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Identifying Numerically Abundant Culturable Bacteria from Complex Communities: an Example from a Lignin Enrichment Culture

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Culturable bacteria that were numerically important members of a marine enrichment community were identified and characterized phylogenetically. Selective and nonselective isolation methods were used to obtain 133 culturable bacterial isolates from model marine communities enriched with the high-molecular-weight (lignin-rich) fraction of pulp mill effluent. The culture collection was screened against community DNA from the lignin enrichments by whole-genome hybridization methods, and three marine bacterial isolates were identified as being numerically important in the communities. One isolate was in the α-subclass of *Proteobacteria*, and the other two were in the γ-subclass of *Proteobacteria*. Isolate-specific 16S rRNA oligonucleotide probes designed to precisely quantify the isolates in the lignin enrichment communities indicated contributions ranging from 2 to 32% of enrichment DNA, values nearly identical to those originally obtained by the simpler whole-genome hybridization method. Two 16S rRNA sequences closely related to that of one of the isolates, although not identical, were amplified via PCR from the seawater sample originally used to inoculate the enrichment medium. Partial sequences of 14 other isolates revealed significant phylogenetic diversity and unusual sequences among the culturable lignin enrichment bacteria, with the *Proteobacteria*, *Cytophaga-Flavobacterium*, and gram-positive groups represented.

There is wide consensus among microbial ecologists that the majority of bacteria in complex natural communities do not form colonies on the rich media traditionally used for enumerating and isolating bacterial species, even though they may be viable (8). Among the bacteria which do grow under laboratory conditions, it is difficult to distinguish the ecologically relevant strains from the opportunistic species which can take advantage of the culture conditions chosen in the laboratory but which are numerically unimportant in the intact natural community. Thus, results of laboratory studies using randomly chosen culturable bacteria generally cannot be widely extrapolated to address functional attributes of the total community.

Results of several recent studies, however, have suggested that the chasm between ecologically important bacterial species and culturable bacterial species may not be as great as previously thought. Greater success in bacterial isolation has been achieved by using culture conditions that more closely approximate natural environments (36) or by using novel tools, such as optical tweezers to physically isolate individual bacteria (26). Furthermore, molecular evidence that some readily culturable bacteria may indeed be abundant, and presumably functionally important, in the environment from which they were isolated has been accumulating. Rehnstam et al. (33) found that several bacterial isolates from coastal seawater were dominant members of the bacterioplankton community, while Fuhrman et al. (17) isolated representative marine bacteria accounting for up to 20% of bacterial DNA in the source environment.

For the vast majority of isolates, however, little is known about their numerical abundance in the complex microbial communities from which they were cultured. Such information would be extremely valuable in order to identify appropriate candidates for further ecological, physiological, and genetic studies and to target native bacteria suitable for bioremediation or biodegradation purposes. In this study, we focus on bacteria involved in lignin degradation, an important biogeochemical function for which the isolation of numerically abundant bacterial strains would be of interest, both because of the abundance of naturally occurring lignins and because of the importance of lignin degradation to the pulp and paper industry. Using a marine pulp mill waste enrichment culture with demonstrated abilities to degrade polymeric lignin (23) as a model bacterial community, we assembled a collection of bacterial isolates and then tested several approaches for screening the isolates to identify those which were numerically abundant within the enrichment community. Methods involving whole-genome hybridizations were found to yield abundance estimates very similar to those based on specifically designed 16S rRNA oligonucleotide probes. Three culturable, numerically dominant members of the lignin enrichment community were identified and characterized from these studies. Although our model communities are likely to be compositionally simpler than communities from unmanipulated natural samples, we demonstrate a protocol successful for screening large culture collections to identify organisms which are numerically abundant members of complex communities.

MATERIALS AND METHODS

Lignin enrichment culture. A black liquor sample which contained lignin-rich by-products from the pulping of southern U.S. hardwood species was obtained from Federal Paper Board Company Inc., Augusta, Ga. The black liquor (4 g) was dissolved in 1 liter of 0.1 N NaOH and then continuously circulated through a tangential flow filtration unit (Pelicon; Millipore Corp., Bedford, Mass.) with a

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1,000-molecular-weight size exclusion membrane for 3 h to isolate the high-molecular-weight fraction; 64% of the original A_{280} was retained in this fraction.

Enrichment cultures were set up in triplicate 6-liter flasks. The medium consisted of 20 mg of C liter of high-molecular-weight black liquor^{-1} diluted in 3 liters of filter-sterilized seawater (20‰) with added inorganic nutrients at concentrations typical of coastal seawater (5 μM N as NH_4NO_3 and 1 μM P as KH_2PO_4). Each flask was inoculated with 20 ml of freshly collected seawater which had been filtered through a 1-μm-pore-size Nuclepore filter to remove detrital particles and larger planktonic organisms; the seawater inoculum contained 5.6×10^5 bacterial cells ml^{-1} as determined by acridine orange direct counting (AODC) (25). The seawater used for the medium and inoculation was collected from a salt marsh on Sapelo Island, Ga., and had no prior exposure to paper industry wastes. After inoculation of the enrichment cultures, three subsequent transfers were carried out (20 ml into 3 liters of fresh medium) at 2-week intervals. The flasks were incubated in the dark at room temperature with shaking at 100 rpm. The pH of the medium was the same as that of the original water (7.9) and did not change during incubation. All glassware used in the enrichment cultures was combusted or treated with 10% HCl to avoid contamination. At the end of the 8-week enrichment period, the cultures contained 4.6×10^6 bacterial cells ml^{-1}.

