APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1996, p. 766–771 Vol. 62, No. 3
0099-2240/96/$04.00+0

Diversity of Uncultured Microorganisms Associated with the Seagrass *Halophila stipulacea* Estimated by Restriction Fragment Length Polymorphism Analysis of PCR-Amplified 16S rRNA Genes

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Received 18 August 1995/Accepted 12 December 1995

The diversity of microorganisms associated with the leaves of the seagrass *Halophila stipulacea* in the northern Gulf of Elat was examined by culture-independent analysis. Microorganisms were harvested by a sonication treatment for total-community genomic DNA isolation. Oligonucleotides complementary to conserved regions in the 16S rRNA-encoding DNA (rDNA) of bacteria were used for PCR amplification. The 16S rDNA PCR products were subcloned and further characterized by a restriction fragment length analysis termed ARDRA (amplified rDNA restriction analysis). These analyses were carried out after reamplifying the cloned fragments with two primers binding symmetrically to the plasmid immediately on both sides of the cloned insert. Computer-aided clustering was performed after separate restriction analysis with enzymes *Hin*fI and *Hpa*II. By this method, 103 cloned 16S rDNA fragments were clustered into a total of 58 different groups. Sequence analysis of clones with an identical ARDRA pattern confirmed that members of an ARDRA group were closely related to each other. The sequenced clones were found to be affiliated with a marine snow-associated plastid-like rRNA clone and with a marine *Hyphomonas* strain, respectively. The method applied in this study could be useful for the routine study of other microbial communities of interest.

Although the analysis of the composition of natural microbial populations has a long tradition in microbial ecology, microbiologists have been constrained by the use of traditional methods, since the component organisms have had to be grown in the laboratory. Consequently, a collection of bacterial isolates of a habitat is unlikely to represent the in situ diversity. In contrast, culture-independent techniques that involve total-community genomic DNA or RNA extraction followed by amplification of 16S rRNA-encoding DNA (rDNA) or 16S rRNA by the PCR have revealed immense phylogenetic diversity within a naturally occurring community (3, 7, 9, 10, 17, 20, 29). 16S rRNAs are valuable phylogenetic marker molecules for microorganisms because they are universally distributed and constant in function and because different positions of their sequences change at very different rates (30). This makes the 16S rRNA gene accessible for many methods, like sequence analysis, ribotyping (11), restriction fragment length polymorphism (RFLP) analysis, and hybridization with oligonucleotide probes (reviewed in reference 1), all useful for the identification and typing of microorganisms. RFLP analysis of PCR-generated rDNA fragments was named ARDRA (amplified rDNA restriction analysis) by Vaneechoutte et al. (27). This method is fast, simple, and requires no sequence information about the amplified 16S rDNA fragments. In studies on collections of cultured type strains, it was shown that the ARDRA method enables the distinction of bacterial species (12, 27).

The present study investigated the analysis of natural microbial communities associated with an aquatic plant in a culture-independent approach. The seagrass *Halophila stipulacea* (Forsskål) Ascherson is one of the most common and ubiquitous seagrasses in the subtidal zone of the shores of the northern Gulf of Elat (18). In a preceding study, the differences in viable counts of heterotrophic bacteria attached to various vegetation parts of *H. stipulacea* were analyzed (28). Pereg et al. examined the characteristics and the contribution of nitrogen fixation in association with various vegetation parts and the sediment of *H. stipulacea* beds (22). We have focused our attention on the diversity of microorganisms associated with the leaves of *H. stipulacea* since both studies demonstrated that these vegetation parts harbor the highest density of microorganisms attached to the plant.

The intention of this study was the improvement of RFLP methods for routine analyses of large numbers of samples of PCR-amplified and cloned 16S rDNA fragments from natural microbial communities. Initially, this approach involved the isolation of microorganisms from the plant by sonication treatment followed by total-community genomic DNA extraction without culturing the participating microorganisms. PCR-generated 16S rDNA fragments of the extracted DNA were cloned and further analyzed by a modified ARDRA method, which was used to distinguish different phylotypes in the 16S rDNA library.

