Journal of Industrial Microbiology (1995) 15, 85–93

Use of polymerase chain reaction (PCR) fingerprinting to differentiate bacteria for microbial products screening

CF Hirsch and JM Sigmund

Department of Natural Products Discovery, Merck Research Laboratories, Rahway, New Jersey 07065, USA

PCR fingerprinting offers a practical molecular means to quickly and reliably differentiate bacteria for microbial products screening. A combination of low resolution and high resolution PCR fingerprinting provides a hierarchical system which allows the discrimination of bacteria at species and subspecies level within 7 h. DNA was extracted from cells by incubating them in water at 95° C for 30 min. A sample of 1 μl of the cell-free aqueous extract then was used as a source of template DNA in the PCR. The PCR products were separated by electrophoresis on an acrylamide gel and visualized by ethidium bromide staining. The band patterns generated for each different culture were unique, reproducible, and independent of cultivation conditions. Band patterns may be compared visually or by using imaging and pattern matching software. In our laboratory, bacteria such as actinomycetes, Gram-negative and Gram-positive soil eubacteria, and photosynthetic non-sulfur bacteria have been differentiated using PCR fingerprinting.

Keywords: polymerase chain reaction; PCR; fingerprinting; bacteria; products; screening

Introduction

An important challenge in microbial products screening programs which deal with bacteria is to develop methods which will allow one to differentiate the cultures being studied at an early stage of the overall process. Ideally, the method of choice should: (i) reproducibly discriminate cultures at different levels of resolution; (ii) be unaffected by the cultivation conditions used to grow the bacteria; (iii) be applicable to a wide diversity of bacteria; and (iv) be rapid and easy to do.

Historically, the differentiation of bacteria in microbial screening programs has relied on the determination of various phenotypic features. These have included colony and cell morphology, cell wall and cell membrane constituents, composition of cellular proteins, and various nutritional and biochemical characteristics. However, it is recognized that phenotypic characteristics are influenced by the cultivation conditions used to grow cells and that these characteristics may change with the age or physiological state of the cells. Therefore, as pointed out by Goodfellow and O'Donnell [6], one must be wary when using phenotypic features to differentiate bacteria that the differences one observes are due to genetic differences and not simply to variations in cultivation conditions.

Procedures involving nucleic acids to distinguish bacteria are generally recognized as offering greater precision and reliability than methods based on phenotypic characteristics [6, 8]. While many different nucleic acid techniques have been developed which will differentiate bacteria, none is practical for routine use in microbial products screening programs either because they are technically demanding or too cumbersome.

Correspondence: CF Hirsch, Department of Natural Products Discovery, Merck Research Laboratories, RY 80Y-330, Rahway, NJ 07065, USA
Revised 31 October 1994; accepted 22 March 1995

The emergence of methods using the polymerase chain reaction (PCR) to generate genomic fingerprints now appears to offer an alternative nucleic acid based technique to discriminate bacteria. We present here studies showing that PCR fingerprinting is an easy, rapid, reliable, and high resolution means to differentiate both traditional and more non-traditional bacteria for microbial products screening.

Materials and methods

Bacteria used in study

The bacteria used in this study were obtained from the ATCC: *Pseudomonas syringae* 10205, *P. syringae* pv *delphinii* 8719, *P. syringae* pv *apii* 8722, *P. syringae* pv *tabaci* 17914, *P. syringae* pv *glycinea* 8727, *P. syringae* pv *tabaci* 11527, *P. aeruginosa* 10145, *P. cepacia* 25416, *P. mesoacidophila* 31433, *P. acidophila* 31363, *P. cocovenenans* 33664, *P. fluorescens* 13525, *P. pyrrocinia* 15958, *Bacillus polymyxa* 842, *B. mycoides* 6462, *B. licheniformis* 12759, *B. subtilis* 6051, *B. circulans* 4513, *B. cereus* 14579, *B. megaterium* 14581, *Rhodospirillum rubrum* 277, *Rhodopseudomonas blastica* 33485, *Rhodobacter sphaeroides* 21455, *Rhb. sphaeroides* 33575, *Rhb. sphaeroides* 17023, *Rhb. sphaeroides* 21286, *Actinomadura citrea* 27887, *Streptoverticillium baldaccii* 23654, *Streptomyces griseus* 23345, *Nocardioides albus* 27980, *Pseudonocardia compacta* 35407, *Nocardiopsis dassonvillei* 23218, *Nocardia asteroides* 19247, *Spirillospora albida* 15331, *Ampullariella lobata* 15350, *Actinoplanes missouriensis* 14538, *Streptosporangium roseum* 12428, *Planomonospora venezuelensis* 23865, *Planobispora longispora* 23867, *Rhodococcus coprophilus* 29080, *Microbispora* sp 55140, *Microtetraspora glauca* 23057, *Dactylosporangium aurantiacum* 23491, *Micromonospora carbonacea* 27114, *Promicromonospora citrea* 15908, *Geodermatophilus obscurus* 25078, *Arthrobacter crystallopoietes* 15481, *Sporichthya polymorpha* 23823, *Oerskovia turbata* 25835, *Intrasporan-*

gium calvum 23552, *Saccharothrix australiensis* 31497, *Kibdelosporangium aridum* 39323, *Kineospora aurantiaca* 29727, *Saccharopolyspora hirsuta* 27875, *Kitasatosporia setae* 33774, *Streptoalloteichus hindustanus* 31217, *Pilimelia terevasa* 25603, and *Amycolatopsis mediterranei* 13685.

Growth of cultures

Eubacteria were grown 14–16 h at 28° C in Brain Heart Infusion broth (Difco); Nutrient broth (Difco); CGY broth consisting of glucose 10 g L^{-1}, casein hydrolysate 10 g L^{-1}, NaCl 5 g L^{-1}, K_2HPO_4 0.35 g L^{-1}, Na_2SO_4 0.14 g L^{-1}, and $MgCl_2 \cdot 6H_2O$ 0.06 g L^{-1} in 100 mM MOPS buffer, pH 7.0; or BASE broth consisting of glucose 2 g L^{-1}, NH_4Cl 1 g L^{-1}, NaCl 5 g L^{-1}, K_2HPO_4 0.03 g L^{-1}, and $MgSO_4 \cdot 7H_2O$ 0.08 g L^{-1} in 100 mM MOPS buffer, pH 7.0. Actinomycetes were grown at 28° C for 1–3 days on the surface of sterile polycarbonate membrane filters (0.1-μm pore diameter, Nuclepore, Pleasanton, CA, USA) overlaid onto ATCC medium #174 [3]. Photosynthetic non-sulfur bacteria were grown anaerobically at 25° C in the light (500 lux) for 14 days on the surface of sterile polycarbonate membrane filters overlaid onto ATCC medium #112 [3].

Extraction of template DNA

Cells were harvested from 1 ml of broth culture by centrifugation and cells grown on filters were harvested by aseptically scraping growth from the filter surface with a sterile spatula. Cells were washed once in 1 ml sterile saline (NaCl 8.5 g L^{-1}), and template DNA was extracted from washed cells by incubating them (2–10 mg wet weight) in 0.1–0.5 ml sterile water at 95° C for 30 min [2]. Following heating, cell debris was removed from the extract by centrifugation and the extract was stored at −20° C until used as a source of template DNA in the PCR. Storage of extracts at −20° C for as long as 2 years did not alter the PCR fingerprints produced.