Bacterial culture collection. Bacterial strains were isolated from the final enrichment flasks by using half-strength YTSS agar plates (4 g of yeast extract [Difco Laboratories, Detroit, Mich.], 2.5 g of tryptone [Difco], 20 g of sea salts [Sigma, St. Louis, Mo.], 18 g of agar, and 1 liter of distilled water) or pulp mill waste plates (enrichment medium plus 2% agar). To obtain the isolates, duplicate series of dilutions were prepared in saline solution from the three final replicate enrichment flasks (designated flasks A, B, and C). An aliquot of 0.1 ml was spread on three plates of each type from the 10^{-2} to 10^{-5} dilutions. All colonies growing on the most dilute plates (10^{-5} for half-strength YTSS plates and 10^{-4} for pulp mill waste plates) were inoculated onto fresh agar. In addition, six morphologically distinct colonies from less dilute plates were also inoculated onto fresh plates. In total, 106 organisms were isolated from the three replicate flasks on solid media (73 on half-strength YTSS plates and 33 on pulp waste plates).

Isolates were also obtained by a liquid extinction dilution technique (6, 36). An aliquot of the enrichment culture from each flask was serially diluted in four replicate series of tubes, resulting in an average of 10^3 to 10^{-1} cells tube^{-1} (based on AODC of the bacteria in the original enrichment culture). The dilution medium was the same medium as was used for enrichment. Each week for the next 4 weeks, a 0.1-ml subsample from each dilution tube was transferred to 9.9 ml of new medium. Growth of bacterial cells in the liquid dilutions was monitored every 3 days by AODC. From every tube which was originally inoculated with a nominal cell density of 0.1 to 10 cells tube^{-1} (36 tubes) and which appeared, on the basis of microscopic examination, to support a pure bacterial culture (32 tubes), a 0.5-ml aliquot was removed and spread-plated on half-strength YTSS; most of these produced colonies on the solid medium, and a total of 27 isolates were obtained. Isolates obtained by all methods were stored in freezing medium at −70°C after purity was confirmed by at least five transfers to fresh solid medium.

Biolog GN MicroPlates (Biolog Inc., Hayward, Calif.) were used for biochemical characterization of isolates suspected on the basis of hybridization results (described below) to be duplicate organisms. Isolates were grown on YTSS for 48 h at room temperature. Colonies were resuspended in 2.5% NaCl, and Biolog plates were inoculated according to the manufacturer's protocol and incubated at 32°C. Results were recorded after 24 and 48 h.

DNA extraction. The community DNA extraction procedure was a modification of the method of Tsai and Olson (40). Briefly, bacteria from 1 liter of the lignin enrichment cultures or 8 liters of the original seawater inoculum (approximately 5×10^9 cells) were collected on 47-mm-diameter, 0.2-μm-pore-size Nuclepore filters and stored at −20°C. To extract DNA, the frozen filters were thawed, cut into small strips, and then treated with 15 mg of lysozyme ml^{-1} in 2 ml of buffer (0.15 M NaCl, 0.1 M EDTA; pH 8.0) in polypropylene tubes. After 2 h at 37°C, 2 ml of sodium dodecyl sulfate (SDS) buffer (0.1 M NaCl, 0.5 M Tris [pH 8.0], 10% SDS) was added, and the tube contents were briefly vortexed and then subjected to three freeze-thaw cycles (−70°C for 30 min followed by 65°C for 5 min). The tube contents were centrifuged for 10 min at 4°C ($6,000 \times g$) to remove cell debris and filters. Saturated phenol (2 ml; Ambion Inc., Austin, Tex.) was added to the supernatant, and the mixture was subjected to gentle shaking and centrifugation for 10 min ($6,000 \times g$). The aqueous fraction was carefully drawn off with a disposable pipette tip that had been cut obliquely to produce a wide hole. A second extraction was carried out using a mixture of phenol, chloroform, and isoamyl alcohol (25:24:1, vol/vol), and a third extraction was carried out with chloroform-isoamyl alcohol (24:1, vol/vol). The DNA was precipitated in 1 volume of isopropanol overnight at −70°C and then washed once with cold 70% ethanol. The dried DNA was resuspended in TE (10 mM Tris, 1 mM EDTA; pH 8.0). The purity, integrity, and RNA content of the extracted DNA were determined by three methods: A_{260} and A_{280}, electrophoresis in 0.8% agarose with or without digestion with the restriction enzyme *Sau*3AI (16), and PCR amplification with universal primers targeting the 16S rRNA gene. DNA was always quantified with Hoechst dye 33258 (Sigma) immediately before use. The estimated DNA extraction efficiency was 20 to 30%, assuming a DNA content of 5.7 fg cell^{-1} (16).

DNA from bacterial isolates was extracted by a modified hexadecyltrimethyl ammonium bromide (CTAB) procedure (12, 13, 31) which reduces interference from extracellular polysaccharides. Briefly, a colony was suspended in 300 μl of STET buffer (50 mM Tris, 50 mM EDTA, 8% sucrose, 0.1% Triton X-100; pH 8.0) with 1 mg of lysozyme ml^{-1} and incubated for 30 min at 37°C. The test tubes were placed in boiling water for 45 s and then rapidly cooled on ice. After centrifugation at $6,000 \times g$ for 2 min, the supernatant with polysaccharides was discarded, and the pellet was resuspended in 200 μl of STET buffer and 300 μl of CTAB solution (100 mM Tris, 20 mM EDTA, 2.45 M NaCl, 3.5% CTAB; pH 8.0). The test tubes were incubated at 55°C for 30 min and finally at 70°C for 1 min. The tube contents were centrifuged at $15,000 \times g$ for 3 min at room temperature. The supernatant was extracted with chloroform-isoamyl alcohol (24:1) and then centrifuged ($15,000 \times g$) for 3 min. DNA was precipitated by adding 1/5 volume of 7.5 M ammonium acetate and 2 volumes of ethanol. After precipitation, the DNA pellet was washed with cold 70% ethanol and resuspended in TE.