MATERIALS AND METHODS

Collection of microorganisms. *H. stipulacea* samples were collected from a bed situated 1.5 km south of the Marine Biology Laboratory of Elat in November 1993. This bed stretched between a depth of 6 and 13 m. An overview of the procedure of the analysis of the microbial community associated with leaves of *H. stipulacea* described below is illustrated in a flowchart in Fig. 1. The plants were washed carefully in sterile seawater, and the leaves were cut off. (Seawater was obtained from the sample site and filter sterilized.) Approximately 4 g (wet weight) of leaves was placed in a 50-ml plastic tube and covered with sterile seawater up to a volume of 50 ml. To harvest the associated microorganisms, the tube was treated five times for 12 s in a sonication bath (Cole-Palmer, model 8851). The supernatant was prefiltered through three layers of cheesecloth. Microorganisms were collected onto a 47-mm-diameter, 0.2-μm-pore-size nitrocellulose membrane (Schleicher & Schuell) with a vacuum pump. Microorganisms were washed off the membrane by sonication treatment (see above) and suspended in 3.5 ml of sterile seawater. Of this suspension, 1.5 ml was centri-

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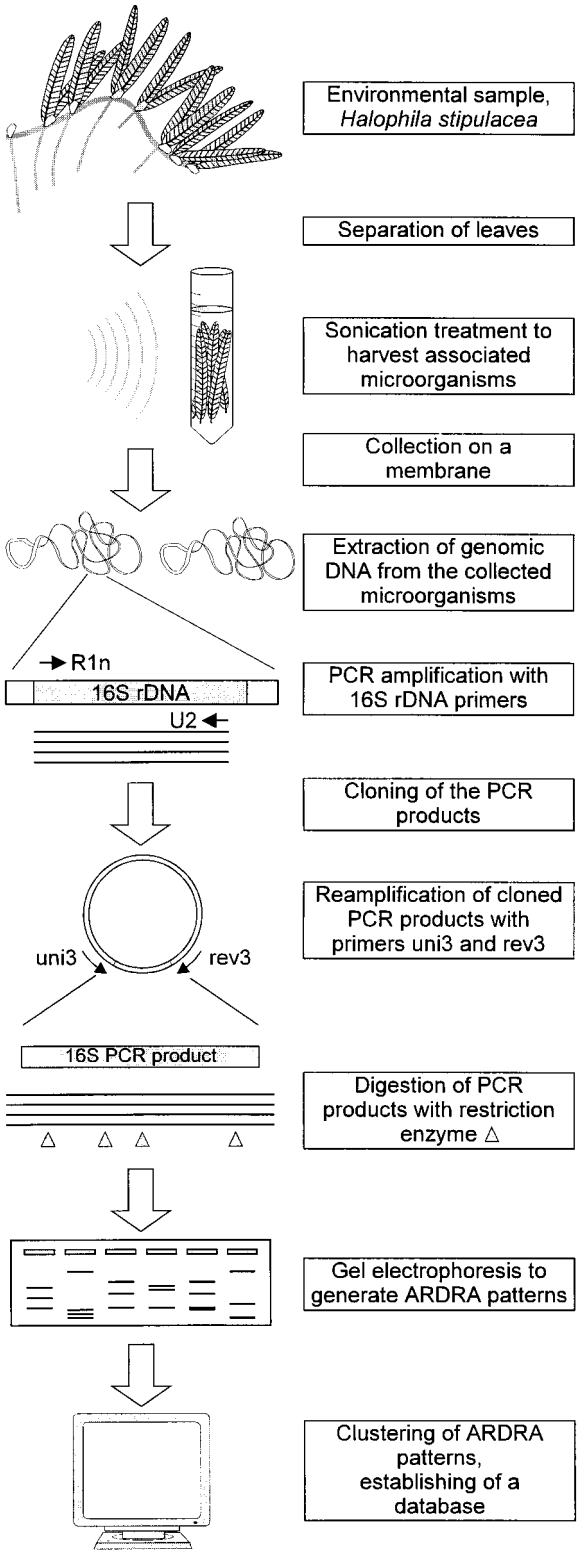

FIG. 1. Flowchart of the procedure of the analysis of microbial communities associated with leaves of the seagrass *H. stipulacea*. Details of the methods used are described in the text.