Primers

The primers used for low resolution PCR fingerprinting were described by Welsh and McClelland [20, 21] and have extensive homology to tRNA genes; resulting in the amplification of regions between adjacent tRNA genes (Figure 1a). For the experiments presented here, the primers T5A (5′-AGTCCGGTGCTCTAACCAACTGAG-3′) and T3B (5′-AGGTCGCGGGTTCGAATCC-3′) were used. The high resolution PCR fingerprinting primers which were used were described by Jensen *et al* [7]. These primers, L1 (5′-CAAGGCATCCACCGT-3′) and G1 (5′-GAAGTCGTAACAAGG-3′), have sequences which are complementary to conserved regions of the 16S and 23S rRNA genes and result in amplification of the variable spacer regions between the genes (Figure 1b). All primers were obtained from Genset (La Jolla, CA, USA).

PCR amplification

Amplification was done in a 50-μl reaction mixture consisting of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM each dATP, dCTP, dGTP, and TTP, 1 μM each primer, and 1.25 units of *Taq* DNA polymerase (AmpliTaq-LD, Perkin-Elmer, Norwalk, CT, USA). Following the addition of 1 μl of cell extract, the reaction mixture was incubated in a Techne PHC-3 thermal cycler equipped with a heated lid (Techne, Inc, Princeton, NJ, USA). For low resolution fingerprinting, a temperature program consisting of 30 cycles of 0.5 min at 94° C, 0.5 min at 50° C, and 72° C for 2 min followed by a final 10-min interval at 72° C was used. For high resolution fingerprinting, a program was used consisting of 30 cycles of 0.5 min at 94° C, 0.5 min at 55° C, and 2 min at 72° C, followed by a final 10-min incubation at 72° C. For each program, the ramping rate which was used between temperatures was the fastest available. Negative controls without added template DNA were routinely run for all amplifications to ensure that the bands which were produced were the result of amplification from added template DNA and not from DNA contaminating the PCR reagents. Following amplification, the reaction mixtures can be stored at 4° C or −20° C prior to electrophoresis.

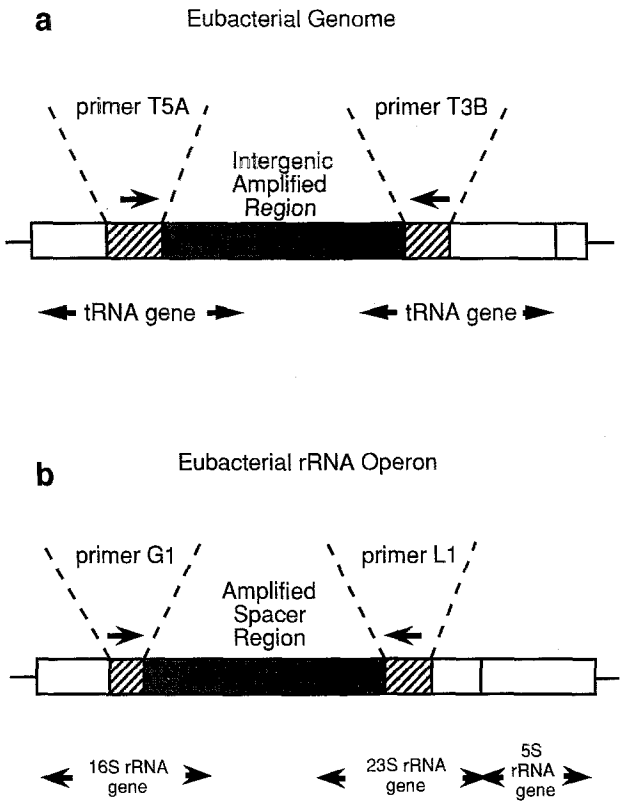

Figure 1 Schematic representation of low resolution and high resolution PCR fingerprinting. (a) Low resolution PCR fingerprinting results in the amplification of regions of DNA located between adjacent tRNA genes using primers which incorporate consensus sequences of tRNA genes. (b) High resolution PCR fingerprinting amplifies the variable DNA spacer regions located between the 16S and 23S rRNA genes of bacteria using primers which are homologous to conserved regions of the eubacterial 16S and 23S rRNA genes

Electrophoresis

A sample of 10 μl of PCR reaction mixture was added to 2 μl of TBE sample buffer (Novex, San Diego, CA, USA). Ten microliters of this mixture was loaded onto a 4–20% acrylamide gradient mini-gel (80 × 80 × 1 mm, Novex) and the PCR products present in the reaction mixture were

separated by electrophoresis at 200 V for 90 min in 1 × TBE buffer. Following electrophoresis, the gel was stained for 20 min in ethidium bromide (0.5 μg ml^{-1} water), and then destained for 10 min in water. Bands were visualized with UV light (300 nm) and photographed using Polaroid type 667 film (f4.5–5.6, 1 s). Band patterns were compared visually with each other using a 50–2000-bp DNA size standard (Bio-Rad, Hercules, CA, USA) as a reference marker.

Determination of DNA concentration in extracts

The concentration of DNA in extracts was estimated spectrophotometrically. A sample of 100 μl of extract was treated with RNase (10 μg ml^{-1}, DNase-free, Boehringer-Mannheim, Indianapolis, IN, USA) for 1 h at 37° C to degrade any RNA which was present. The extract then was filtered through an ultrafiltration membrane (50 NCO, Microcon 30, Amicon, Beverly, MA, USA) to remove low molecular weight constituents. The DNA retained on the filter was washed once with 400 μl of sterile water. The DNA was then resuspended in 100 μl of sterile water and the absorbance of the solution at 260 nm was determined. The DNA concentration in the extract was calculated according to the formula: DNA concentration (mg ml^{-1}) = A_{260}/20 [9].

Results

Evaluation of PCR fingerprinting

The primary requirement for PCR fingerprinting to be used effectively as a tool to differentiate cultures for microbial products screening would be that the data generated are reproducible. This would require that the fingerprints not be affected by the cultivation conditions used to grow cells. An experiment was done to determine the effect of growing cells in different media on the reproducibility of low resolution and high resolution fingerprints. Cells of *Pseudomonas cepacia* ATCC 25416 and *Bacillus subtilis* ATCC 6051 were grown overnight in Brain Heart Infusion broth, Nutrient broth, CGY broth, and BASE broth. The cells were harvested and extracts were prepared. A sample of each extract was used as a source of template DNA to generate low resolution and high resolution fingerprints. As shown in Figure 2a, the low resolution fingerprint pattern for each different culture was identical for the cells grown in each of the four different media. Figure 2b shows that the high resolution fingerprint pattern for each of the different cultures grown in each of the four different media also were the same. The greatest difference observed among both the low resolution and high resolution fingerprints for each organism was a slight variation in the intensity of some of the bands.

