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Separation of Bacterial Cells by Isoelectric Focusing, a New Method for Analysis of Complex Microbial Communities

ELKE JASPERS AND JÖRG OVERMANN*

Institut für Chemie und Biologie des Meeres, Universität Oldenburg, D-26111 Oldenburg, Germany

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A simple isoelectric focusing (IEF) method for whole bacterial cells was developed. In a pH gradient of 2 to 10 and an electric field of 11.5 V cm^{-1}, mixtures of cells from the three different bacterial strains *Chlorobium limicola* 6230, *Pseudomonas stutzeri* DSM 50227, and *Micrococcus luteus* DSM 20030 could be separated. A density gradient of Ficoll prevented convective currents in the system. The method was tested with a concentrated mixture of bacteria from a shallow eutrophic lake and yielded up to 10 different bands. Species composition in each IEF band was analyzed by PCR plus denaturing gradient gel electrophoresis (DGGE). Each IEF band exhibited a different species composition. After the separation of cells by IEF three times more 16S ribosomal DNA signals could be detected by DGGE than in the unfractionated natural bacterial community. It is concluded that the resolution of these molecular biological methods is significantly enhanced if cells are first separated by IEF. At the same time, the IEF fractions are enriched for certain species, which can be used in subsequent cultivation experiments.

Many natural bacterial communities are extremely diverse. In soil, the bacterial community can consist of 4,000 completely different genomes (18). In a marine sediment, the number of different genomes even exceeded 10,000 (19). These estimates are based on the reassociation kinetics of isolated chromosomal DNA as an indirect measure for genetic diversity.

Currently, only a fraction of the microbial diversity can be investigated by direct methods. A minute fraction of the bacterial cells present in a natural environment grow in laboratory media. Culturability ranges from 0.001 to 3% for oxic aquatic habitats (1); similar values were reported for anoxic waters (0.13 to 0.2%) (17) and for sediments and soils (0.25 to 0.5%) (1, 18). In order to identify a larger fraction of the bacteria in the environment, molecular biological methods have been introduced into ecology. Such culture-independent methods mostly target the 16S ribosomal DNA (rDNA) molecule, separating base sequences of different organisms either by an initial cloning step (20) or according to their different melting behavior in denaturing gradient gel electrophoresis (DGGE) (8).

Application of molecular biological methods has led to the discovery of novel 16S rDNA sequences (20), but the resolution of these methods still appears insufficient with respect to the high bacterial diversity in nature. If DGGE is used to separate different 16S rDNA sequences directly after PCR, usually fewer than 15 DNA bands (corresponding to approximately the same number of different sequences) can be distinguished (8, 17). If 16S rDNA sequences are separated by cloning instead of DGGE, the degree of resolution can be much higher (4). Drawbacks of the cloning approach are cloning biases and a rather tedious screening procedure (15). However, a major shortcoming of both molecular biological methods is that the bacteria corresponding to a certain 16S rDNA fingerprint are not available for further studies. Otherwise, the phylogenetic information could be employed directly to improve cultivation of these bacteria.

* Corresponding author. Mailing address: Institut für Chemie und Biologie des Meeres, Universität Oldenburg, Postfach 2503, D-26111 Oldenburg, Germany. Phone: 49-441-970-6376. Fax: 49-441-798-3583. E-mail: j.overmann@palmikro.icbm.uni-oldenburg.de.

Electrophoretic mobility has been used in the past to isolate unialgal fractions from mixed phytoplankton suspensions (2) or to purify cyanobacterial cultures (11). Compared to conventional electrophoresis, isoelectric focusing (IEF) has a much higher resolving power. To our knowledge, this latter technique has been applied in only one case to separate bacterial cells of laboratory cultures (7).

In the present study, we combined the fractionation of whole bacterial cells by IEF with a molecular biological analysis of the resulting fractions by DGGE. With this new approach, the resolution of DGGE is enhanced considerably while at the same time providing living cells for cultivation experiments.

MATERIALS AND METHODS

Cultivation of bacterial strains. *Pseudomonas stutzeri* DSM 50227 and *Micrococcus luteus* DSM 20030 were grown in modified YT medium (10 g of Bacto Tryptone, 5 g of yeast extract, 5 g of NaCl [pH 7.0]) at 37°C for 12 h. *Chlorobium limicola* 6230 was grown in the basal medium described by Overmann and Pfennig (10) at 22°C with illumination at 100 microeinsteins m^{-2} s^{-1} from a tungsten lamp.

Preparation of bacterial cells. Pure cultures were harvested by centrifugation (10 min, 7,700 × *g* at 4°C).