For bacterial isolate DNA used in hybridizations with community DNA, we used the same extraction method as was used for the community DNA (i.e., the Tsai and Olson method [40]) to avoid the possibility that differences in the DNA extraction method might influence the extent of hybridization (46). Isolates were grown in half-strength YTSS, and the cells were then centrifuged and washed in saline solution. The Tsai and Olson method was followed as described above except that the period for the lysozyme incubation was 30 min.

Whole-genome probing. (i) Colony hybridizations. Colony lifts were performed as described by Sambrook et al. (34). Isolates were grown on half-strength YTSS plates, and a nylon membrane (Hybond; Amersham Corporation, Arlington Heights, Ill.) was pressed down onto the plates to transfer cells from the colonies to the membrane. The membrane was then treated with NaOH, and the DNA was bound by UV cross-linking.

The hybridization conditions were a modification of those of Voordouw et al. (42, 44). The membrane was prehybridized for 4 h at 68°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.5)–10× Denhardt solution (1× Denhardt solution is 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone)–0.2% SDS–10% 500,000-molecular-weight dextran sulfate–100 ng of salmon sperm DNA ml^{-1}, all at 50 μl cm^{-2}. Community DNA from one of the flasks (flask B) was labeled with a random priming kit (Stratagene, La Jolla, Calif.) using [α-^{32}P]dCTP (Amersham Corporation) to a specific activity of 10^9 cpm μg^{-1}. Unincorporated nucleotides were removed by precipitation of the probe with ethanol, and the pellet was washed in cold 70% ethanol before use. The probe was denatured by boiling and added to the hybridization solution to a concentration of 10^7 cpm ml of buffer^{-1}. After 16 h of hybridization at 68°C, the membrane was washed with 2× SSC–0.1% SDS at room temperature for 10 min, then with 6× SSC–0.2% SDS at 68°C for 1 h, and then with 0.1× SSC–0.1% SDS at room temperature for 10 min. The membrane was dried and exposed to X-ray film with intensifying screens for 0.5 to 24 h at −70°C.

(ii) Dot blot hybridizations. Twenty-five isolates giving the strongest signals in the colony hybridizations (10 isolated from half-strength YTSS plates, 9 isolated from pulp mill waste plates, and 6 isolated from liquid dilutions) were selected for reverse dot blot hybridizations with community DNA from flasks A and B (Fig. 1). A volume of DNA extract containing 100 ng of DNA from an isolate or from one of several American Type Culture Collection (ATCC) standard cultures was denatured by the addition of 1.85 μl of 5 N NaOH to obtain a final concentration of 0.35 N NaOH. After 10 min at room temperature, the sample was diluted with 208 μl of ice-cold distilled water to give a final concentration of 0.04 N NaOH, and 1 volume of cold 2 N ammonium acetate was added (modified from the procedure of Kafatos et al. [28]). The DNA was spotted onto positively charged nylon membranes and allowed to air dry.

On the basis of the results of these hybridizations, 10 isolates giving strong dot blot hybridization signals (6 isolated from half-strength YTSS plates, 2 isolated from pulp mill waste plates, and 2 isolated from liquid dilutions) were again hybridized with community DNA from all the flasks and original seawater inoculum by quantitative dot blot hybridizations, in which community DNA served as the target and the isolate DNA served as the probe (Fig. 1). DNAs from enrichment communities and the original seawater inoculum were spotted on a nylon membrane (1 μg and 500 ng). The filter also contained standards ranging from 50 to 500 ng of DNA of the isolate to be quantified; the DNA in each standard dot was supplemented with salmon sperm DNA to give a total of 1 μg per spot. For negative controls, community DNA samples treated with DNase were spotted on the membrane. DNA from each of the 10 isolates was labeled with [α-^{32}P]dCTP by random priming, and hybridization was carried out as described above. The signal intensity was quantified with a laser densitometer (Molecular Dynamics, Sunnyvale, Calif.). A linear regression of the quantity of isolate DNA versus hybridization signal was used to quantify the amount of isolate DNA within the community (r^2 ranged between 0.990 and 1 in all cases). Three isolates, all originally from YTSS plates, were chosen for further study on the basis of the strength of the hybridization signal.

Sequencing and design of 16S rRNA oligonucleotide probes. 16S rRNA gene sequences were determined for the three selected isolates, designated IRE-31, E-37, and KW-40 (Fig. 1). We determined the sequence of approximately 1,400 bp for each isolate as described elsewhere (22).

The sequences were aligned with those in the Ribosomal Database project (30), GenBank, and EMBL databases by using the Genetics Computer Group

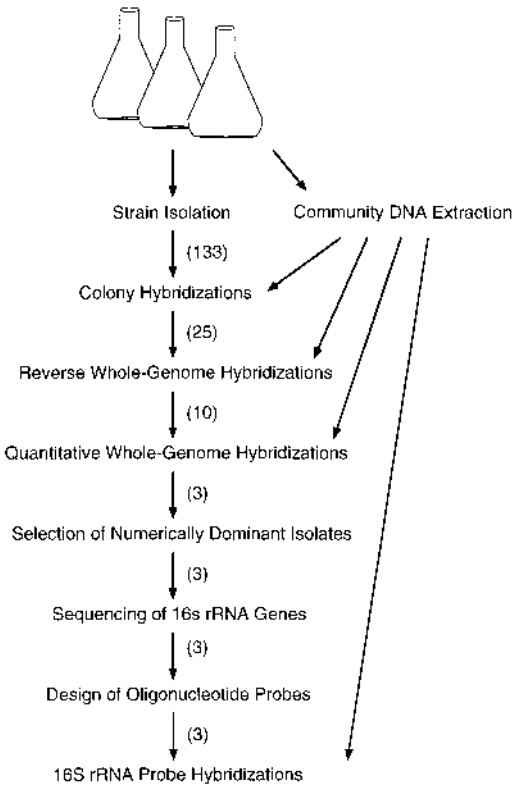

FIG. 1. Flow diagram of methods used to screen the isolate collection for numerically abundant organisms. Values in parentheses indicate the number of strains carried through each step of the procedure.