A potential problem one might anticipate in applying PCR fingerprinting to a variety of different bacteria would be variation in the amount of DNA which would be released from different cultures by the hot aqueous extraction procedure. This would result in variable amounts of template DNA subsequently being added to the PCR. An experiment was done to compare the extraction of DNA from a Gram-negative bacterium (*P. cepacia* ATCC 25416) and a Gram-positive bacterium (*B. subtilis* ATCC 6051).

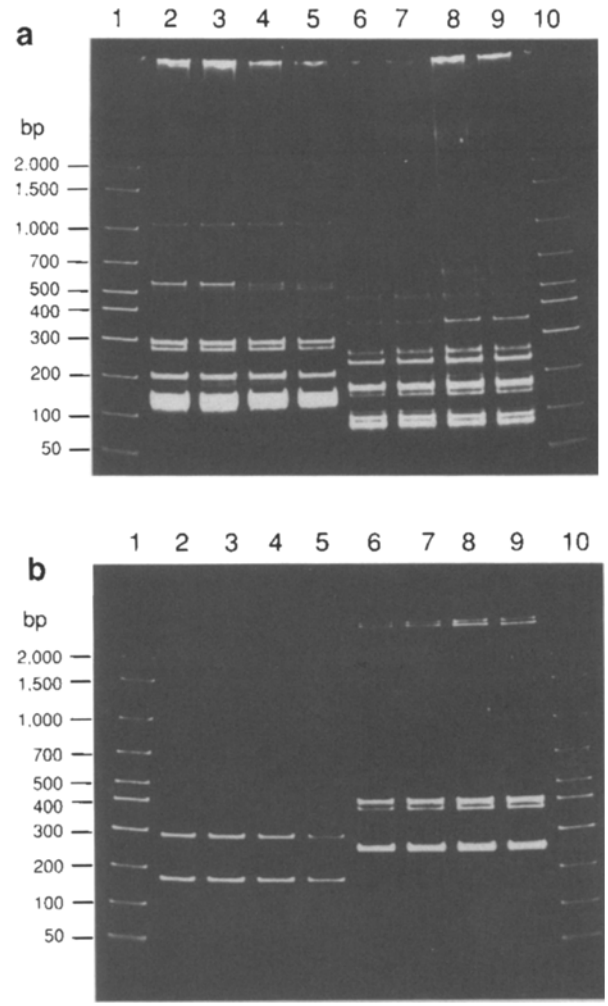

Figure 2 Effect of different growth media on the reproducibility of PCR fingerprints for *P. cepacia* ATCC 25416 and *B. subtilis* ATCC 6051. (a) Low resolution PCR fingerprinting. (b) High resolution PCR fingerprinting. Lanes 1 and 10, marker; *P. cepacia* grown in (2) BHI medium, (3) NB medium, (4) CGY medium, and (5) BASE medium; *B. subtilis* grown in (6) BHI medium, (7) NB medium, (8) CGY medium, and (9) BASE medium

Cells of *P. cepacia* and *B. subtilis* were grown 14–16 h in Nutrient broth medium and extracts prepared. The number of cells present in each of the cultures was determined by direct microscopic count (Petroff–Hauser) and the concentration of DNA present in each of the extracts was determined spectrophotometrically (Table 1). The concentration of DNA in the extract from *P. cepacia* was more than 7-fold greater than that for *B. subtilis*. When the amount of

Table 1 Comparison of DNA extraction from *Pseudomonas cepacia* ATCC 25416 and *Bacillus subtilis* ATCC 6051

Organism	DNA concentration in extract (ng μl^{-1})	DNA released cell^{-1} (fg)
B. subtilis	6.5	2.3
P. cepacia	48	12

DNA that was released per cell was compared for the two different bacteria, the efficiency of extraction of DNA for cells of *P. cepacia*, using the hot aqueous extraction procedure, was more than five times that observed for cells of *B. subtilis*.

The effect of different template DNA concentrations on the reproducibility of PCR fingerprints was studied. Samples of the crude aqueous extracts from *P. cepacia* and *B. subtilis* were 2-fold serially diluted and 1-μl aliquots of the dilutions were used as a source of template DNA for PCR fingerprinting. As shown in Figure 3a, the low resolution fingerprint of *P. cepacia* which was produced using extract which was diluted 1 : 64 was essentially the same as that produced using undiluted extract. Figure 3b shows the results for *B. subtilis* and it was observed that with increasing dilution of the extract, the bands present in the low resolution fingerprint became less intense; possibly reflecting the lower initial DNA concentration of the extract. However, it is clear that the low resolution PCR fingerprinting pattern produced for each of the dilutions of the *B. subtilis* extract is identical to the others. Figure 3c shows the effect of diluting the extract on the high resolution fingerprint of *P. cepacia*. With increasing dilution, the intensity of the bands present in the high resolution fingerprint rapidly declined. The effect of diluting the extract on the band intensity was such that the fingerprint generated from the 1 : 32 dilution was barely discernible, however, the banding pattern remained identical to that seen in the fingerprints produced from less diluted extract. Figure 3d shows the effect of diluting the extract on the high resolution fingerprint of *B. subtilis*. The bands in the *B. subtilis* high resolution fingerprint which was produced using the extract diluted 1 : 64 were only slightly less intense than those present in the fingerprint from the undiluted extract.

In a separate experiment (data not shown), the low resolution and high resolution fingerprints which were produced using hot aqueous cell extracts as a source of template DNA were identical to those which were produced using purified template DNA which was isolated from cells by the method of Visuvanathan *et al* [19].

The effect of using different cell extracts and different PCR amplifications on the reproducibility of PCR fingerprints was evaluated. Cells of *P. cepacia* and *B. subtilis* were grown in nutrient broth in four separate experiments. Each time, cell extracts were prepared and samples of the extracts were used for low resolution and high resolution fingerprinting. Figure 4a compares the low resolution fingerprints which were generated for *P. cepacia* and *B. subtilis* from four different extracts which were used in four separate PCR amplifications. It was seen that the fingerprints generated for each different culture from each of the four experiments were essentially identical; with only some slight variation in the intensity of some of the minor bands observed. The results for the high resolution fingerprints are shown in Figure 4b and reveal that for each of the cul-