TABLE 1. Oligonucleotides corresponding to conserved regions of the 16S rRNA gene used for hybridization and sequencing

Oligonucleotide	Sequence[a]	Positions in *E. coli* 16S rRNA	Reference
R1n	GCTCAGATTGAACGCTGGCG	22–41	—[b]
U2	ACATTTCACAACACGAGCTG	1085–1066	—
358f	AGACTCCTACGGGAGGCAGCAGT	336–358	14[c]
536f	GTGCCAGCMGCCGCGGTAATWC	515–536	15[c]
928f	TAAAACTYAAAKGAATTGACGGGG	905–928	15[c]
336r	ACTGCTGCSYCCCGTAGGAGTCT	358–336	14[c]
515r	GWATTACCGCGGCKGCTGGCAC	536–515	15[c]
907r	GCCCCCGTCAATTCMTTTRAGTTT	930–907	15[c]

[a] K = G or T; M = A or C; R = A or G; S = G or C; W = A or T; Y = C or T.

[b] —, V. Tichy (Freiburg, Germany).

[c] Sequence elongated for the purpose of more efficient annealing of fluorescent primers for sequencing.

fuged at 8,000 × *g* for 6 min. The pellet was suspended in 1 ml of 1 M NaCl and used immediately for the extraction of genomic DNA to avoid a shifting of the natural composition of the collected microorganisms as described for marine bacterioplankton during confinement (8).

Genomic DNA extraction. The microorganism suspension was placed on ice for 20 min. After spinning down, the pellet was suspended in 250 μl of sucrose lysis buffer (20% [wt/vol] sucrose, 1 mM EDTA, 10 mM Tris-HCl; pH 8.0). For enzymatic cellular lysis, 250 μl of lysozyme-RNase solution (5 mg of lysozyme per ml, 1 mg of RNase A per ml) was added before incubation at 37°C for 60 min. After adding 100 μl of proteinase buffer (5% [vol/vol] *N*-laurylsarcosine, 5 mg of pronase E per ml, 1 mM EDTA, 10 mM Tris-HCl; pH 8.0), the preparation was incubated at 37°C for an additional 60 min. Proteins were removed by adding 70 μl of 3 M Na acetate and 200 μl of phenol and then shaking cautiously. After centrifugation at 13,000 × *g* for 10 min, the aqueous phase was extracted with phenol-chloroform (1:1) twice and once with chloroform to remove the residual phenol. The genomic DNA was allowed to precipitate at −20°C for 8 h after the addition of 700 μl of ice-cold isopropanol and was collected by centrifugation at 13,000 × *g* for 20 min. The genomic DNA pellet was washed twice by adding 500 μl of 80% ethanol, incubated for 15 min at room temperature, and centrifuged again at 13,000 × *g* for 10 min. Finally, the pellet was suspended in 30 μl of Tris-EDTA buffer (1 mM EDTA, 10 mM Tris-HCl; pH 8.0). The concentration of the DNA was 50 ng/μl as measured by A_{260}, with a yield of 872 ng/g (wet weight) of *H. stipulacea* leaves.

Oligonucleotide synthesis and purification. The oligonucleotides used in this study were synthesized on a Gene Assembler Special (Pharmacia LKB Biotechnology AB [Pharmacia Biotech], Uppsala, Sweden). Oligonucleotides were purified by use of NAP-10 columns (Pharmacia Biotech) as described by the manufacturer.

Amplification of 16S rRNA genes. The amplification of partial 16S rDNA was carried out selectively from genomic DNA by PCR (23) with oligonucleotide primers annealing to conserved regions of bacterial 16S rRNA genes (26a). The forward primer R1n corresponded to positions 22 to 41 of *Escherichia coli* 16S rRNA (4), whereas the reverse primer U2 corresponded to the complement of positions 1085 to 1066 (the sequences of both primers are shown in Table 1). The conditions for PCR amplification were as follows: 10 ng of genomic DNA, 10 μl of 10× reaction buffer, 2 U of *Taq* DNA polymerase, 200 μM each of the four deoxynucleotides (all components from Pharmacia Biotech), and 200 nM each primer were combined in a total volume of 100 μl. As negative controls, reactions without DNA were carried out. The reaction mixture was overlaid with mineral oil (Perkin-Elmer Cetus, Norwalk, Conn.) and incubated in a thermal cycler (model 480; Perkin-Elmer Cetus). The cycling program was as follows: initial denaturation at 94°C for 120 s; 35 rounds of 94°C for 80 s, 54°C for 60 s, and 72°C for 90 s; a final extension step at 72°C for 180 s. Amplified PCR products were analyzed by Tris-acetate-EDTA (TAE)-agarose gel electrophoresis (24). Negative controls showed no amplification. For purification, the amplified DNA was extracted with phenol-chloroform and precipitated at −70°C for 20 min after the addition of 300 μl of 98% ethanol and 4 μl of 4 M LiCl to the aqueous phase. The DNA was centrifuged at 13,000 × *g* for 15 min. Finally, the DNA pellet was suspended in 10 μl of water.