Water samples for the investigation of a natural bacterial community were obtained on 15 February and 15 May 1996 in the southeastern bight of the Zwischenahner Meer, a shallow (mean depth, 3 m) eutrophic lake. In February, the lake was covered by 30 cm of solid clear ice. Forty liters of water was obtained from a depth of 0.5 m and filtered through a 20-μm-diameter plankton net. Afterwards, bacterial cells were concentrated over a 0.2-μm-mesh-size filter in a tangential flow system (Filtron, Karlstein, Germany). The resulting concentrate (440 ml) was filtered through an 8-μm-pore-size filter (cellulose nitrate; Sartorius, Göttingen, Germany) to remove larger filamentous cyanobacteria, which interfered with the subsequent IEF. In a final concentration step, the bacterial suspension was centrifuged for 15 min at 48,400 × *g* at 5°C. Bacterial pellets were resuspended in 1 ml of sterile Ficoll (density, 1.075 g $liter^{-1}$; Pharmacia Biotech, Freiburg, Germany) and either used immediately or stored in liquid nitrogen.

In order to investigate the influence of divalent cations on the IEF of bacterial cells, pellets were incubated for 17 min in 40 ml of either 5 mM $CaCl_2$ or 0.1% sodium polyphosphate (Aldrich, Dorset, England). Afterwards, cells were harvested by centrifugation (10 min at 7,700 × *g* for pure cultures, 15 min at 48,400 × *g* for natural samples) and used immediately.

IEF of bacterial cells. Based on the small-scale apparatus described by Godson (5), a simple device for IEF was constructed (Fig. 1). The 13-mm (inner diameter) glass tube is surrounded by a cooling jacket. Near the bottom, the central glass tube is connected to a smaller side arm. The whole device can be autoclaved.

For the preparation of the IEF column, the bottom opening was closed with a rubber stopper and the tube was filled with 18 ml of heavy anode solution (1%

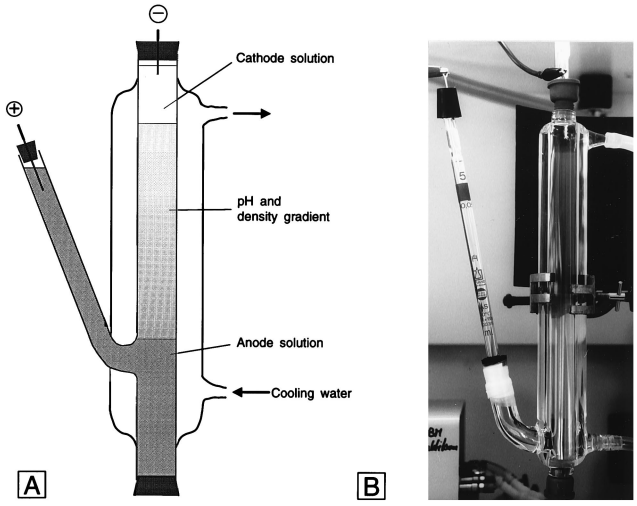

FIG. 1. Schematic (A) and photograph (B) of apparatus for IEF of whole bacterial cells. The cathode (⊖) and anode (⊕) consist of platinum wires which are connected to a power supply.

H_3PO_4 in Ficoll with a density of 1.100 g liter^{-1} [pH 1.0]). Separation of bacterial cells took place in the central 25-ml portion of the column. This section contained 0.1% of the carrier ampholytes (Serva, Heidelberg, Germany), which, during electrophoresis, generated a pH gradient with pH values decreasing towards the bottom of the tube. To prevent convective currents during electrophoresis, the separation medium also contained a density gradient of Ficoll, which, in contrast to Percoll, is compatible with the extreme pH values of the cathode and anode solutions. The gradient was poured with a model 385 gradient former (Bio-Rad Laboratories, Munich, Germany) using 12.5 ml each of a light (density, 1.020 g liter^{-1}; containing 0.1% ampholyte) and a heavy (density, 1.100 g liter^{-1}; containing 0.1% ampholyte) Ficoll solution. During the filling of the central tube of the device, the separation medium gradually forced the anode solution into the side arm.

In some of the experiments, Tween 20 was added to the separation medium to final concentrations of 0.0015 to 0.005%. In some cases, bacterial cells were suspended in the light solution before the separation medium was poured.

Finally, the separation medium was overlaid with 2 ml of a cathode solution consisting of 250 mM NaOH (pH 12). Rubber stoppers holding 5-cm-long and 1-mm-thick platinum wires as electrodes were inserted into the anode and cathode solution.