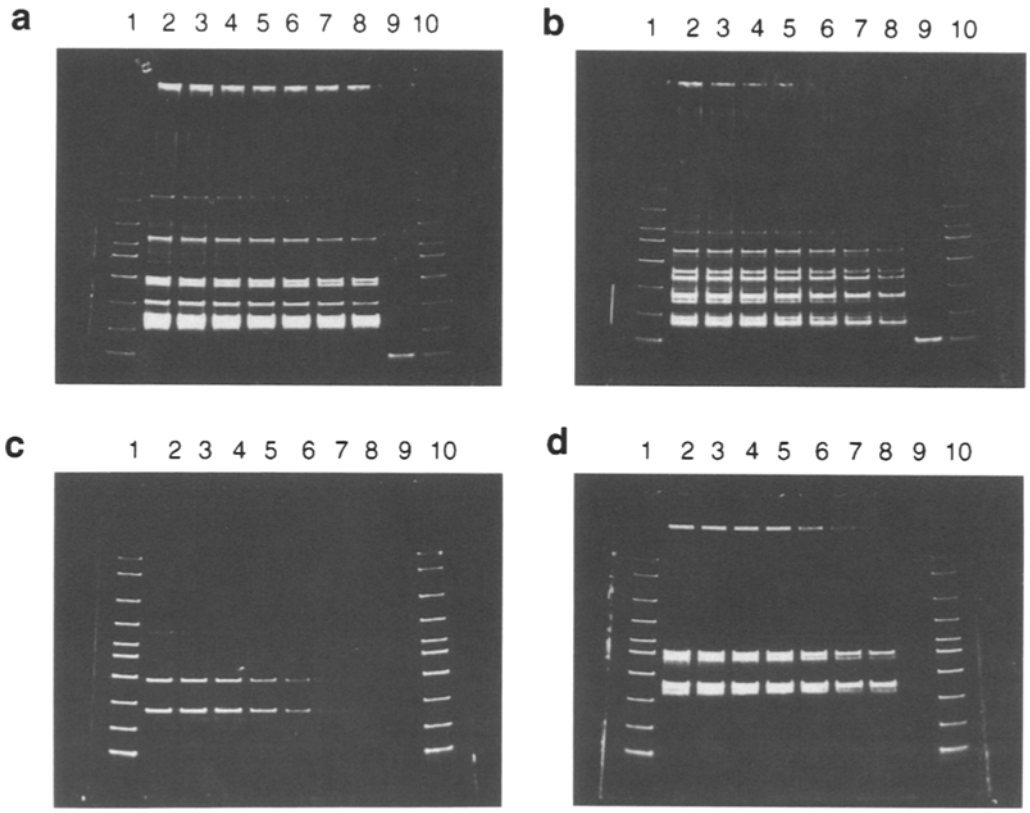

Figure 3 Effect of diluting cell extract on the reproducibility of PCR fingerprints for *P. cepacia* ATCC 25416 and *B. subtilis* ATCC 6051. (a and b) Low resolution PCR fingerprinting. (c and d) High resolution PCR fingerprinting. (a and c) *P. cepacia*; (b and d) *B. subtilis*. Lanes 1 and 10, markers; (2) undiluted extract; (3) extract diluted 1 : 2; (4) extract diluted 1 : 4; (5) extract diluted 1 : 8; (6) extract diluted 1 : 16; (7) extract diluted 1 : 32; (8) extract diluted 1 : 64; (9) control—no template

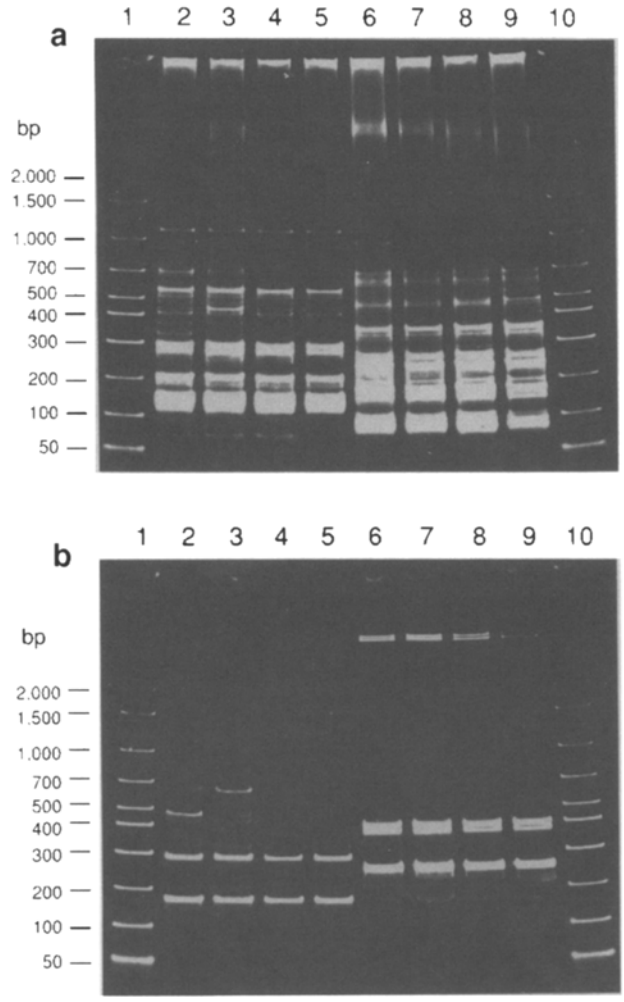

Figure 4 Reproducibility of PCR-fingerprints for *P. cepacia* ATCC 25416 and *B. subtilis* ATCC 6051 from different experiments. (a) Low resolution fingerprinting. (b) High resolution fingerprinting. Lanes 1 and 10, markers; *P. cepacia* (2) exp #1; (3) exp #2; (4) exp #3; (5) exp #4; and *B. subtilis* (6) exp #1; (7) exp #2; (8) exp #3; and (9) exp #4

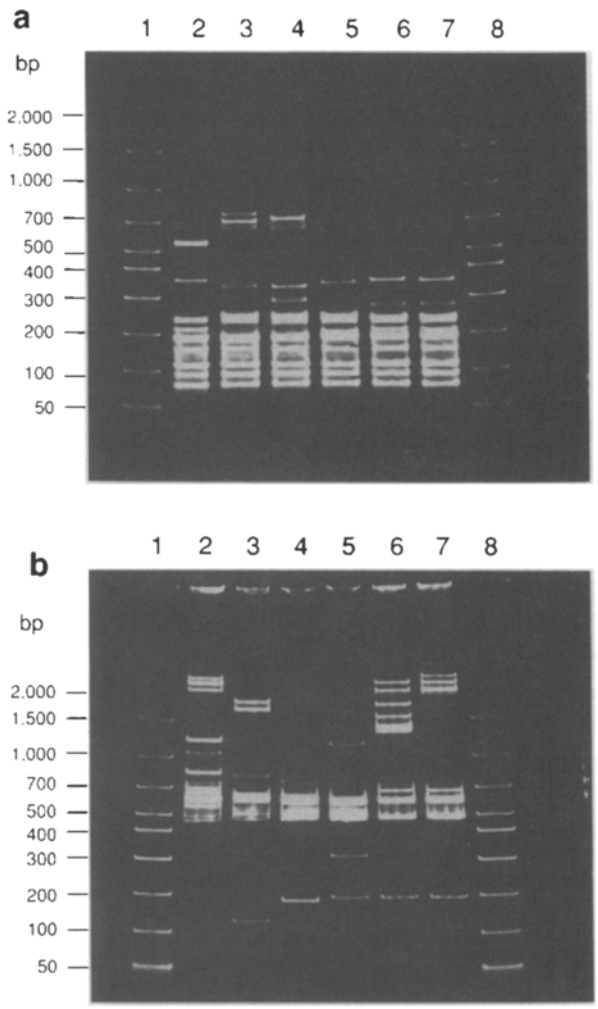

Figure 5 PCR fingerprinting of *Pseudomonas syringae* strains. (a) Low resolution fingerprinting. (b) High resolution fingerprinting. Lanes 1 and 8, markers; (2) *P. syringae* ATCC 10205; (3) *P. syringae* pv *delphinii* ATCC 8719; (4) *P. syringae* pv *apii* ATCC 8722; (5) *P. syringae* pv *glycinea* ATCC 8727; (6) *P. syringae* pv *tabaci* ATCC 11527; and (7) *P. syringae* pv *tabaci* ATCC 17914

tures the major bands of the fingerprints were reproduced from each of the four different experiments.