Cloning of 16S rDNA PCR products. The 10 μl of purified PCR product was made blunt-ended with Klenow polymerase, and the 5′ end was phosphorylated with T4 polynucleotide kinase (Pharmacia Biotech) by standard methods. The purified DNA was used in a blunt-end ligation reaction with a *Sma*I-digested, dephosphorylated pUC18 vector (pUC18 *Sma*I bacterial alkaline phosphatase; Pharmacia Biotech). Competent *E. coli* XL1-Blue cells (5) were transformed by the method of Morrison (19). Clones were screened for α-complementation with

X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as a substrate (24) on Penassay broth (PA) agar plates supplemented with ampicillin (150 μg/ml).

Reamplification of the cloned 16S rDNA PCR products. Crude lysates of clones of the library were obtained by suspending the amount of a small colony grown on a PA agar plate supplemented with ampicillin (150 μg/ml) in 50 μl of lysis buffer (0.05 M NaOH, 0.25% sodium dodecyl sulfate) and heated at 95°C for 15 min. The lysates were diluted 1:10 with water, and cell debris was removed by centrifugation at 8,000 × *g* for 5 min. Cloned PCR fragments were reamplified by PCR with primers uni3 and rev3 binding symmetrically on both sides of the *Sma*I site in the pUC18 vector (primer uni3, 5′-GTCGACTGTAGAGGATC CCC-3′; primer rev3, 5′-CGAATTCGAGCTCGGTACCC-3′). The oligonucleotide uni3 was mismatched in one position compared with the sequence of pUC18 to eliminate one *Hin*fI site in the primer part of the resulting PCR product. The conditions for PCR amplification were as follows: 1 μl of the diluted lysate, 2.5 μl of 10× reaction buffer, 0.5 U of *Taq* DNA polymerase, 200 μM each of the four deoxynucleotides (all components from Pharmacia Biotech), and 200 nM each primer were combined in a total volume of 25 μl and incubated in a thermal cycler (model PTC-100; MJ Research, Inc., Watertown, Mass.). The cycling program was as follows: initial denaturation at 94°C for 120 s; 35 rounds of 94°C for 40 s, 54°C for 60 s, 72°C for 60 s; and a final extension step at 72°C for 180 s. Amplified DNA was controlled by TAE-agarose gel electrophoresis (24).

Hybridization of the cloned 16S rDNA fragments. The clones of the library were further examined by hybridization experiments to verify their authenticity as 16S rDNA clones. The reamplified cloned PCR products were hybridized in three dot blots with the three oligonucleotides 358f, 536f, and 928f (Table 1) corresponding to conserved regions of the 16S rRNA gene. One hundred picomoles of each oligonucleotide was labeled with the digoxigenin oligonucleotide 3′-end labeling kit (Boehringer GmbH, Mannheim, Germany). One microliter of each cloned and reamplified PCR product was spotted on three nylon membranes (Hybond-N; Amersham), denaturated with 0.5 M NaOH–1.5 M NaCl, and renaturated with 1.5 M NaCl–1 M Tris-HCl (pH 7.5). Finally, the membranes were soaked with 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), dried, and UV cross-linked for 5 min. Prehybridization was carried out at 68°C, and hybridization was done at 40°C as described in the manufacturer's protocol (digoxigenin DNA detection kit; Boehringer).

16S rDNA ARDRA. Reamplified cloned 16S rDNA PCR products were digested separately with the restriction enzymes *Hin*fI and *Hpa*II (Pharmacia Biotech), both recognizing tetranucleotide sequences. Separation of the digested PCR fragments was performed by polyacrylamide gel electrophoresis (PAGE) with the Multiphor II system (Pharmacia Biotech). Polyacrylamide gels (6% polyacrylamide [19:1], 6.5 M urea, 2× Tris-borate-EDTA) of 0.5-mm thickness were poured between two glass plates on a carrier film (GelBond-PAG-Film; Pharmacia Biotech). Electrophoresis was carried out for 30 min at 500 V at 12°C, followed by silver staining as described by Bassam et al. (2). Stained gels were scanned and analyzed with the Wincam version 2.1 and Fingerprint Analyzer version 2.0 software (Cybertech, Berlin, Germany). The similarity between individual 16S rDNA ARDRA patterns was estimated for both of the restriction enzymes used by applying the Sørensen-Dice coefficient (13). The similarity matrices of all 16S rDNA fragments for both enzymes were combined by calculating the average between both similarity values for all pairings in the matrices. Cluster analyses were performed by the unweighted pair group method with arithmetic means (25). A maximum band position error of 3% and a similarity level of 96% were used to group individual clones.