IEF proceeded at a constant voltage of 300 V from a Bio-Rad Power Pac 3000. Current was limited to 4 mA, power was limited to 2 W, and the temperature was kept constant at 4°C. The formation of the pH gradient proceeded during the initial 1.5 h of the run, accompanied by a drop in current from 4 to 1 mA.

Typically, separation of bacterial cells lasted 10 to 11 h. Afterwards, a syringe needle with a Tygon tube attached to it was inserted through the bottom rubber stopper until the needle tip reached the separation part of the column, and the IEF column was fractionated into aliquots of 1 ml with a peristaltic pump. The aliquots were used for subsequent molecular biological analysis and for determination of the pH gradient. Samples were stored at −80°C.

Plateability and efficiency of separation. Cells of *P. stutzeri* and *M. luteus* were subjected to IEF. The fraction of plateable cells was determined for cell suspensions before the treatment and for the bacterial bands harvested from the IEF column after separation. Plate counts were performed on modified YT medium (containing 1.4% agar), and total cell numbers were determined by epifluorescence microscopy with 4′,6-diamidino-2-phenylindole (DAPI) (12). The plateability was calculated as the ratio of colony counts to total cell number.

For the determination of the efficiency of separation, *P. stutzeri* and *M. luteus* cells were mixed and applied to an IEF column. These species could be distinguished readily according to their morphology. After separation into bacterial bands, cell numbers of both species were counted separately for each band.

Lysis of bacterial cells. The bacterial fractions obtained by IEF contained Ficoll, which, due to its high density, would prevent the sedimentation of bacterial cells during centrifugation. Therefore, the density of the medium in the samples was decreased by the addition of four volumes of phosphate-buffered saline (PBS) (117.5 mM NaCl, 1.5 mM KCl, 5 mM Na_2HPO_4, 1 mM KH_2PO_4 [pH 7.4]) and the samples were centrifuged at 48,400 × *g* for 1 h at 5°C. Cell pellets were resuspended in 67 μl of double-distilled water and transferred to a 0.5-ml PCR tube. Bacterial cells were lysed by six freeze-thaw cycles (1 min in a −80°C ethanol bath, followed by 1 min in a 50°C water bath) (3). A final heating step (5 min at 85°C) was used to inactivate nucleases and proteinases.

PCR amplification. Bacterial sequences present in the lysates were amplified in a GeneAmp PCR System 9600 (Perkin-Elmer, Überlingen, Germany). We devised a two-step PCR protocol to increase the sensitivity of amplification. In the first step, a 919-bp fragment of the 16S rDNA was generated, which served as a template for the amplification of a 626-bp fragment in the second step. Because of the high sensitivity of our method (detection limit, 10 fg of DNA [see Results]), traces of contaminating DNA in the conventional Ampli*Taq* DNA polymerase (Perkin-Elmer, Weiterstadt, Germany) led to false positives. Therefore, a low-DNA preparation of the enzyme (Ampli*Taq* DNA polymerase LD; Perkin-Elmer) was used.

In the first amplification step, 10 μl of cell lysate, 12.5 pmol of each primer (8f [5′-AGAGTTTGATCCTGGCTCAG-3′] and 907r [5′-CCGTCAATTCCTTTGAGTTT-3′] [6]), 10 μmol of each deoxyribonucleoside triphosphate, and 5 μl of 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl [pH 8.3], 15 mM $MgCl_2$, 0.01% [wt/vol] gelatin; Perkin-Elmer) were combined and adjusted to a final volume of 50 μl with sterile double-distilled water. After an initial denaturation (94°C, 4 min), 1.25 U of Ampli*Taq* DNA polymerase LD was added at a temperature of 80°C. Forty-five thermal cycles followed, with the annealing temperature set to 40°C for 2 min. Primer extension was carried out at 72°C for 2 min, and denaturation was done at 94°C for 1.5 min. In addition, we used a 1-min ramp for the temperature shifts from 94 to 40°C and from 40 to 72°C.

Amplification products of the first PCR step served as a template in the subsequent PCR, in which primers GM5F (341f) (5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCCTACGGGAGGCAGCAG-3′ [9]) and 907r were employed. In order to enhance the specificity of this second PCR, it was necessary to change the PCR protocol described above. Amplification products of the first PCR were diluted by 10^{-4}, and 1 μl of the dilution was used for reamplification. A second type of PCR buffer [10× buffer containing 670 mM Tris-HCl (pH 8.3), 10 mM $MgCl_2$, 166 mM $(NH_4)_2SO_4$, and 100 mM β-mercaptoethanol] was employed, and acetamide was added to each PCR tube to a final concentration of 4% in order to minimize nonspecific annealing of the primers (13). Furthermore, the PCR cycle was modified. Twenty-five thermal cycles were run, with the annealing temperature set to 60°C for the first cycle and lowered by 0.2°C for each consecutive cycle (touch-down). Annealing time was set to 20 s. Primer extension was carried out at 72°C for 20 s, and denaturation was done at 94°C for 0.5 min. Finally, five cycles at a constant annealing temperature of 55°C were performed.