Differentiation of bacteria using PCR fingerprinting

An experiment was done to demonstrate the resolving power of low resolution and high resolution PCR fingerprinting. Fingerprints were generated for *P. syringae* and five pathovars of *P. syringae*. Figure 5a presents the low resolution fingerprints and it is seen that the fingerprints are very similar among the different strains. The banding pattern between 50 and 200 bp for each of the cultures is virtually identical. Above 200 bp, the banding patterns begin to exhibit differences for each of the different cultures, except for the two *tabaci* pathovars, ATCC 11527 (lane 6) and ATCC 17914 (lane 7). The low resolution fingerprints for each of these pathovars are indistinguishable. However, examination of the high resolution fingerprints for each of these cultures (Figure 5b, lanes 6 and 7) shows that they are different. While the banding patterns for the high resolution fingerprints of *P. syringae* pv *tabaci* ATCC 11527 and ATCC 17914 are identical between 100 and 700 bp, they are very different above 1000 bp. Between 1000 and 2000 bp, the high resolution fingerprint of *P. syringae* pv *tabaci* ATCC 11527 (lane 6) exhibits a group of four intense bands which are absent from the high resolution fingerprint of *P. syringae* pv *tabaci* ATCC 17914 (lane 7). In addition, the fingerprint of *P. syringae* pv *tabaci* ATCC 17914 (lane 7), includes a large (>2000 bp) product which is not seen in the fingerprint of *P. syringae* pv *tabaci* ATCC 11527 (lane 6).

The low resolution fingerprints for seven different species of *Pseudomonas* are shown in Figure 6a and demonstrate that each of the different species has a unique fingerprint. Each of the high resolution fingerprints for each culture (Figure 6b) is different, which supports the differentiation of the cultures based on the low resolution fingerprints.

The PCR fingerprints for seven different species of *Bacillus* are presented in Figure 7. Comparison of the low

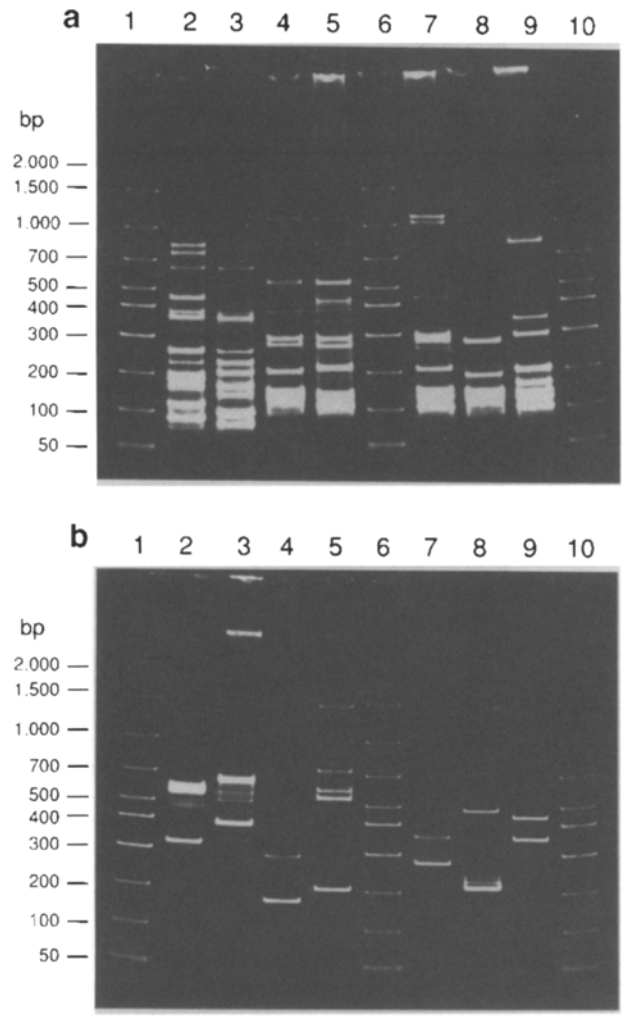

Figure 6 PCR fingerprinting of *Pseudomonas* species. (a) Low resolution fingerprinting. (b) High resolution fingerprinting. Lanes 1, 6, and 10, markers; (2) *P. aeruginosa* ATCC 10145; (3) *P. fluorescens* ATCC 13525; (4) *P. cepacia* ATCC 25416; (5) *P. pyrrocinia* ATCC 15958; (7) *P. mesoacidophila* ATCC 31433; (8) *P. acidophila* ATCC 31363; and (9) *P. cocovenenans* ATCC 33664

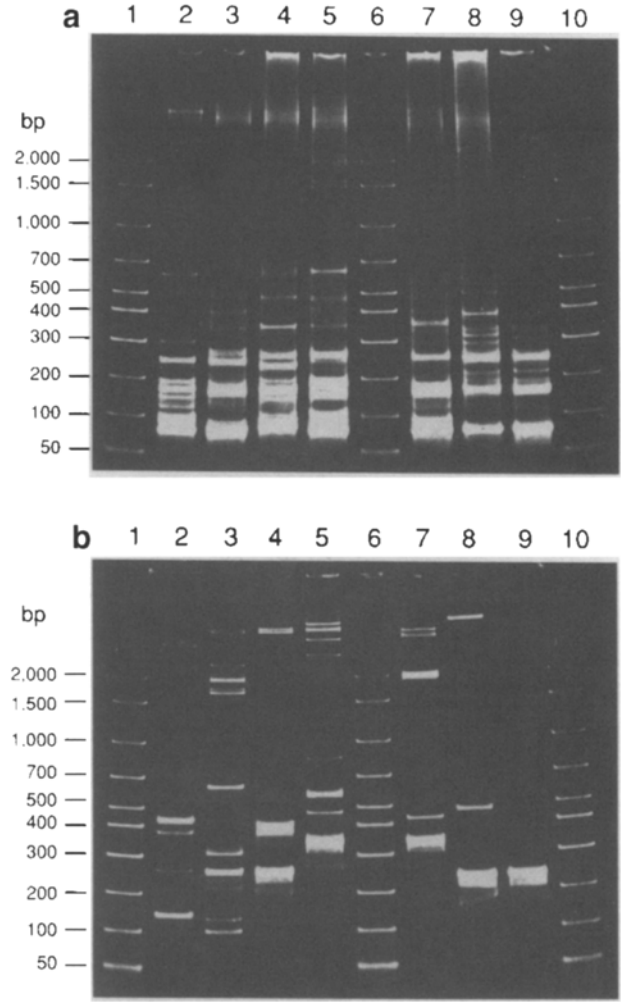

Figure 7 PCR fingerprinting of *Bacillus* species. (a) Low resolution fingerprinting. (b) High resolution fingerprinting. Lanes 1, 6, and 10, markers; (2) *B. polymyxa* ATCC 842; (3) *B. licheniformis* ATCC 12759; (4) *B. subtilis* ATCC 6051; (5) *B. circulans* ATCC 4513; (7) *B. megaterium* ATCC 14581; (8) *B. mycoides* ATCC 6462; and (9) *B. cereus* ATCC 14579