Sequencing of cloned 16S rDNA PCR fragments. Plasmid DNA for sequencing was isolated with the Qiagen plasmid kit (Diagen GmbH, Hilden, Germany). Sequencing of cloned 16S rDNA PCR fragments was performed with an automated laser fluorescent DNA sequencer (Pharmacia Biotech) as described by the manufacturer. Sequencing reactions were carried out with the AutoRead sequencing kit (Pharmacia Biotech) as described by Zimmermann et al. (31) with the fluorescent labeled primers 358f, 536f, 928f, 336f, 515r, and 907r (Table 1) corresponding to conserved regions of the 16S rRNA gene and with primers M13forward and M13reverse corresponding to regions in plasmid pUC18.

Analysis of sequence data. The 16S rDNA sequences were aligned with sequences from the RDP database (16). The SUGGEST-TREE tool of the RDP database was used to search the RDP database for close evolutionary relatives.

Nucleotide sequence accession number. The GenBank accession numbers for the sequences discussed in this report are U41088 to U41091.

RESULTS AND DISCUSSION

Collection of microorganisms and preparation of an rDNA library. After collecting *H. stipulacea* plants, we harvested the microorganisms associated with the leaves by sonication treatment. To investigate whether this treatment was harmful to the bacteria, e.g., by lysing them, a part of a liquid culture of *E. coli* was treated in the same way as a control. The cell titers of the treated and the nontreated parts of the culture were the same, indicating that this method was sufficiently gentle so that we would not expect it to lyse cells in the sample (data not shown). By this method, bacteria and possibly other attached epiphytic or faunal communities were isolated.

After extracting genomic DNA from the collected microorganisms and PCR amplification with 16S rRNA primers, we found the blunt-end cloning strategy most suitable to clone the PCR products, because of its independence from possible 3′-end overhangs in the PCR products (6). Additionally, there was no risk of losing clones because of internal restriction sites as reported in studies using PCR primers with restriction site-containing linkers for cloning (9).

Hybridization of the cloned 16S rDNA fragments. Clones of the PCR-generated library were reamplified by PCR with primers uni3 and rev3 complementary to sequences of the plasmid vector. The use of these primers for reamplification instead of the 16S rDNA primers allowed PCR amplification with crude lysates, which can be performed in large numbers in a short time. In previously described methods, cloned PCR products were cut from the prepared plasmid vector and excised from agarose gels (20), or insert PCR products were reamplified with rDNA primers from small-scale plasmid preparations (7). In the case of the reamplification with rDNA primers, background bands resulting from contaminating *E. coli* DNA in the plasmid preparation were reported (7).

A total of 143 β-galactosidase-negative recombinant clones were reamplified. One hundred three PCR fragments were between 1,040 and 1,120 bp in size (72% of the screened clones). Hybridization with three oligonucleotides corresponding to conserved regions of the 16S rRNA gene was positive for all 103 PCR fragments, indicating that all clones contained intact 16S rDNA inserts. Hybridization against smaller and larger fragments was negative except for one smaller fragment, 760 bp in size, which showed hybridization to primers 536f and 928f but no signal in the case of primer 358f. Sequence analysis of this clone revealed that the 16S rDNA fragment started at bp 362 (according to *E. coli* 16S rRNA gene). This might indicate that during the cloning reaction a part of the fragment including the complementary sequence to primer 358f was deleted. All of the following analyses were performed with the 103 intact 16S rDNA clones.

16S rDNA ARDRA. The diversity of recovered rDNA PCR fragments in our leaf library was examined by comparative ARDRA. Primers uni3 and rev3 for the reamplification of the cloned fragments corresponded to regions in the plasmid vector immediately adjacent to the cloning site and showed no recognition sequence for the restriction enzymes *Hin*fI, *Hpa*II, *Hha*I, *Dde*I, and *Hae*III, all recognizing tetranucleotide sequences. Therefore, both orientations of a cloned fragment resulted in identical ARDRA patterns for these enzymes. If necessary, the sequence of the primers can be adapted easily to other enzymes that also can be used for the ARDRA method.

