DNA Fingerprinting Techniques for Microorganisms

A Proposal for Classification and Nomenclature

Mario Vaneechoutte

Abstract

A whole array of DNA-fingerprinting techniques, which provide indirect access to DNA sequence polymorphism in order to assess species or clonal identity of bacterial organisms or in order to study bacterial genome composition, have been described during past decades. Nomenclature has been sometimes erroneous and/or confusing, also because of hybrid techniques that combine different approaches. It can be shown that most techniques study the sequence polymorphism of only the chromosome, or only the plasmid(s) or only a gene or gene fragment and that the sequence polymorphism is revealed by AFLP (amplified fragment length polymorphism) or by RFLP (restriction fragment length polymorphism) or by special electrophoresis techniques. Starting from these considerations, some taxonomy of techniques, which enables more appropriate nomenclature, can be developed.

Index Entries: DNA-fingerprinting; bacteria; restriction; amplification; identification; typing; chromosome; plasmid; RFLP; AFLP; SSCP; SRFA; DGGE; PFGE.

The most direct and most informative approach to studying DNA-sequence polymorphism is DNA-sequence determination. Since this approach is currently still laborious and tedious, and since it does not allow analysis of larger parts of the genome or of complete chromosomes, a whole array of DNA-fingerprinting techniques has been described during past decades, which enable the study of the DNA-sequence polymorphism or the genetic composition of a plasmid or a chromosome in an indirect way. These techniques have been applied for typing, i.e., assignation of bacterial isolates to clonally related lineages (clones, strains) within a single species, for identification, i.e., assignation of isolates to different species, for chromosome mapping, and for taxonomic studies.

More recently, because of the possibility of amplifying DNA-fragments enzymatically *(1,2)*, the scientific community has been overwhelmed by an avalanche of new PCR-based DNA-fingerprinting techniques. This has added to the already existing confusion about the designation of DNA-fingerprinting techniques, since the same names are used for different techniques, the same technique has several names, and some designations actually refer to another technique or to a nonrelevant part of the technique, or refer to a collection of techniques, or are not very informative. This often makes it impossible to retrieve literature or to know which technique a colleague is referring to.

This contribution is a preliminary attempt to weed out this Babylonian confusion of tongues by setting out some guidelines which might allow a more logical organization of the classification and designation of DNA-fingerprinting techniques.

1. Some Examples of Terminology that May Lead to Confusion

1.1. The Same Designation Used for Different Techniques

rDNA-restriction analysis can be done by amplification of (part of) the rRNA-cistron (rDNA)

Address to whom all correspondence and reprint requests should be addressed: Department of Clinical Chemistry, Microbiology & Immunology Blok A, University Hospital, B 9000 Ghent, Belgium, E-mail: Mario.Vaneechoutte@rug.ac.be

followed by restriction analysis of the amplicon (*see* Section 4 2.3.3). However, the designation "ribosomal nucleic acid gene restriction analysis" has already been used in the original description of a technique *(3)* that is commonly known as ribotyping. Ribotyping consists of restriction digestion of the complete chromosome, followed by hybridization with a probe complementary to the rDNA. Ribotyping makes use of the rDNA, not because of the intrinsic species-specific sequence information of this region, as is the case for rDNA-restriction analysis (*see* Section 4.2.3.3., item 1), but because the rDNA is present in multiple copies in most bacteria. Thus, hybridization with this repeat allows highlighting of only a few of the chromosomal DNA restriction fragments and enhances the interpretability of the obtained fingerprint. Perfect ribotyping patterns can be obtained without a single restriction event within the rDNA. A more appropriate name for this technique would be "rDNA-selective restriction fragment hybridization (rDNA-SRFH)" (*see* Section 4.2.2.3., item 3d).

1.2. Several Designations in Use for a Single Technique

Arbitrarily primed PCR fingerprinting (AP-PCR) *(4)* has also been called "randomly amplified polymorphic DNA" (RAPD) *(5)*, "DNA-amplified fingerprinting" (DAF) *(6)*, "arbitrary primer-PCR" *(7,8)*, "multiple arbitrary amplicon profiling" (MAAP) *(9)*, and "PCR-mediated genotyping" *(10)*.