package (20) and the National Center for Biotechnology Information package (1). Oligonucleotide probes specific for each of the isolates were designed by choosing a 20-bp region in the case of IRE-31 and KW-40 and 18 bp in the case of E-37, for which variability was high with respect to the closest phylogenetic groups and for which there was no match with any other sequence in the data banks (Table 1).

Community hybridization with oligonucleotide probes. The oligonucleotide probes were checked for specificity by hybridization with cultured organisms closely related to the three isolates, as indicated by sequence analysis. *Roseobacter litoralis* and *Roseobacter denitrificans* were used to check the specificity of the probe for E-37; *Pseudomonas putida* and *Pseudomonas aeruginosa* were used to check the specificity of the probe for IRE-31, and these two species were also used to check the specificity of the probe for KW-40 because although the complete 16S rRNA sequence of KW-40 was most similar to that of *Halomonas* spp. and other marine bacteria, the 20-base region of the probe matched the pseudomonad sequences most closely. DNA from the isolates was arrayed on a nylon membrane along with DNA from 28 to 36 other isolates from flask C (1 μg of DNA per dot).

The hybridization conditions for the oligonucleotide probes were similar to those described for whole-genome probing except for changes in the temperatures of hybridizations and washes (Table 1). Hybridizations were performed at 5°C below the calculated melting temperature for the sequence as described by Braun-Howland et al. (4). The universal probe 1406R (29) (Table 1) served as a positive control. Oligonucleotide probes were labeled with terminal transferase (Boehringer Mannheim Corp., Indianapolis, Ind.) by using [α-^{32}P]dATP to a specific activity of approximately 10^8 dpm μg^{-1}. After removal of unincorporated nucleotides and desalting, probes were added to the hybridization solution at a concentration of 10^6 cpm ml of buffer^{-1}. Hybridization was carried out for 16 h, after which the membrane was washed with three changes of 2× SSC with 0.1% SDS for 1 h, then dried, and exposed to X-ray film.

Following experimental confirmation of the specificity of the 16S rRNA-based probes, the abundance of isolates IRE-31, E-37, and KW-40 in the enrichment communities was determined by dot blot hybridizations. Community DNA from each of the three flasks was spotted onto a nylon membrane (1 μg per dot), along with isolate standards ranging from 50 to 500 ng of DNA, again with salmon sperm DNA added to adjust the total amount of DNA to 1 μg. The membranes were hybridized with [α-^{32}P]dATP-labeled oligonucleotide probes as described above. The contribution of each isolate was calculated as for total-genome hybridization. Hybridization signals with the universal probe (not shown) were identical for all dots, confirming that the total quantities of DNA spotted were similar.

Comparative analysis of 16S ribosomal DNA. The phylogenetic diversity of the enrichment culture bacterial community was studied by sequencing a fragment of the 16S rRNA gene from nine morphologically distinct bacterial isolates giving strong signals in the community DNA hybridizations plus five other strains chosen on the basis of unique morphology (seven isolates originally cultured on half-strength YTSS plates, three cultured on pulp mill waste plates, and four from liquid dilutions). A 300-bp sequence was generated with the universal primers 926F and 1406R (29). Evolutionary distances were calculated using Jukes-Cantor corrections, not considering base ambiguities, by means of the PHYLIP package (15). Phylogenetic trees were constructed by the neighbor-joining algorithm using the PHYLIP package.

Detection of E-37 in seawater. Primers were designed for detection of E-37 or related organisms in coastal Georgia seawater. PCR amplification of DNA extracted from seawater was carried out by using a primer pair consisting of the oligonucleotide probe specific for E-37 (ROS137-37) and a primer we designed to target a conserved region in the phylogenetic group of the α-subclass of *Proteobacteria*, in which E-37 is located (MALF-1; Table 1). The PCR mixture contained 0.5 μg of DNA, 0.1 mM each deoxynucleoside triphosphate, 100 nM each primer, 1 U of *Taq* DNA polymerase, and $MgCl_2$ ranging from 1 to 10 mM. The final volume was 100 μl. The PCR program was identical to the one described above except that the annealing temperature was 54°C. The size of the PCR product was confirmed, and the fragment was then sequenced as described above.

Following amplification of a sequence similar, but not identical, to that of E-37 (the new sequence was designated 37SW-1), PCR was repeated with the substitution of a new primer, designated MALF-2, for MALF-1. The new primer overlapped partially with MALF-1 and was designed so that the first base at the 3′ end had a mismatch to the sequence retrieved with the ROS137-37–MALF-1 primer pair but not to the E-37 sequence. A second sequence, designated 37SW-2, was retrieved with this primer.

Nucleotide sequence accession numbers. The 16S rRNA sequences have been deposited in the GenBank nucleic acid sequence database under accession no. U58338 to U58356.