Initially, all 103 reamplified 16S rDNA fragments were digested with the endonuclease *Hin*fI and separated by PAGE. The use of PAGE and silver staining allowed resolution even of short fragments of 50 bp, produced in some clones by the restriction enzymes used. ARDRA with *Hin*fI digestion resulted in 39 different pattern types (Fig. 2). An ARDRA with *Hpa*II of some groups containing more than one member demonstrated that the *Hin*fI groups can be subdivided further. For example, *Hin*fI group 1 consisting of 20 members could be divided into six *Hpa*II subgroups (Fig. 3). To obtain a comprehensive collection of *Hpa*II ARDRA patterns, all of the clones of the library were digested with *Hpa*II. Clustering of the library was performed after combining the similarity results of both ARDRAs. The initially determined 39 *Hin*fI patterns

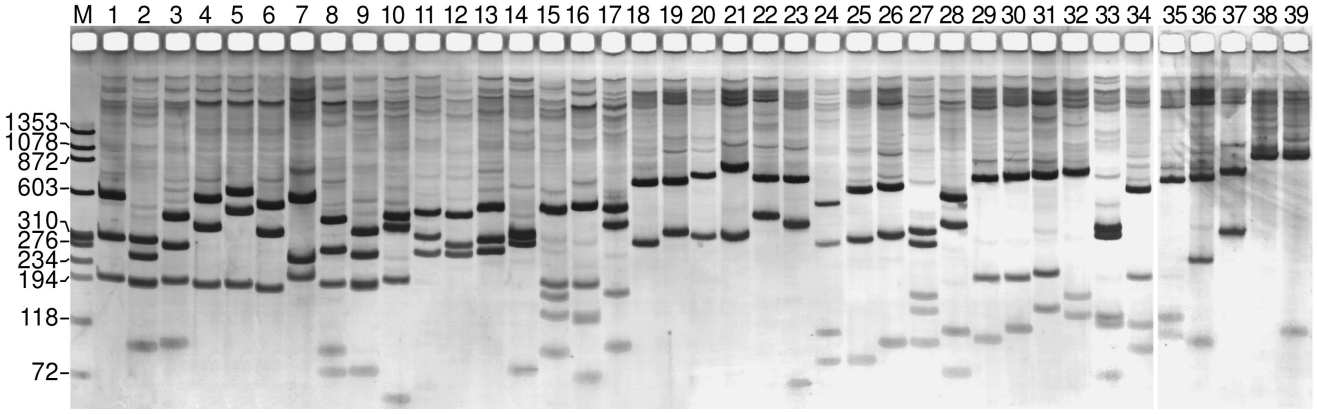

FIG. 2. ARDRA patterns of 16S rDNAs from uncultured microorganisms associated with leaves of the seagrass *H. stipulacea*. The patterns were obtained from a PCR-generated 16S rDNA library after ARDRA with the restriction enzyme *Hin*fI. A total of 103 cloned fragments were separated into 39 different groups of identical or very similar *Hin*fI ARDRA patterns. Lanes: M, size marker ϕX174RF DNA digested with *Hae*III; 1 to 39, *Hin*fI ARDRA patterns of groups 1 to 39, respectively. The numbers on the left indicate the molecular sizes in base pairs.

were expanded to 58 patterns after *Hpa*II digests were performed (Fig. 4).

The distribution of all of the clones of the leaf library among these groups was determined. Group 1 included 13.6% (14 members) and group 3 included 6.8% (7 members) of all 16S rDNA clones. A total of 36 groups (35% of the clones in the library) were represented by only a single 16S rDNA clone.

Obviously, the analysis of separate digestions with two restriction enzymes instead of a double digest gives more precise results. Small bands, which often occur in a double digest, are difficult to detect in the gel, and in a double digest, it is not possible to discriminate which band results from a specific enzyme. Because both enzymes recognize a tetranucleotide sequence, they statistically cut a given fragment with similar frequencies. Therefore, the combination of the similarity values from both ARDRAs in one matrix was performed with a 50% weight for each enzyme.

Visual analysis of the generated ARDRA patterns is time-consuming and impossible with increasing pattern numbers. With the aid of appropriate software, the grouping of similar ARDRA patterns can be automated, and the similarity of the patterns can be computed. The programs used also allow the generation of a growing database of ARDRA patterns. This is essential for further comparative analysis of clones generated from other natural communities.

