with bulk extracted rRNA (43, 44), involved extracting total RNA directly from field samples and probing each RNA extract for the presence of *S. putrefaciens* rRNA. The colony hybridization and TSI agar methods were identical in terms of their ability to identify *S. putrefaciens* colonies in a field of total colonies formed on isolation medium. In this study, only the SPN3-positive colonies had a TSI agar-positive phenotype. This was somewhat surprising, since microbially catalyzed cysteine degradation can also result in a TSI agar-positive phenotype (5). It is highly unlikely that *S. putrefaciens* is the only S^{2-}-producing bacterial species that inhabits the lake sediments. One possible explanation for this finding is that the initial isolation step (i.e., aerobic growth on nutrient agar) selected against other S^{2-}-producing eubacterial species. Another possible explanation is that microbially catalyzed Fe^{3+} reduction is also required for iron sulfide production on TSI agar. Barrett and Clark (5) have suggested that the Fe^{2+} supplement in TSI agar is reoxidized during autoclaving and, therefore, must be microbially reduced in order for an iron sulfide precipitate to form on TSI agar. A TSI agar-positive phenotype would then indicate that a colony possesses the ability to simultaneously produce S^{2-} and reduce Fe^{3+}. This appears to be the case since *S. putrefaciens* mutant strains that are not able to form iron sulfide precipitates on TSI agar are also deficient in the ability to grow anaerobically in the presence of either Fe^{3+} or $S_2O_3^{2-}$ as a sole terminal electron acceptor (16). In this study, the ability to simultaneously produce S^{2-} and reduce Fe^{3+} may be a phenotypic trait unique to *S. putrefaciens*. The TSI agar method may therefore be used as a simple, preliminary screening technique for detecting the presence of *S. putrefaciens* in natural water samples. The colony hybridization method may subsequently be used to verify the identity and abundance of *S. putrefaciens*.

At the time of sampling in this study, *S. putrefaciens* rRNA was not detectable in either the lake water column or deeper lake sediments. However, *S. putrefaciens* rRNA was detected in the surficial sediments of Oneida Lake and its major southern tributary, Chittenango Creek. Several physical and chemical properties of the lake and its surrounding drainage area may be important factors in determining the ecological niche occupied by *S. putrefaciens*. Oneida Lake is a shallow, highly eutrophic freshwater basin located on the fertile Lake Ontario floodplain of central New York State (Fig. 1). The combination of a large lake surface area (207 km^2), shallow mean depth (7 m), and nearly constant wind mixing keep the lake water column well oxygenated throughout most of the year (30, 31). The major southern tributaries of the lake originate in foothills rich in both Fe and Mn, and, as a result, these metal-rich tributaries contribute a high flux of nutrients to the lake. The lake experiences intense algal blooms during the summer months, and the sinking organic detritus acts as a biological pump transporting large amounts of Fe and Mn to the lake sediments (10, 11). The organic compound-rich, Fe- and Mn-containing sediments are generally anaerobic, with only a thin (1- to 2-mm) surficial layer separating the well-oxygenated lake water column from the anaerobic sediments (33). These suboxic, surficial sediment layers are thought to contain a large population of Fe^{3+}- and Mn^{4+}-reducing bacteria (33). The results of our study indicate that such a population does indeed exist, as shown by the *S. putrefaciens* rRNA signals detected in the surficial layers of the lake sediments. Other factors, such as the identity and concentration of organic carbon and the availability of other terminal electron acceptors, may also influence the distribution of *S. putrefaciens* in Oneida Lake. In addition to Fe^{3+} and Mn^{4+}, *S. putrefaciens* is capable of respiring anaerobically with a wide range of other terminal electron acceptors, including nitrate (NO_3^-), nitrite (NO_2^-), sulfite (SO_3^{2-}), thiosulfate ($S_2O_3^{2-}$), trimethylamine-*N*-oxide, fumarate, and perhaps several others (27, 32).

A dominant feature of the floor topography of Oneida Lake is the shallow midlake region, Shackleton Shoals (Fig. 1). This rocky outcropping is located in the middle of the largest freshwater deposit of ferromanganese nodules yet reported (10). Shackleton Shoals is located in a 20-km^2 midlake region where approximately 25% of the lake floor is covered by ferromanganese concretions. The overall Fe and Mn budget values for the lake waters, sediments, and major tributaries indicate that almost all of the Mn lost from the lake water can be accounted for in the nodules, while most of the Fe lost from the lake water is incorporated into the sediments (10, 11). Whether *S. putrefaciens* is directly involved in ferromanganese nodule formation is not known; however, the highest *S. putrefaciens*-targeted rRNA signals detected in this study were in the surficial sediments of Shackleton Shoals. Metal-reducing bacteria such as *S. putrefaciens* may contribute indirectly to ferromanganese nodule formation via one of several potential mechanisms. By reducing Mn^{4+} oxides directly, *S. putrefaciens* may effectively remobilize recently buried Mn from deeper sediment layers to the (anoxic-oxic) sediment-water interface, where Mn^{2+} can accumulate and subsequently be incorporated into ferromanganese concretions. Alternately, *S. putrefaciens* may reduce the more readily available Fe^{3+} oxides to their soluble Fe^{2+} forms, which then act as chemical reductants of the Mn^{4+} oxide particles. Abiotic Fe^{2+}-catalyzed Mn^{4+} reduction would then drive Mn accumulation at the sediment-water interface. Considerable work will be required to confirm these hypotheses.

The results of preliminary in situ hybridization experiments performed with fluorescently labeled oligonucleotide probe SPN3 and various laboratory-grown cultures indicate that epifluorescence microscopy may be useful for differen-

tiating individual cells of *S. putrefaciens* from cells of other microorganisms in complex microbial assemblages (Fig. 3). Future ecological studies involving the use of in situ fluorescently labeled probes for identification of individual *S. putrefaciens* cells in field samples should aid in determining the spatial and temporal distribution, and possibly the metabolic activity (13, 39), of metal-reducing bacteria in redox-stratified natural water systems.

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