RESULTS

Enrichment. The enrichment cultures were inoculated with 1.1×10^8 cells to give an original concentration of 3.7×10^3 cells ml^{-1}. Cell numbers increased to 2.2×10^6 cells ml^{-1} at the time of the first transfer, 2.5×10^6 cells ml^{-1} at the time of the second transfer, and 4.6×10^6 cells ml^{-1} at the time of the

TABLE 1. Sequences and hybridization conditions for 16S rRNA oligonucleotide probes and primers

Probe or primer	Target	Sequence	Hybridization temp (°C)	Wash temp (°C)
PSE470-31[a]	IRE-31	5′-CTTGCAGGATTGACGTTACC	62	53
ROS137-37[a]	E-37	5′-TTCTGTTGAGGATAGCCC	58	49
PSE464-40[a]	KW-40	5′-AATACCCGCTTGCTGTGACG	65	56
Universal 1406R	All organisms	5′-ACGGGCGGTGTGTRC	55	52
MALF-1[b]	Group of α-subclass of *Proteobacteria*	5′-GCCGGGGTTTCTTTACCA		
MALF-2[c]	Group of α-subclass of *Proteobacteria*	5′-CGGGGTTTCTTTACCAGG		

[a] Probe name indicates the phylogenetic group closest to each isolate based on 16S rRNA sequences (*Pseudomonas* or *Roseobacter*), and numbers indicate the position of the first base in the 16S rRNA gene (*Escherichia coli* numbering system) which the probe was designed to target.
[b] Probe targets *E. coli* positions 488 to 505.
[c] Probe targets *E. coli* positions 486 to 503.

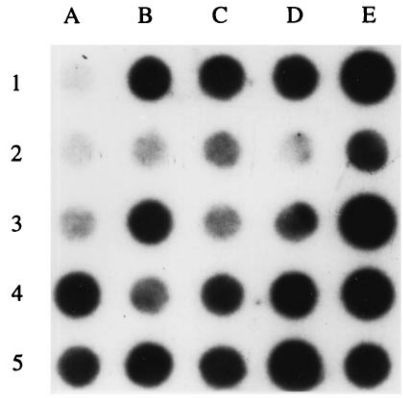

FIG. 2. Dot blot hybridizations of 25 isolates from flask B probed with community DNA from flask B. The membrane includes DNA from six isolates obtained by liquid extinction dilution (positions A1 to A5 and B1). The remaining isolates were obtained on solid media (YTSS or pulp mill waste plates), including isolates IRE-31 (D2), E-37 (E4), and KW-40 (D4). Biolog patterns and cellular and colony morphology all indicate that the isolates in positions A5 and E2 are identical, the isolates in positions D5, E1, E3, and E4 are identical, and the isolates in positions D4 and E5 are identical.

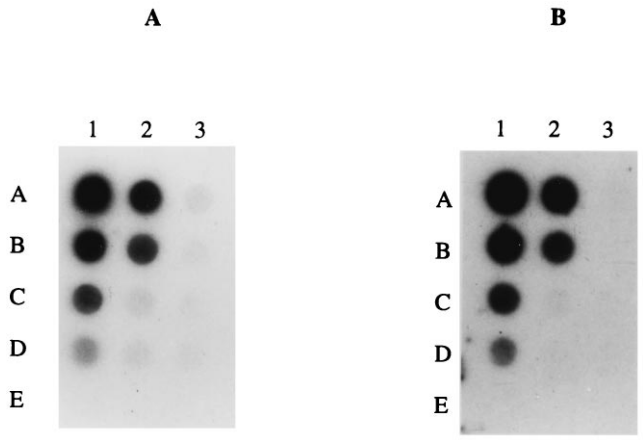

FIG. 3. Quantification of isolate IRE-31 in four communities using whole-genome hybridization (A) and oligonucleotide probe PSE470-31 (B). Column 1 contains isolate IRE-31 DNA in decreasing amounts (row A, 500 ng; row B, 200 ng; row C, 100 ng; row D, 50 ng; and row E, 0 ng). Positions 2A and 2B contain 1 μg and 500 ng, respectively, of community DNA from flask A. Positions 2C and 2D contain 1 μg and 500 ng, respectively, of community DNA from flask B. Positions 3A and 3B contain 1 μg and 500 ng, respectively, of community DNA from flask C. Positions 3C and 3D contain 1 μg and 500 ng, respectively, of DNA from the original seawater inoculum. Positions 2E and 3E contain 1 μg of salmon sperm DNA.

6-week enrichment. Cell densities were very similar among the replicates. A total of 133 isolates were obtained from the three replicate flasks: 73 were isolated on YTSS plates, 33 were isolated on pulp waste plates, and 27 were obtained from liquid extinction dilution cultures in pulp waste medium.

Numerically abundant cultures. Two whole-genome approaches were used to identify culturable bacteria making an important numerical contribution to the pulp mill waste enrichment communities. The simpler of these methods was based on colony hybridizations in which bacterial cells lifted from an agar plate onto a membrane were hybridized with enrichment community DNA as the probe. Of the 133 colonies screened by this method, 25 were found to give strong hybridization signals relative to others (data not shown), although the nonquantitative nature of this approach (the amount of DNA from each colony fixed to the membrane is unknown) makes rigorous comparisons of numerical abundance between isolates impossible.

The second whole-genome approach was based on the reverse sample genome probing method of Voordouw et al. (41, 43). DNA was extracted from 25 isolates, spotted in known quantities onto the membranes, and subjected to hybridizations using community DNA from flasks A and B as the probe (41, 43). The intensities of the hybridization signals were found to vary significantly among the isolates (Fig. 2). Results of the two whole-genome screening methods were in good agreement: isolates giving the strongest hybridization signals in the colony hybridization screen also gave the strongest signals in reverse sample genome probing.