16S rDNA sequence analysis. Other studies have illustrated that the ARDRA method resolves species differences in the sequence of amplified 16S rRNA genes (12, 27). In these studies, the ARDRA method was used to distinguish cultured and characterized type strains. In our work, the grouping of the cloned 16S rDNA fragments could not be proved by other characteristics of the microorganisms from which they originated. Therefore, sequence analyses of some clones of the library were carried out to prove the grouping of the clones by ARDRA. Two 16S rDNA sequences of groups 1 and 3 (Fig. 4) were determined completely. The two clones HstpL1 and HstpL4 of group 1 showed a 94% DNA sequence homology and most closely resembled the same sequence entry in the database. The two sequences HstpL3 and HstpL6 of group 3 displayed 99% DNA sequence homology and were also both most closely related to an identical database sequence.

Because of the difficulties in translating rRNA similarity values into nomenclatural divergence, it was suggested that similarity values below 95% to known sequences be regarded as good evidence of the discovery of novel species (1). Thus, a similarity of about 99% between the mentioned sequences of group 3 clearly signifies that both belong to the same species. Additionally, the similarity value of about 94% between the two sequenced clones of group 1 indicates that both were closely related, but whether both sequences originated from organisms of the same species cannot be determined.

The accuracy of distinction between different 16S rDNA fragments by ARDRA depends on the number of restriction enzymes used. This relationship was discussed in detail by Nei and Li (21). If close relationships between 16S rDNAs are to be analyzed, the use of two restriction enzymes is not sufficient. But, obviously, the type of restriction enzyme used also influences the accuracy of differentiation. As can be deduced from their DNA sequences, clones HstpL1 and HstpL4 of group 1 would not be differentiated by an additional ARDRA with *Hha*I, *Dde*I, or *Rsa*I but would be distinguished by *Hae*II or *Taq*I.

Phylogenetic relationships of group 1 and group 3. Database comparisons indicated that group 3 was affiliated with the *Hyphomonas* group of alpha *Proteobacteria*. The most similar database entry was *Hyphomonas* sp. strain MHS3 (26), with a

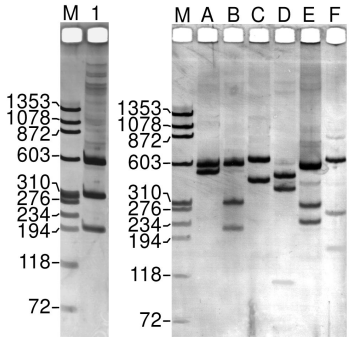

FIG. 3. Subdivision of a *Hin*fI group (group 1 in Fig. 2) consisting of 20 members by ARDRA with the restriction enzyme *Hpa*II. After digestion with *Hpa*II, the group could be subdivided further into six different groups (A to F). Lanes: M, size marker ϕX174RF DNA digested with *Hae*III; 1, *Hin*fI pattern of group 1 from Fig. 2; A to F, *Hpa*II ARDRA patterns of all six subgroups of this *Hin*fI group. Patterns A, B, C, D, E, and F correspond to groups 1, 18, 32, 50, 55, and 28, respectively. The numbers on the left indicate molecular sizes in base pairs.