Amplification products were visualized by gel electrophoresis on 1.5% (wt/vol) agarose gels in 1× TAE buffer (40 mM Tris-acetate [pH 8.0], 1 mM EDTA). Gels were stained with ethidium bromide (0.5 μg ml^{-1}) and photographed under UV light.

DGGE. The 626-bp-long 16S rDNA fragments from different bacterial species can be separated by DGGE (8, 9) based on their different melting behavior. The GC-rich sequence at the 5′ end of primer GM5F results in a stable melting behavior of the generated DNA fragments during the subsequent DGGE.

DGGE was carried out in the Bio-Rad D Gene system. PCR samples were applied directly onto 6% (wt/vol) polyacrylamide gels (acrylamide/N,N′-methylene bisacrylamide ratio, 37:1 [wt/wt]) in 1× TAE buffer (pH 7.4). Gels contained a linear gradient of 30 to 60% denaturant (100% denaturant = 7 M urea plus 40% [vol/vol] formamide). Electrophoresis proceeded for 5 h at 200 V and 60°C. Afterwards, gels were stained for 20 min with ethidium bromide and photographed.

RESULTS

Development of the IEF method. Initial experiments demonstrated that the binding of Ca^{2+} to the bacterial cell surface is crucial to the separation of the different species during IEF. A separation of *P. stutzeri* and *M. luteus* cells was reproducible after a pretreatment of the cells with $CaCl_2$. In contrast, separation of the species was not possible after Ca^{2+} ions had been removed from the cells by a treatment with polyphosphate (Fig. 2). Consequently, bacterial cells were preincubated in 5 mM $CaCl_2$ in all subsequent experiments.

The cells of each of the three species formed macroscopic aggregates when they reached their isoelectric points (IEPs) in the pH gradient. Aggregation could be prevented by adding Tween 20 (0.005% final concentration) to the separation medium. Also, interspecies aggregation occurred during the migration of bacterial cells and was most pronounced for *P. stutzeri* and *C. limicola*. Interspecific aggregation occurred if the highly concentrated mixed cell suspensions were applied to the top of an established pH gradient but was not observed if bacterial cells were incorporated in the separation medium before the IEF was started. Therefore, bacterial mixtures were

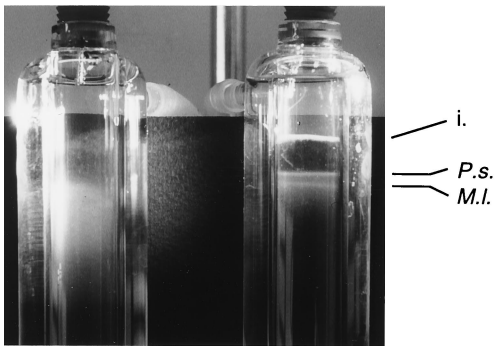

FIG. 2. IEF of a mixture of *P. stutzeri* (*P.s.*) and *M. luteus* (*M.l.*) cells after pretreatment with 0.1% sodium polyphosphate (left IEF column) and of an untreated mixture (right column). The pH gradient ranged from 7 to 2. i., interface between the cathode solution and the separation medium.

added routinely to the light solution before the gradient was poured.

When ampholytes for a pH range of 2 to 7 were employed, *C. limicola* cells formed aggregates at the beginning of the IEF run. These aggregates did not disappear during IEF and interfered with the separation. The initial pH value of the pH 2 to 7 separation medium was 4.53, which is close to the IEP of *C. limicola* (4.19; see below). Therefore, we used an ampholyte mixture for the pH range 2 to 10 during the subsequent IEF experiments. The ampholyte mixture contained 0.33% ampholytes for the range pH 2 to 4 plus 0.67% ampholytes for the range pH 3 to 10. With this mixture, the initial pH value was significantly higher (5.69) and no initial aggregation of *C. limicola* occurred.