resolution fingerprints of the *Bacillus* species (Figure 7a) indicates that all are different. However, two of the species, *B. mycoides* ATCC 6462 (Figure 7a, lane 8) and *B. cereus* ATCC 14579 (Figure 7a, lane 9), did exhibit low resolution fingerprints which were very similar. This would be expected since DNA/DNA hybridization studies demonstrated a high degree of homology between *B. cereus* and *B. mycoides* [16] and phenotypic studies suggest that *B. mycoides* may be a subspecies of *B. cereus* [13]. The difference between the patterns for *B. cereus* ATCC 14579 and *B. mycoides* ATCC 6462, however, was evident when their high resolution fingerprints were compared (Figure 7b, lanes 8 and 9). The high resolution fingerprint for *B. mycoides* (Figure 7b, lane 8) exhibited three major bands; a very intense band located between 200 and 300 bp, a less intense band at about 500 bp, and another band located at >2000 bp. In comparison, the high resolution fingerprint of *B. cereus* (Figure 7b, lane 9) exhibited only the intense band located between 200 and 300 bp.

The PCR fingerprints for some photosynthetic non-sulfur bacteria are presented in Figure 8 and show that low resolution fingerprinting (Figure 8a) readily distinguishes between species from three different genera. Comparison of the low resolution fingerprints for each of the four strains of *Rhodobacter sphaeroides*, however, shows that three of them (Figure 8a, lanes 5–7) were very similar. The low resolution patterns of strains ATCC 21455 (Figure 8a, lane 6) and ATCC 21286 (Figure 8a, lane 7) in fact were identical, suggesting that these cultures may be the same. An examination of the high resolution fingerprints for these two cultures (Figure 8b, lanes 6 and 7) shows that the strains are related, but not identical and that each of them is different from ATCC 17023 (Figure 8b, lane 5).

The ability of PCR fingerprinting to be applied and to discriminate across broad groups of bacteria is demonstrated in Figure 9. Low resolution PCR fingerprints were generated for a representative species from each of 32 different genera of bacteria, including many of the acti-

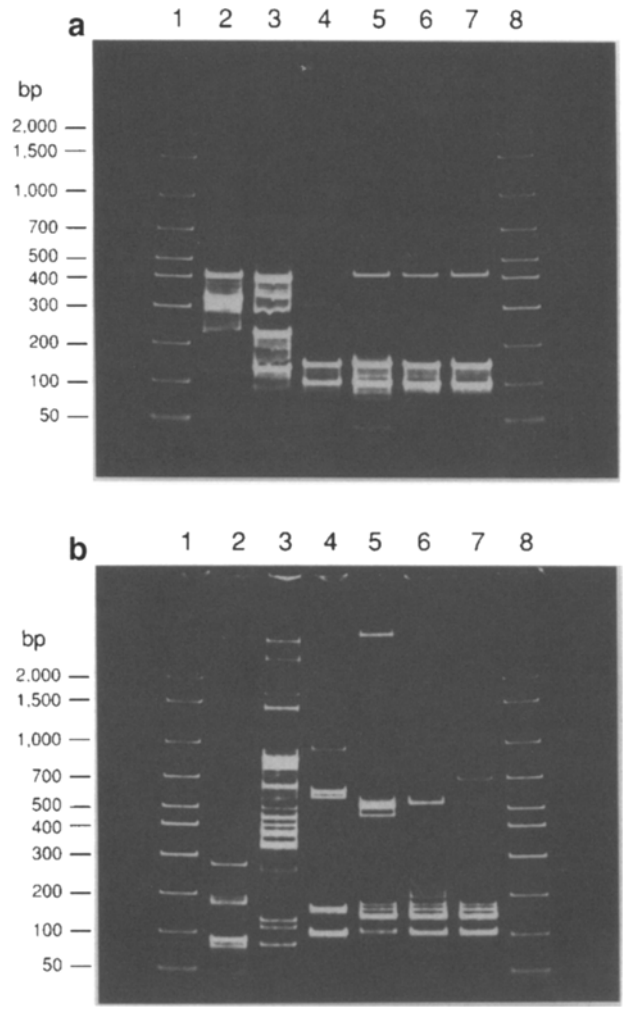

Figure 8 PCR fingerprinting of photosynthetic non-sulfur bacteria. (a) Low resolution fingerprinting. (b) High resolution fingerprinting. Lanes 1 and 8, markers; (2) *Rhodospirillum rubrum* ATCC 277; (3) *Rhodopseudomonas blastica* ATCC 33485; (4) *Rhodobacter sphaeroides* ATCC 33575; (5) *Rhodobacter sphaeroides* ATCC 17023; (6) *Rhodobacter sphaeroides* ATCC 21455; and (7) *Rhodobacter sphaeroides* ATCC 21286

nomycete genera which historically have been dealt with in microbial products screening programs. A comparison of the low resolution fingerprints showed that each of the different cultures produced a unique fingerprint.

Discussion

Low resolution and high resolution PCR fingerprinting appear to offer particular advantages for use in differentiating bacteria for microbial products screening. The fingerprint patterns generated for each strain are reproducible and independent of cultivation conditions. The techniques are easy to perform and, since few experimental steps are required, the chance for the introduction of experimental error should be low. This would facilitate interlaboratory comparison of data. The speed with which PCR fingerprinting can be done allows the differentiation of a culture to species and subspecies level in about 7 h and requires only a small amount of biomass (eg, a single colony). Most of the steps involved in PCR fingerprinting would be amenable to automation, so PCR fingerprinting would be able to handle the large numbers of cultures which normally are dealt with in microbial products screening programs. Finally, as illustrated by results with *P. syringae* pathovars, the combination of low resolution and high resolution PCR fingerprinting offers a hierarchical system of differentiation; with low resolution fingerprinting resolving cultures at the species level and high resolution fingerprinting differentiating at subspecies level. For screening, the ability to group cultures at a lower level of resolution, such as is possible with low resolution fingerprinting, should be useful, particularly for the design and application of fermentation conditions and the development of specific culture isolation methodology. The ability of high resolution fingerprinting to discriminate cultures at the subspecies level provides a means for determining if isolates which appear the same by low resolution fingerprinting are identical. For microbial products screening, such information would be useful in evaluating which cultures should be selected for screening so that repeated testing of the same culture could be avoided.

Recent reports [1, 5, 17] have described the use of another PCR technique, randomly amplified polymorphic DNA (RAPD), for the differentiation of microorganisms for microbial products screening. The authors reported the variability of the RAPD fingerprints with variation in the concentration of template DNA [5]. Other investigators made similar observations [4, 14] and the variability of RAPD fingerprints with other parameters has been noted [14, 15]. In contrast to RAPD, low resolution and high resolution PCR fingerprinting appear to be more applicable methods for use in differentiating bacteria for microbial products screening. As shown in this study, these techniques generate reproducible fingerprints with varying concentrations of template DNA. As a result of the ability of low resolution and high resolution fingerprinting to generate reproducible fingerprints with varying concentrations of template DNA, there is no need to extract, purify, and standardize the template DNA for each culture which one wishes to investigate such as is required for RAPD fingerprinting. The simple and rapid method of using a hot aqueous cell extract as a source of template DNA appears to be sufficient. In those rare cases where a low efficiency of extraction leads to a template DNA concentration in the extract which is too low to produce an intense fingerprint, we have found that by increasing the concentration of cells in the extract a sufficient template DNA concentration can be obtained (unpublished data).