Ten selected bacterial strains giving strong hybridization signals in both colony and reverse genome screens were subsequently used in quantitative whole-genome hybridizations, with enrichment community DNA serving as the target and isolate DNA as the probe. Three isolates were found to account for a significant fraction of one or more of the enrichment communities. Isolate IRE-31 accounted for 22% of the community DNA from flask A, although it was not detected in the DNA from the other flasks (Fig. 3A and Table 2). Isolate E-37 accounted for about one-third of the community DNA in flask A, 11% in flask B, and 4% in flask C (Table 2). Isolate KW-40 accounted for 3% of the enrichment community DNA in flask B but was not detected in the other flasks (Table 2). Of the remaining seven bacterial isolates, one was a duplicate of E-37 and one was a duplicate of KW-40. The other five isolates (A4, B1, B2, C3, and E2 in Fig. 2) gave hybridization signals indistinguishable from the background (i.e., 1% or less of the self-hybridization signal).

We used whole-genome hybridizations to estimate the relative abundance of IRE-31, E-37, and KW-40 in the seawater initially used to inoculate the pulp mill waste enrichment cultures. DNA extracted from the original seawater sample and probed with whole-genome probes made from these isolates did not produce hybridization signals above background level (Fig. 3A).

Specificity of whole-genome approaches. We investigated the extent of cross-reactivity occurring between distinct isolates when whole-genome hybridization techniques are used. DNA from 28 to 36 isolates from flask C and from ATCC bacterial species most closely related to the three isolates generally did not hybridize with the whole-genome probes made from IRE-31, E-37, or KW-40 (Fig. 4A); at most, only two of the flask C isolates hybridized with any of the probes, and even ATCC species closely related to the three probe organisms did not

TABLE 2. Percent contributions of three isolates to DNA in enrichment flasks A, B, and C[a]

Isolate	% Contribution to DNA ±1 SD					
	Flask A		Flask B		Flask C	
	Genome	16S	Genome	16S	Genome	16S
IRE-31	22 ± 0.2	23 ± 2.9	ND[b]	ND	ND	ND
E-37	33 ± 7	32 ± 3.2	11 ± 1	14 ± 2.3	4 ± 0.4	3.7 ± 1.9
KW-40	ND	ND	3 ± 0.01	2.0 ± 1.2	ND	ND

[a] The contribution of each strain was measured by whole-genome hybridizations (Genome) and by isolate-specific 16S rRNA-based oligonucleotide probes (16S) (n = 2). No contribution to the DNA in the seawater inoculum was detected.

[b] ND, not detected.

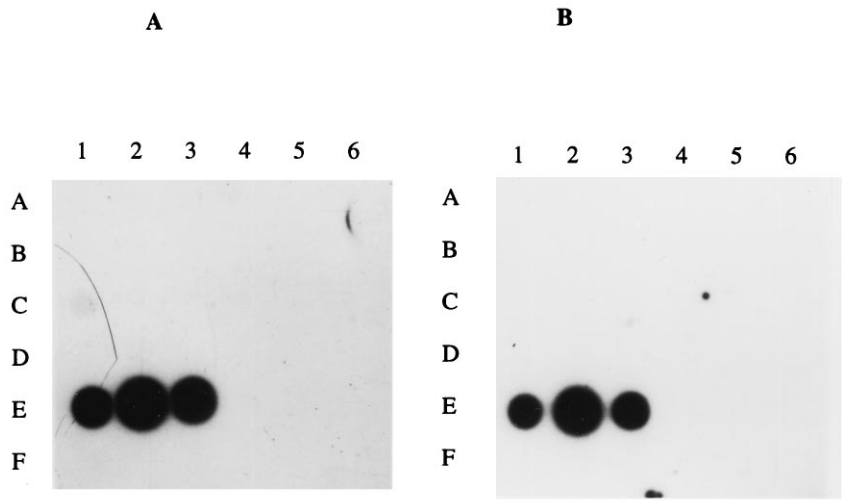

FIG. 4. Specificity of whole-genome hybridizations with isolate IRE-31 DNA (A) and with oligonucleotide probe PSE470-31 specific for IRE-31 (B) tested by hybridizing DNAs from closely related culturable organisms (A1, *R. litoralis*; B1, *R. denitrificans*; A2, *P. putida*; and B2, *P. aeruginosa*) and 28 flask C isolates. Position C1 is isolate KW-40, D1 is isolate E-37, and E1 is isolate IRE-31; position F6 is TE buffer. Similar membranes were also hybridized with DNA from E-37 and KW-40 as probes and their corresponding isolate-specific oligonucleotide probes.

produce measurable signals. For those few flask C isolates producing strong hybridization signals (for example, positions E2 and E3 in Fig. 4A), the Biolog patterns, colonial morphology, and cellular morphology of the target and probe organisms were identical, suggesting duplicate isolations of the same strain.

When whole-genome hybridization was subsequently used to investigate further whether isolates IRE-31, E-37, and KW-40 were represented multiple times in the culture collection, we found that all three isolates (originally obtained from YTSS plates) had been isolated more than once but only on solid medium. These organisms were not found among the isolates obtained by liquid dilution.

Specificity of 16S rRNA-based oligonucleotide probes. The specificity of all three isolate-specific oligonucleotide probes was checked with flask C isolates and ATCC organisms most closely related to the isolate for which each oligonucleotide probe was designed. The three probes were found to discriminate between the target organism and all nontarget organisms tested (Fig. 4B).

16S rRNA-based quantification of representative isolates. The percent contributions of isolates IRE-31, E-37, and KW-40 as determined by hybridizations with oligonucleotide probes were very similar to those obtained by whole-genome hybridization (Table 2 and Fig. 3). Isolates IRE-31 and E-37 were again found to be very important components of the community in flask A (23 and 32% of the pool of DNA coding for rRNA). Isolate KW-40 was detected only in flask B (2% of the total). Oligonucleotide probing of DNA extracted from the original seawater inoculum, in agreement with whole-genome results, also showed that all three of the isolates were present at undetectable levels in the seawater inoculum (Table 2).