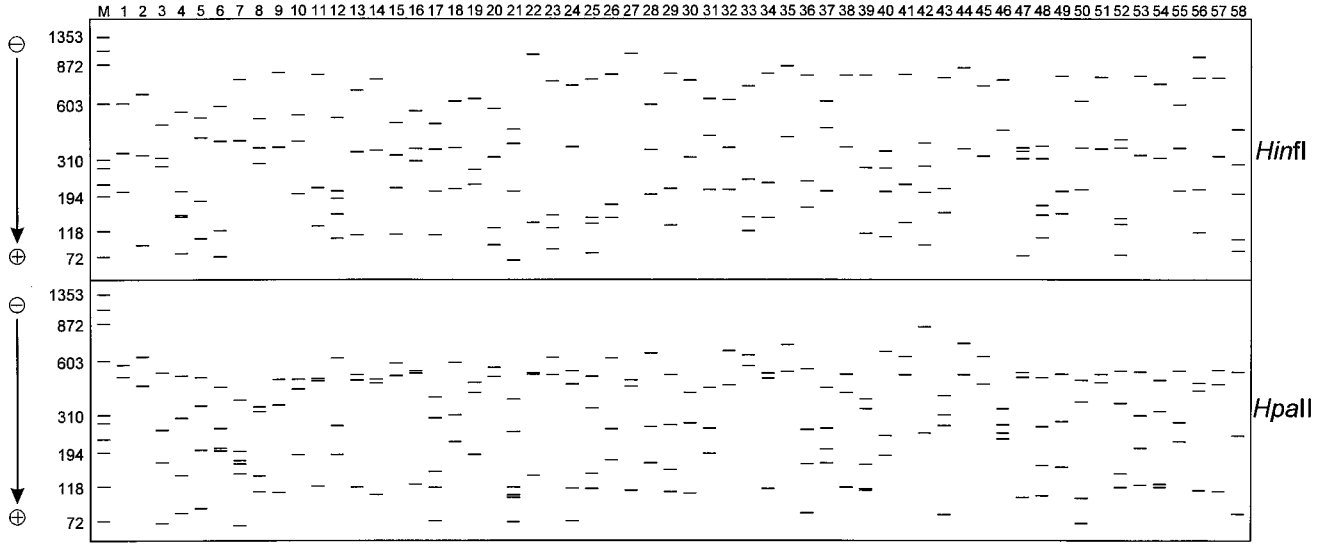

FIG. 4. Schematic presentation of all 58 ARDRA patterns of 103 cloned 16S rDNA PCR fragments from uncultured microorganisms associated with leaves of the seagrass *H. stipulacea*. ARDRA patterns after separate digestion with *Hin*fI (upper panel) and *Hpa*II (lower panel) are shown. The direction of electrophoresis is indicated by an arrow. Lane designations indicate the ARDRA cluster number in the order of initial detection. Lane M, size marker ϕX174RF DNA digested with *Hae*III (molecular sizes in base pairs are indicated by the numbers on the left).

DNA sequence homology of 94%. These budding and/or prosthecate nonphototrophic bacteria adhere to surfaces and are found in diverse locations, like oligotrophic environments and bacterial biofilm communities (26).

Group 1 was affiliated with the oxygenic phototroph lineage including cyanobacteria, chloroplasts, and cyanelles. The sequences HstpL1 and HstpL4 most closely resembled rRNA from the chloroplast of eukaryotic algae. In detail, they showed a 94 and a 95% homology, respectively, to the marine snow-associated clone agg56, which was also suggested to originate from rRNAs of plastids (7). Clone agg56 was recovered in a culture-independent, PCR-based study focusing on the phylogenetic diversity of aggregate-attached versus free-living marine bacteria. The authors suggested that these rDNA sequences originated from intact plastids contained in phytodetrital material (7). In the case of *H. stipulacea*, plastid-like 16S rDNA sequences possibly originated from eukaryotic algae attached to the surface of the plant. These algae were coisolated by the applied sonication method for the collection of associated microbial populations from the surface of the leaves.

Potential biases in the study. The methods described in this report bear potential biases, like any sampling and analytical scheme does. Sample collection, cell lysis, DNA extraction, PCR amplification, and the cloning process are potential sources of bias. Our points of interest were microbial communities associated with the plant, but the applied sampling and PCR amplification obviously also included algae. The amount of 16S rDNA sequences originating from sources other than associated microbial communities on the surface of the plant remains to be detected.

Conclusions. In this report, we described the improvement of the ARDRA method for the routine analysis of natural microbial communities without cultivation. Grouping of clones of 16S rDNA libraries by the reported methods is a useful tool to reduce the number of clones that have to be analyzed further to determine their phylogenetic affiliation and to examine the diversity of natural microbial communities. These methods will be useful to study temporal changes or other parameters in microbial communities and to examine other parts of the *H. stipulacea* plant. We believe that these methods are applicable to many natural habitats and allow the routine evaluation of large numbers of cloned 16S rDNA fragments.

ACKNOWLEDGMENTS

We thank M. Fahima and N. Sar from the Department of Food Engineering and Biotechnology, Technion Institute, Haifa, Israel, for helpful assistance and technical expertise. We also gratefully acknowledge B. Bayer for helpful technical assistance. We thank V. Tichy from TÜV Energie und Umwelt GmbH, Freiburg, Germany, for kindly providing 16S rDNA primers R1n and U2.

This research was supported by grants from the Bundesministerium für Forschung und Technologie (03F0056C). The project was performed in the framework of the German-Israeli Foundation for Scientific Research and Development (G.I.F.).

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