The targets for low resolution PCR fingerprinting and high resolution PCR fingerprinting, prokaryotic tRNA genes and rRNA genes, respectively, are present in all bacteria. Thus, PCR fingerprinting would be applicable to all bacteria. The PCR products which were generated by low resolution fingerprinting of *Staphylococcus* species have been studied by Welsh and McClelland [21]. They have shown by DNA sequencing of some of the low resolution PCR fingerprint bands that the PCR products produced represent polymorphic intergenic spacer regions between

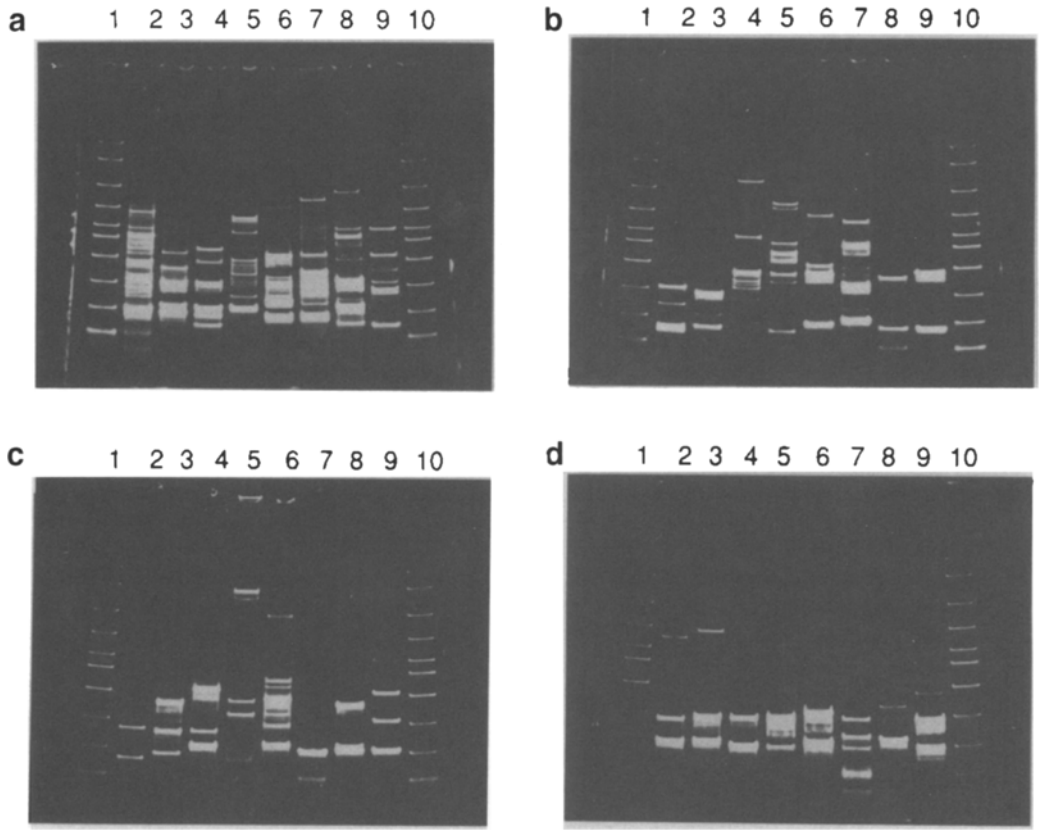

Figure 9 Low resolution PCR fingerprinting of species representing 32 different genera of bacteria. Lanes 1 and 10, markers. (a); (2) *Actinomadura citrea* ATCC 27887; (3) *Streptoverticillium baldaccii* ATCC 23654; (4) *Streptomyces griseus* ATCC 23345; (5) *Nocardioides albus* ATCC 27980; (6) *Pseudonocardia compacta* ATCC 35407; (7) *Nocardiopsis dassonvillei* ATCC 23218; (8) *Nocardia asteroides* ATCC 19247; (9) *Spirillospora albida* ATCC 15331. (b); (2) *Ampullariella lobata* ATCC 15350; (3) *Actinoplanes missouriensis* ATCC 14538; (4) *Streptosporangium roseum* ATCC 12428; (5) *Planomonospora venezuelensis* ATCC 23865; (6) *Planobispora longispora* ATCC 23867; (7) *Rhodococcus coprophilus* ATCC 29080; (8) *Microbispora* sp ATCC 55140; (9) *Microtetraspora glauca* ATCC 23057. (c); (2) *Dactylosporangium aurantiacum* ATCC 23491; (3) *Micromonospora carbonacea* ATCC 27114; (4) *Promicromonospora citrea* ATCC 15908; (5) *Geodermatophilus obscurus* ATCC 25078; (6) *Arthrobacter crystallopoietes* ATCC 15481; (7) *Sporichthya polymorpha* ATCC 23823; (8) *Oerskovia turbata* ATCC 25835; (9) *Intrasporangium calvum* ATCC 23552. (d); (2) *Saccharothrix australiensis* ATCC 31497; (3) *Kibdelosporangium aridum* ATCC 39323; (4) *Kineospora aurantiaca* ATCC 29727; (5) *Saccharopolyspora hirsuta* 27875; (6) *Kitasatosporia setae* ATCC 33774; (7) *Streptoalloteichus hindustanus* ATCC 31217; (8) *Pilimelia terevasa* ATCC 25603; (9) *Amycolatopsis mediterranei* ATCC 13685

adjacent tRNA genes. Welsh and McClelland postulated that, during amplification, the tRNA consensus primers pick the best pairs of matches in a particular genome and, since tRNA gene sequences and their organization are unlikely to differ significantly between closely related species, similar sized PCR products are produced. The PCR products produced using high resolution PCR fingerprinting represent the intergenic regions between the 16S and 23S prokaryotic rRNA genes [7, 12]. Thus, the pattern of bands produced by high resolution fingerprinting is determined by a combination of the number of rRNA operons present in a particular genome and the number of heterogeneous spacer sequences existing among the different 16S–23S rRNA genes. For example, *Escherichia coli* contains seven rRNA operons [11] and within these there exist two classes of intergenic spacer regions. Three of the operons have spacer regions containing the tRNA genes for isoleucine and alanine and the remaining four operons contain the tRNA gene for glutamic acid [10]. The degree to which the 16S–23S intergenic spacer region appears to vary among bacteria is quite great since Jensen *et al* [7] reported that they were able to distinguish 300 strains of bacteria belonging to 28 different species or serotypes using high resolution fingerprinting. The dependence of the high resolution PCR fingerprinting band pattern on the number of rRNA operons present in a genome may account for the rapid decline in band intensity of the PCR-ribotyping fingerprint of *P. cepacia* that was observed by diluting the extract (Figure 3c). If the number of rRNA operons present in *P. cepacia* ATCC 25416 is low, then the targets for high resolution PCR fingerprinting would be diluted out much faster than if a larger number of rRNA operons were present. Based on the high resolution fingerprint of *P. cepacia* ATCC 25416, one would expect, however, at least two rRNA operons to be present in the genome of this bacterium.