Enrichment community diversity. Full 16S rRNA sequence analysis of the three dominant isolates placed E-37 in the α-subclass of *Proteobacteria* and IRE-31 and KW-40 in the γ-subclass of *Proteobacteria*. Attempts to use PCR to amplify part of the 16S rRNA gene of E-37 from a seawater sample used as the original inoculum resulted in sequences from very closely related organisms but not E-37. Sequence 37SW-1 (96% similar to that of E-37) was generated with the primer pair consisting of ROS137-37 and MALF-1. Sequence 37SW-2 (98% similar to that of E-37 and 98% similar to 37SW-1) was generated with the primer pair ROS137-37–MALF-2.

Sequence analysis of an additional 14 isolates placed 8 within the α-subclass of *Proteobacteria*, 1 within the γ-subclass of *Proteobacteria*, 3 within the *Cytophaga-Flavobacterium* group, and 2 in the gram-positive bacteria (Fig. 5). One of the isolates of the α-subclass of *Proteobacteria* obtained from pulp mill waste plates, designated EE-36, had a 16S rRNA sequence that was 96% similar to that of E-37. However, subsequent checks of cross-reactivity between these two isolates in whole-genome hybridizations gave only background signal, and the ROS137-37 oligonucleotide probe did not hybridize with EE-36. Three other isolates placed within the α-subclass of *Proteobacteria* (one from half-strength YTSS, one from pulp mill waste plates, and one from liquid dilution medium) had 16S rRNA sequences with similarities of >98% (isolates EE-34, L-85, and E-43 in Fig. 5). Subsequent checks of cross-reactivity between these three isolates in whole-genome hybridizations gave up to 60% of the self-hybridization signal.

DISCUSSION

Recent analyses of 16S rRNA sequences retrieved directly from natural samples have yielded considerable information about the composition of natural bacterial communities (3, 5, 10, 19, 21, 45). Bacteria identified via their 16S rRNA sequences have been placed within the existing phylogenetic framework for prokaryotic organisms, and, in some cases, in situ abundance of these organisms has been subsequently determined by design of oligonucleotide probes (17, 21). Yet although it provides a new perspective on the diversity of bacteria in nature, the sequence analysis approach cannot yield the actual organisms themselves; thus, physiological traits and functional roles are unknown and can, at best, only be inferred from phylogenetic affinities (2, 10). The need to culture ecologically relevant bacteria from complex natural and seminatural communities still exists.

Traditional culturing approaches have proven inadequate for retrieving representative bacteria from most natural bacterial communities, as is evident from the numerous differences between 16S rRNA sequences retrieved from natural samples

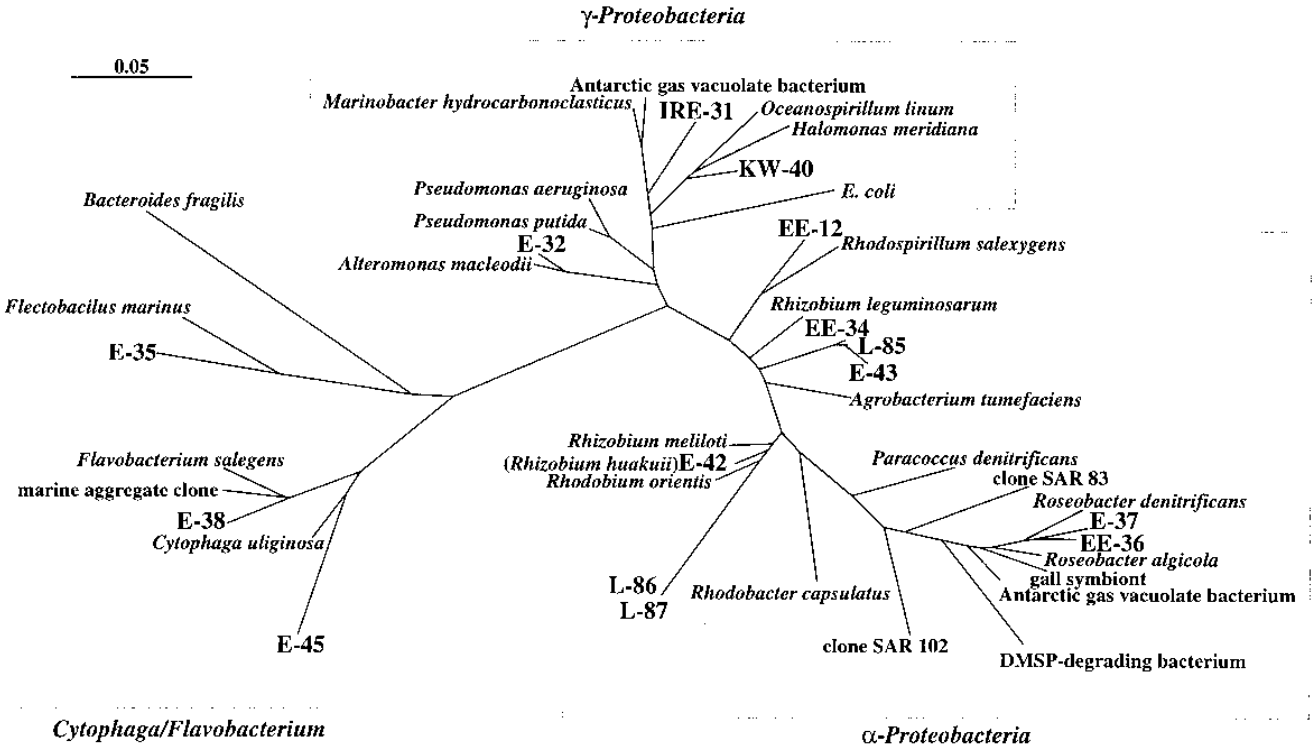

FIG. 5. Phylogenetic tree showing affiliations of isolates IRE-31, E-37, and KW-40 and 12 other isolates from flask B with the most closely related sequences (based on a 300-bp sequence generated with the primer 926F). Only sequences in the *Proteobacteria* and *Cytophaga-Flavobacteria* phyla are shown. Bar, Jukes-Cantor distance. DMSP, dimethylsulfoniopropionate.