Separation of defined mixtures of bacterial strains. After optimizing the conditions for IEF as described in the previous section, a mixture of *C. limicola*, *P. stutzeri*, and *M. luteus* cells could be separated. Within 6 h, the three bands separated (Fig. 3 and 4A), reaching a constant vertical position after 10.5 h (Fig. 4A). The ampholyte mixture employed did not generate a linear pH gradient (Fig. 4B). As a result, the migration velocity of all bacterial bands increased during the initial 5 h of the IEF and slowed down afterwards.

In a series of experiments, IEPs of the three species were determined. The IEP was highly reproducible for a given species (*C. limicola*, 4.19 ± 0.04; *P. stutzeri*, 3.83 ± 0.02; *M. luteus*, 3.23 ± 0.18) and did not change after the treatment with $CaCl_2$ or in the presence of Tween 20 (data not shown).

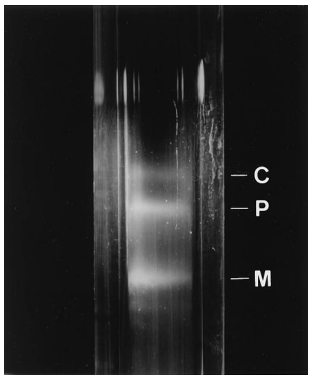

FIG. 3. Separation of cells of *C. limicola* (C), *P. stutzeri* (P), and *M. luteus* (M) after 6 h of IEF in a gradient of pH 2 to 10. The inner diameter of the IEF column is 13 mm.

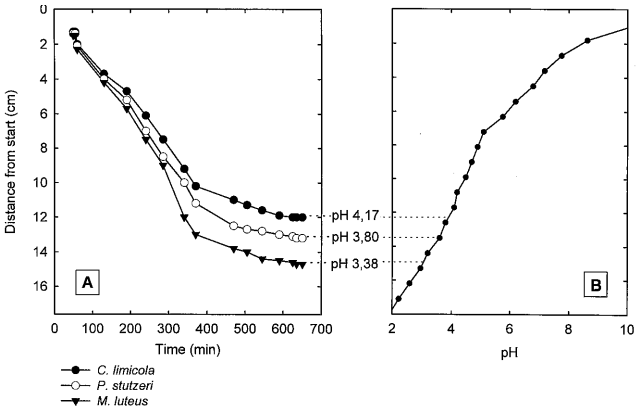

FIG. 4. (A) Time course of vertical migration of the three bacterial species during IEF. The experiment is the same as that shown in Fig. 3. (B) Vertical pH gradient in the separation medium of the IEF column. The IEPs of the three species are indicated.

In another IEF run, *P. stutzeri* and *M. luteus* cells were separated and both bacterial bands were checked for cross-contamination with cells of the other species. A total of 11.5% of the cells in the *P. stutzeri* band were identified as *M. luteus*, while the contamination of the *M. luteus* band was 25.5%. Clearly, cross-contamination still represents a potential limitation of our method.

The 12-h exposure to 300 V had a varying effect on the plateability of *P. stutzeri* and *M. luteus*. If freshly grown cultures were employed, the plateability of *M. luteus* cells remained unchanged after IEF, while only 2.7% of the initial plateability was detected for *P. stutzeri*. If cells stored in liquid nitrogen were applied to the IEF, plateability of both species decreased (*P. stutzeri*, 0.5% remaining; *M. luteus*, 60% remaining).

Sensitivity and specificity of the PCR method. In the present study, a PCR protocol was developed which allows a rapid amplification of 16S rDNA fragments from a small number of bacterial cells. The low detection limit is a prerequisite for the phylogenetic identification of minor components of a bacterial community, which would not form dense, visible bands during IEF.

In the first amplification step, a weak amplification signal was obtained at a template concentration of 100 fg of genomic DNA (Fig. 5A). In the reamplification step, the detection limit was decreased further to 10 fg (Fig. 5B, lane 9′). Reamplification of the negative control yielded a very faint signal (Fig. 5B, lane 10′), which most likely resulted from contamination of the DNA polymerase. This signal in the reamplified control could be eliminated by adding acetamide to the reamplification samples without affecting the detection limit of the PCR.

Analysis of a natural bacterial community. Cells of the natural bacterial community sampled in February 1996 in Zwischenahner Meer remained distributed over most of the IEF column. Against this background of turbidity, a total of six bacterial bands could be distinguished, based on their white, reddish, greenish, or yellow color (Fig. 6A). From each band, 300 μl was harvested with a syringe fitted with a 0.8- by 120-mm needle; the bacterial cells were lysed, and 16S rDNA fragments were amplified by two-step PCR. This yielded highly specific amplification products (Fig. 7) suited for the separation of 16S rDNA sequences by DGGE. For comparative purposes, a second sample from Zwischenahner Meer was obtained on 15 May 1996 and separated by IEF. In this case, bacteria in the sample accumulated in 10 different bands (Fig.