We have used low resolution and high resolution PCR fingerprinting routinely in our laboratory for the past year to differentiate non-actinomycete eubacteria isolated from soil and have found it to offer an effective means for differentiating a variety of bacteria. In our experience, two situations in particular demonstrate the value of PCR fingerprinting. The first, and most common, has been the ability

to determine if morphologically identical isolates are or are not different. The second has been the ability of PCR fingerprinting to determine if morphologically different cultures are or are not different. This situation has arisen most often with cultures that spontaneously generate morphological variants. By using PCR fingerprinting, we have been able to establish the presence of variants because, despite their sometimes considerable morphological differences, their low resolution and high resolution fingerprints are identical. In a situation where the morphological 'variants' actually represent a mixed culture, different PCR fingerprints for each of the 'variants' is observed.

We have encountered very few problems using PCR fingerprinting. One of the most commonplace has been the presence in some extracts of substances which inhibit the amplification reaction. Our studies indicate that the polysaccharides produced by certain bacteria are effective inhibitors of the PCR amplification (unpublished data). The easiest solution which we have found for this problem is to suppress or minimize polysaccharide formation by using media which do not contain or contain only low levels of carbohydrate.

In using PCR fingerprinting, we have found that small numbers of fingerprints may be readily compared with each other by visual inspection. However, the best approach for analyzing large numbers of fingerprints would be the use of software that has been developed for imaging, storing, and comparing electrophoretic band patterns [18]. An inherent problem in using visual analysis to compare fingerprints is the inability to reliably normalize patterns within and between gels. It also is difficult to correct for any distortions of the lanes, such as is observed in the outermost lanes of the 4–20% acrylamide gradient gels which we use. We have investigated the use of single concentration acrylamide gels, where there is no distortion in the outermost lanes, however, we have found that 4–20% acrylamide gradient gels provide the best resolution of the bands. Recently, we have begun to analyze fingerprint patterns using the Gel Compar software package from Applied Maths (Risquons-Toutstraat 38, B-8511 Kortrijk, Belgium). We have found that this software allows normalization within and between gels and that it readily corrects for any distortions of the lanes. We anticipate that computerized analysis of PCR fingerprints will provide a very powerful, reliable, and easy to use tool for microbiologists involved in microbial products screening. With computerized fingerprint analysis, one could easily monitor the diversity of the bacteria being tested in a screening program; flag rare, novel, or otherwise interesting cultures; target cultures with specific fingerprints for testing in specific assays; design and evaluate new culture isolation methodology; and group cultures for specific fermentation conditions. In effect, it could provide a tool for screening programs to expand to unexplored areas of the bacterial world in a logical and rational way and, in so doing, help lead to the discovery of new and interesting microbial products.

References

1 Anzai Y, T Okuda and J Watanabe. 1994. Application of the random amplified polymorphic DNA using the polymerase chain reaction for efficient elimination of duplicate strains in microbial screening. II. Actinomycetes. J Antibiot 47: 183–193.
2 Brousseau R, A Saint-Onge, G Prefontaine, L Masson and J Cabana. 1993. Arbitrary primer polymerase chain reaction, a powerful method to identify *Bacillus thuringiensis* serovars and strains. Appl Environ Microbiol 59: 114–119.
3 Cote R (ed). 1984. ATCC Media Handbook. American Type Culture Collection, Rockville, MD.
4 Devos KM and MD Gale. 1992. The use of random amplified polymorphic DNA markers in wheat. Theor Appl Genet 84: 567–572.
5 Fujimori F and T Okuda. 1994. Application of the random amplified polymorphic DNA using the polymerase chain reaction for efficient elimination of duplicate strains in microbial screening. I. Fungi. J Antibiot 47: 173–182.
6 Goodfellow M and AG O'Donnell. 1993. Roots of bacterial systematics. In: Handbook of New Bacterial Systematics (Goodfellow M and AG O'Donnell, eds), pp 3–54, Academic Press, New York, NY.
7 Jensen MA, JA Webster and N Straus. 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. Appl Environ Microbiol 59: 945–952.
8 Johnson JL. 1984. Nucleic acids in bacterial classification. In: Bergey's Manual of Systematic Bacteriology (Krieg NR and JG Holt, eds), 1, pp 8–11, Williams and Wilkins, Baltimore, MD.
9 Johnson JL. 1994. Similarity analysis of DNAs. In: Methods for General and Molecular Bacteriology (Gerhardt P, ed), pp 655–682, American Society for Microbiology, Washington, DC.
10 Kenerly ME, EA Morgan, L Post, L Lindahl and M Nomura. 1977. Characterization of hybrid plasmids carrying individual ribosomal ribonucleic acid transcription units of *Escherichia coli*. J Bacteriol 132: 931–949.
11 Kiss A, B Sain and P Venetainer. 1977. The number of rRNA genes in *Escherichia coli*. FEBS Lett 79: 77–79.
12 Kostman JR, TD Edlind, JJ LiPuma and TL Stull. 1992. Molecular epidemiology of *Pseudomonas cepacia* determined by polymerase chain ribotyping. J Clin Microbiol 30: 2084–2087.
13 Logan NA and RCW Berkeley. 1984. Identification of *Bacillus* using the API system. J Gen Microbiol 130: 1871–1882.
14 MacPherson JM, PE Eckstein, GJ Scoles and AA Gajadhar. 1993. Variability of the random amplified polymorphic DNA assay among thermal cyclers, and effects of primer and DNA concentration. Molec Cell Probes 7: 293–299.
15 Meunier J-R and PAD Grimont. 1993. Factors affecting reproducibility of random amplified polymorphic DNA fingerprinting. Res Microbiol 144: 373–379.
16 Sneath PHA. 1986. Endospore-forming gram-positive rods and cocci. In: Bergey's Manual of Systematic Bacteriology (Sneath PHA, ed), 2, p 1131, Williams and Wilkins, Baltimore, MD.
17 Tanaka H, S Sawairi and T Okuda. 1994. Application of the random amplified polymorphic DNA using the polymerase chain reaction for efficient elimination of duplicate strains in microbial screening. III. Bacteria. J Antibiot 47: 194–200.
18 Vauterin L, J Swings and K Kersters. 1993. Protein electrophoresis and classification: In: Handbook of New Bacterial Systematics (Goodfellow M and AG O'Donnell, eds), pp 251–280, Academic Press, New York, NY.
19 Visuvanathan S, MT Moss, JL Stanford, J Hermon-Taylor and J McFadden. 1989. Simple enzymic method for isolation of DNA from diverse bacteria. J Microbiol Methods 10: 59–64.
20 Welsh J and M McClelland. 1991. Genomic fingerprints produced by PCR with consensus tRNA gene primers. Nucleic Acids Res 19: 861–866.
21 Welsh J and M McClelland. 1992. PCR-amplified length polymorphisms in tRNA intergenic spacers for categorizing staphylococci. Mol Microbiol 6: 1673–1680.