without cultivation and those from the culture collections assembled from these same environments (32, 39, 45). In studies of marine ecosystems, most bacteria identified by 16S rRNA sequence analysis are phylogenetically distant from typical isolates such as *Vibrio*, *Alteromonas*, *Deleya*, and *Oceanospirillum* spp. (5, 9–11, 17–19, 21, 35). Furthermore, for most environmental isolates, little or nothing is known about numerical abundance within their native environment. Thus, there is a need to develop protocols for screening large and repetitive culture collections for ecologically relevant bacterial isolates.

In this study, whole-genome hybridizations were used to identify numerically important members of a lignin enrichment community by quantitative (dot blot hybridization) or nonquantitative (colony hybridization) approaches. Cross-hybridization of chromosomal DNA has long been used as a tool in bacterial systematics to assess relationships among closely related bacteria and is currently a widely accepted standard for the definition of a bacterial species (47). Adaptation of this technique for use with complex natural communities has met with skepticism, however, because of the likelihood of cross-hybridization arising from the homology between genes that are highly conserved between species (14, 24, 27). Furthermore, hybridization kinetics are likely to be complex when nucleic acid with as much sequence complexity as is found in community DNA is used. Nonetheless, the results of the whole-genome hybridizations in this study were easily interpreted and unambiguous. Background hybridizations between two phylogenetically distinct isolates or between an isolate and community DNA were very low.

When whole-genome approaches were compared with the use of specifically designed 16S rRNA probes (Table 2), the quantitative estimates were very similar, generally matching within a few percent. Use of these two molecular approaches in combination, in which whole-genome hybridization is used to screen culture collections for isolates abundant in the parent community and 16S rRNA probes are then designed for the isolates determined to be numerically important in the whole-genome screening, proved to be a fruitful approach to identifying abundant isolates. We have recently used this protocol to identify an isolate which is a numerically dominant member of a bacterial community in groundwater (23), demonstrating that it is a useful approach for screening isolates obtained directly from natural samples as well as from model microbial systems or enrichment cultures.

Phylogenetic analysis based on full 16S rRNA sequences placed E-37 among the group of bacteriochlorophyll *a*-containing bacteria in the genus *Roseobacter* of the α-subclass of *Proteobacteria* (37, 38), although E-37 does not contain bacteriochlorophyll *a* (23). Isolates IRE-31 and KW-40 belonged to the γ-subclass of *Proteobacteria*, closely related to the true pseudomonads and several groups of marine bacteria (*Marinomonas* spp., *Halomonas* spp., *Oceanospirillum* spp., and *Marinobacter hydrocarbonoclasticus*).

Most of the 14 partially sequenced isolates belonged to the α-subclass of *Proteobacteria* (Fig. 5). Only one of these isolates, E-42 (obtained from YTSS plates), gave a 100% match to a previously cultured organism, *Rhizobium huakuii*; this nitrogen-fixing organism was first described as a component of the natural bacterial flora of the leguminous plant *Astragalus sinicus* (7). Two other isolates (obtained from liquid extinction dilution) were also placed within the α-subclass of *Proteobacteria* and formed a deep branch close to *R. huakuii* (L-86 and L-87). Three other α-subclass *Proteobacteria* isolates formed a deep cluster close to *Agrobacterium tumefaciens* and *Rhizobium leguminosarum* (EE-34, L-85, and E-43), while another was found to have 93% similarity to the phototrophic bacterium

Rhodospirillum salexygens (E-12). Another (EE-36) had 96% similarity with E-37, clustering close to it and to *Roseobacter* spp. Isolate EE-36 and the several sequences closely related to E-37 retrieved via PCR from the seawater inoculum together suggest the possibility that E-37 is one of a number of phylogenetically related organisms present in coastal Georgia seawater.

An isolate that placed within the γ-subclass of *Proteobacteria* (E-32) was found to have 97% similarity with *Alteromonas macleodii*, a bacterium isolated from marine environments. Two others were found to have 99% similarity with previously cultured bacteria in the gram-positive phylum; one was close to *Staphylococcus aureus*, and the other was close to *Microbacterium laevaniformans* and *Microbacterium dextranolyticum.*

The partial sequences of the pulp mill waste isolates that were placed within the *Flavobacterium* group were generally quite distant from those of previously sequenced members of this group (Fig. 5). Two of them had similarities of approximately 91% to the closest cultured *Cytophaga* spp. or related organisms (E-35 and E-45). The third isolate (E-38) was 95% similar to a sequence obtained from an uncultured organism associated with particulate organic matter in a coastal marine environment (10).

We found that most of the lignin enrichment isolates were quite distinct from previously isolated and sequenced bacteria, and many have 16S rRNA sequences distant enough to be considered representative of new taxa. Moreover, we have yet to consider the phylogenetic diversity of those enrichment members which are nonculturable or were not amenable to culture with our chosen method. Each new environment or bacterial community subjected to 16S rRNA sequence analysis is likely to yield novel organisms for some time to come, as our knowledge of the diversity of culturable and nonculturable members of natural prokaryotic communities continues to expand.

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