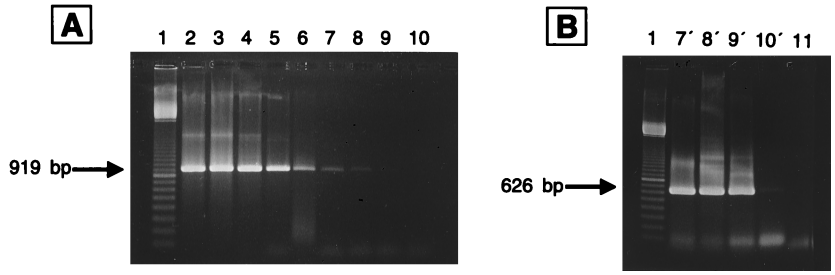

FIG. 5. (A) Amplification of different amounts of *E. coli* genomic DNA. Lanes: 1, molecular size markers (375 ng of 100-bp ladder); 2, 50 ng; 3, 10 ng; 4, 1 ng; 5, 100 pg; 6, 10 pg; 7, 1 pg; 8, 100 fg; and 9, 10 fg of template DNA; and 10, no DNA. (B) Reamplification of the PCR samples shown in panel A. Lanes: 1, molecular size markers (375 ng of 100-bp ladder); 7′ to 10′, samples 7 to 10; 11, no template. No acetamide was added to the PCR samples. Arrows indicate the position of 919-bp and 626-bp DNA fragments.

6B), which were more distinct than those obtained with the winter sample.

The sequence-specific separation of the DNA fragments by DGGE revealed distinct patterns for the six different IEF fractions (Fig. 8). The unfractionated bacterial community yielded only three DNA bands on the DGGE gel. By comparison, five additional bands (Fig. 8, bands a to e) were detected in the IEF fractions. Some of these additional signals were amplified from two fractions positioned next to each other (e.g., Fig. 8, bands c and e in IEF fractions 3 and 4 and band d in fractions 5 and 6). Other signals (e.g., Fig. 8, band b in fraction 6) displayed a peak intensity in one IEF fraction but were also present in other fractions.

DISCUSSION

In the present study, we combined the fractionation of whole bacterial cells by IEF with a molecular biological analysis of the resulting fractions by DGGE. For each bacterial strain the IEP was highly reproducible. The IEP, therefore, is a cytological property well suited for the separation of defined mixtures of bacteria.

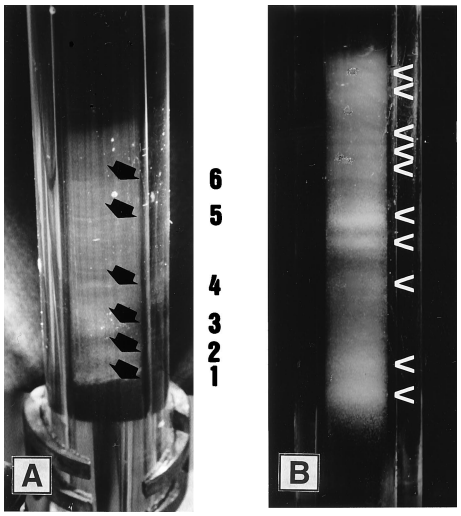

FIG. 6. IEF of bacterial samples from a depth of 0.5 m in the Zwischenahner Meer run at 300 V for 10 h in a gradient of pH 2 to 10. (A) Sample obtained on 15 February 1996. The arrows and numbers indicate fractions of the column which were sampled and which correspond to the six colored bands. (B) Sample obtained on 15 May 1996. The white arrows indicate positions of the bacterial bands.

The significant decrease in plateability of *P. stutzeri* during IEF could be the result of either increased clumping of the cells, irreversible cell damage, or a decrease in culturability. Microscopic examination confirmed that no clumps of *P. stutzeri* cells had formed after IEF. IEF had little or no effect on the plateability of the gram-positive *M. luteus* cells. It remains to be tested if the stability of the cell envelope is important for the effect of high voltage. In IEF, an electric field of 11.5 V cm^{-1} is applied. Gram-negative bacteria survive even an (albeit short) exposure to 6,250 V cm^{-1} during electrotransformation (14), with a survival rate significantly higher than the values obtained for *P. stutzeri* after IEF. Therefore, it appears possible that *P. stutzeri* cells are not irreversibly damaged. In addition, the low pH to which bacterial cells are exposed at the end of IEF (see the IEP values above) represents a stress factor which could affect plateability. Again, the high value for *M. luteus* indicates that, at least for some bacterial species, such low pH values are not critical. Obviously, further work is needed to improve the cultivability of bacterial cells after their separation by IEF. Presently, experiments are being performed to test various methods for resuscitation of *P. stutzeri* after IEF.