1.3. Designations Referring to Another Technique

In the case of rDNA-SRFH, the designation "ribosomal nucleic acid gene restriction analysis" actually refers to true restriction analysis of (part of) the rDNA, which is only possible since the availability of PCR. The designation "ribotyping" has been used also in the description of techniques that are completely different from rDNA-SRFH itself: "PCR-ribotyping" for DNA-fingerprinting based on the amplification of the rRNA 16S-23S spacer region *(11)* (*see* Section 4.2.3.5., item 4) and "long PCR ribotyping" for restriction analysis of the complete rDNA *(12)* (*see* Section 4.2.3.3., item 2).

1.4. Designations Referring to a Nonrelevant Part of the Technique

DNA-restriction analysis with rare cutting restriction enzymes (i.e., low-frequency restriction analysis) (*see* Section 4.2.2.3., item 3a) is most often designated as "pulsed field gel electrophoresis (PFGE)" (e.g., *13–15*). This designation describes only a part of the technique and even refers to a part of the technique which is not crucial in revealing the polymorphism. PFGE is necessary only to enable separation of the large restriction fragments. Moreover, other electrophoresis techniques (like clamped homogeneous electric fields [CHEF] electrophoresis) can be used to achieve this aim. Finally, PFGE and related techniques can also be used for other purposes, like separation of complete chromosomes (*see* Section 4.2.2.2.).

1.5. Designations that Refer to a Collection of Techniques

Restriction fragment length polymorphism (RFLP) analysis is the collection of techniques that use restriction digestion to study DNA-sequence polymorphism. However, "RFLP" is often used to refer to SRFH techniques alone (e.g., 14), while SRFH is only one of several possible RFLP analysis approaches (*see* Sections 4.2.1.3., 4.2.2.3., and 4.2.3.3.). Designations like "PCR-mediated genotyping" *(10)* and "DNA amplified fingerprinting" *(6)*, which have been used for AP-PCR, are designations that apply to all amplified fragment-length polymorphism (AFLP) analysis techniques. "DNA-fingerprinting," which has been used for IS6110-SRFH *(16)*, refers to all techniques reviewed here.

1.6. Noninformative Designations

"Ribotyping" (e.g., *17,18*) is a well-known designation for rDNA-SRFH, but it is not descriptive. Designations like "polymerase chain reaction fingerprinting" *(19)* and "ribosomal DNA-fingerprinting" *(20)* do not provide a detailed description of the concept of the technique.

1.7. Other Remarks

Designations for techniques should refer to a technique or a method. For example, a designation like "RAPD" refers to DNA; designations

like "RFLP" or "AFLP" refer to a polymorphism. If these designations are used, preferably, this should be done in conjunction with "analysis." Also, the term "polymorphism" is often used to refer to a fingerprint, although polymorphism is a natural characteristic of a population. This leads to the erroneous use of the plural form of this substantive (e.g., *5,21–29*).

2. Delineation of the Collection of DNA Fingerprinting Techniques and Account of Its Designation

As is the case for the techniques, the collection of DNA-fingerprinting techniques has many names, like DNA-typing, genotyping, molecular typing, and genomic fingerprinting. Since the techniques dealt with here use DNA to produce one-dimensional graphs, i.e., DNA fingerprints, we suggest using "DNA-fingerprinting" as the most descriptive designation.

First, "DNA" seems to be most fit for general use, since "genomic" refers too specifically to the complete genomic information of a cell. For bacteria, this includes chromosomes and plasmids, while most of the techniques discussed here aim at studying only the plasmid, only the chromosome, or only a gene (*see* Section 4.1.).

"Molecular" is meant as an abbreviation of "molecular biology based," but is rather confusing. Moreover, the definition of what is meant by "molecular biology" is not always clear, since for some it refers to the study of only nucleic acids, while for others it includes also proteins, lipopolysaccharides, and fatty acids.