Cells collected from Zwischenahner Meer were used to discover whether bacterial species from natural samples can also be separated by IEF. The bacterial community present under the ice in winter segregated into six visible zones of cell accumulation which differed in color. An analysis of these six IEF fractions by DGGE revealed different patterns of 16S rDNA fragments for each of the fractions. Obviously the various fractions consisted of different bacterial species. The nonrandom distribution of DGGE bands c, d, and e over the IEF fractions (Fig. 8) indicates that at least some of the species in natural bacterial communities have a distinct IEP and thus are sepa-

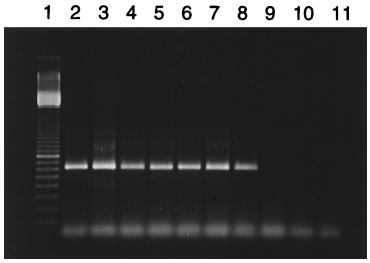

FIG. 7. 16S rDNA fragments amplified after lysis of IEF fractions. Lanes: 1, molecular size markers (375 ng of 100-bp ladder); 2, unfractionated bacterial sample; 3 to 8, fractions 1 to 6 of the IEF column (Fig. 5); 9, control for lysis reagents (bacterial cells omitted); 10, reamplified negative control for first PCR step; 11, negative control for second PCR step.

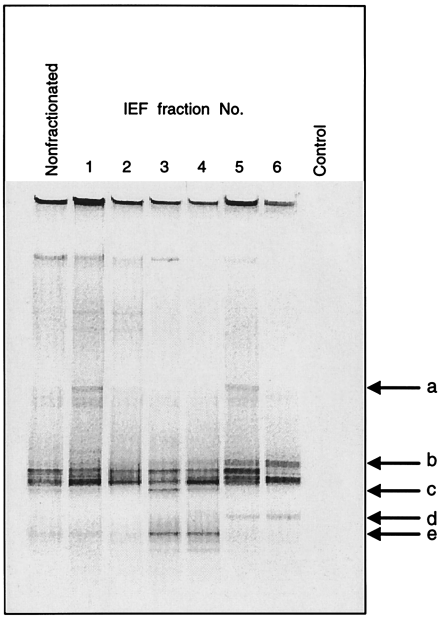

FIG. 8. Separation by DGGE of the 16S rDNA fragments amplified from IEF fractions 1 to 6 and from an unfractionated bacterial sample. A negative image of an ethidium bromide-stained gel is shown. Control, control for lysis reagents (compare Fig. 7, lane 9). The arrows labelled a to e indicate DNA bands which are not present in the unfractionated sample.

rated during IEF (e.g., the species with the 16S rDNA signature d from that with the signature e).

A considerable number of cells were present between the six zones of cell accumulation after the separation of the February sample. Obviously, the bacterial assemblage present during winter comprised cells with a whole range of IEP values. In comparison, IEF of the May sample yielded an accumulation of bacteria in 10 different bands. Also, these bands were much more distinct than those obtained with the winter sample. This indicates that bacterial cells with higher physiological activity during spring also had a better-defined IEP, which in turn facilitated their separation during IEF. However, even the incomplete separation of bacteria from the winter sample significantly improved the molecular biological analysis of the bacterial community by DGGE.

Only three distinct DNA bands were found in the unfractionated winter bacterial sample. This poor recovery of bacterial 16S rDNA sequences again demonstrates that only part of the bacterial species present in a natural sample can be analyzed by conventional DGGE. After the separation of bacterial cells by IEF, a significantly higher number of DGGE bands (eight compared to three) could be detected. Biases due to cell lysis cannot be the reasons for the very low diversity observed in the unfractionated sample because a variety of bacterial species are lysed quantitatively with the method employed in the present study (3). Rather, a bias towards low diversity may be caused by a different amplification efficiency for different 16S rDNA sequences. It has been shown that in some cases a bias effect of PCR can result in microbial diversity being overrepresented (16). We hypothesize that, in our experiments, differential amplification might have led to a preferential amplification of a few sequences, which was further aggravated by the two-step amplification protocol employed.

The detection limit of 10 fg of genomic DNA corresponds to about 2 to 3 cells (one *Escherichia coli* genome represents 4.5 fg of DNA). This low detection limit of our two-step PCR method allows phylogenetic analyses of very small numbers of bacterial cells, for instance, of species which represent only a small fraction of the total bacterial community in a habitat. At least in some environments, species diversity has a very high degree of eveness, with each species accounting for only a small fraction of the total bacterial community. As little as 4% of 124 sequenced clones were redundant in a southern Wisconsin pasture soil (4). This indicates that, in this case, most species represent less than 1% of the total cell number. After further improvement, our approach may allow the selective enrichment of at least some of these species in IEF fractions, permitting an analysis of their 16S rDNA sequences and at the same time providing bacterial cells for cultivation experiments.

In conclusion, the separation of whole cells by IEF can significantly facilitate the analysis of natural microbial communities because it can accomplish a taxon-specific enrichment of bacterial cells which can be used in subsequent cultivation experiments and, at the same time, the phylogenetic composition of the different IEF fractions can be rapidly analyzed by DGGE.

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REFERENCES

1. **Amann, R. I., W. Ludwig, and K.-H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.
2. **Bayne, D. R., and J. M. Lawrence.** 1972. Separating constituents of natural phytoplankton populations by continuous particle electrophoresis. Limnol. Oceanogr. **17:**481–489.
3. **Bej, A. K., M. H. Mahbubani, J. L. Dicesare, and R. M. Atlas.** 1991. Polymerase chain reaction-gene probe detection of microorganisms by using filter-concentrated samples. Appl. Environ. Microbiol. **57:**3529–3534.
4. **Borneman, J., P. W. Skroch, K. M. O'Sullivan, J. A. Palus, N. G. Rumjanek, J. L. Jansen, J. Nienhuis, and E. W. Triplett.** 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Environ. Microbiol. **62:**1935–1943.
5. **Godson, G. N.** 1969. A simple apparatus for rapid isoelectrofocusing of multiple samples on a micro scale. Anal. Biochem. **35:**66–76.
6. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley, Chichester, England.
7. **Longton, R. W., J. S. Cole, and P. F. Quinn.** 1975. Isoelectric focusing of bacteria: species location within an isoelectric focusing column by surface charge. Arch. Oral Biol. **20:**103–106.
8. **Muyzer, G., E. C. de Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:**695–700.
9. **Muyzer, G., S. Hottenträger, A. Teske, and C. Waver.** 1995. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA—a new molecular approach to analyse the genetic diversity of mixed microbial communities, p. 3.4.4.1–3.4.4.22. *In* A. D. L. Akkermans, J. D. van Elsas, and F. J. de Bruijn (ed.), Molecular microbial ecology manual, 2nd ed. Kluwer, Dordrecht, The Netherlands.
10. **Overmann, J., and N. Pfennig.** 1989. *Pelodictyon phaeoclathratiforme* sp. nov., a new brown-colored member of the Chlorobiaceae forming net-like colonies. Arch. Microbiol. **152:**401–406.
11. **Palinska, K. A., and W. E. Krumbein.** 1995. Electrophoretic separation of two unicyanobacterial strains leading to purification. J. Microbiol. Methods **24:**41–48.
12. **Porter, K. G., and Y. S. Feig.** 1980. Use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. **25:**943–948.
13. **Reysenbach, A.-L., L. J. Giver, G. S. Wickham, and N. R. Pace.** 1992. Differential amplification of rRNA genes by polymerase chain reaction. Appl. Environ. Microbiol. **58:**3417–3418.
14. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
15. **Schmidt, T. M., E. F. DeLong, and N. R. Pace.** 1991. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. J. Bacteriol. **173:**4371–4378.

16. **Suzuki, M. T., and S. J. Giovannoni.** 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. **62:**625–630.
17. **Teske, A., C. Wawer, G. Muyzer, and N. B. Ramsing.** 1996. Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. Appl. Environ. Microbiol. **62:**1405–1415.
18. **Torsvik, V., J. Goksøyr, and F. L. Daae.** 1990. High diversity in DNA of soil bacteria. Appl. Environ. Microbiol. **56:**782–787.
19. **Torsvik, V., J. Goksøyr, F. L. Daae, R. Sørheim, J. Michalsen, and K. Salte.** 1993. Diversity of microbial communities determined by DNA reassociation technique, p. 375–378. *In* R. Guerrero and C. Pedrós-Alió (ed.), Trends in microbial ecology. Spanish Society for Microbiology, Barcelona, Spain.
20. **Ward, D. M., R. Weller, and M. M. Bateson.** 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature **344:**63–65.