Furthermore, we would opt to restrict the use of "typing" to techniques for differentiation of strains of a single species (corresponding to e.g., biotyping, serotyping, phage typing). Therefore, the use of "typing" would exclude techniques that yield species- or genus-specific information or that enable the study of taxonomy and chromosomal organization.

DNA-fingerprinting techniques then could be considered as belonging to a class of nucleic acid-based fingerprinting techniques (together with the RNA-fingerprinting techniques that have been described for studying differential gene expression [e.g., ref. *30*]). Nucleic acid-based fingerprinting, together with, e.g., DNA-DNA hybridization, belongs to a class of comparative techniques for genotypic polymorphism analysis, as opposed to analytical sequence polymorphism analysis techniques like DNA- sequencing and chromosomal GC-content analysis (Table 1).

In conclusion, DNA-fingerprinting techniques are composed of those techniques that allow study of DNA-sequence polymorphism by the production of DNA-fingerprints. DNA-fingerprints consist of one or more DNA-fragments separated from each other by electrophoresis. The pattern may be a real image, e.g., a stained gel, or a digital graph, e.g., as obtained after fluorescent fragment electrophoresis (*see* Section 5.4.1.). "Typing" preferably is used for differentiation between strains of a single species and not for fingerprinting.

3. Proposal for Classification of DNA-Fingerprinting Techniques

3.1. Introduction

Since names should refer to the right category level, they can only be given after the category levels have been defined. Thus, one needs a classification of techniques and the names will depend on which approach is followed to categorize the techniques. Careful analysis of the techniques we want to classify here makes possible the delineation of some classification guidelines.

3.2. Classification Approach

All DNA-fingerprinting techniques could be said to have two technical aspects in common: isolation of the part of the genome one wants to study; and subjection of the isolated part of the genome to some technique that may allow us to reveal whether and to what extent DNA-sequence polymorphism is present in the population. In general, three parts of the genome are isolated for further study. Isolation of the plasmid(s), of the chromosome, and of genes or gene fragments are discussed in Section 4.2.

The best known and most widely applied approaches for studying polymorphism in a bacterial population are restriction and amplification, whereby electrophoresis enables the separation of

Table 1
Proposal for a Classification
of Most of the Currently Used DNA-Fingerprinting Techniques with Proposal of Nomenclature

DNA sequence polymorphism analysis techniques[a]
Phenotypic polymorphism analysis [phenotyping][1]
Genotypic polymorphism analysis [genotyping][1]
I. Analytical characterization: DNA sequence determination (sequencing), GC-content determination
II. Comparative characterization:
• Hybridization based analysis:
DNA-DNA hybridization
DNA-RNA hybridization
Heteroduplex formation *(88,89)*
• Nucleic acid-based fingerprinting (genotypic fingerprinting)
[genomic fingerprinting],[2] [genotyping],[1,3] [molecular typing],[1,4] [DNA-fingerprinting][5]
A. RNA-fingerprinting
RNA-arbitrarily primed PCR (RAP) *(30)*: Gene expression
B. DNA-fingerprinting
[genomic fingerprinting],[2,3] [genotypic fingerprinting],[3] [genotyping],[1,3] [molecular typing],[1,4] [nucleic acid-based fingerprinting][3]
1. Plasmid DNA fingerprinting
1.1. E[b]: Plasmid DNA content polymorphism analysis[c] (plasmid profile analysis) *(18,35,37)*: strain[d]
1.2. R: Plasmid DNA RFLP analysis (plasmid DNA restriction analysis) *(33,35,38,39)*: plasmid characterization, strain
1.3. A: Plasmid DNA AFLP analysis
2. Chromosomal DNA fingerprinting
2.1. E: Chromosomal DNA content polymorphism analysis (chromosomal DNA profile analysis) *(40)*: only for organisms with more than one chromosome[e]
2.2. R: Chromosomal DNA RFLP analysis[f]
2.2.1. Chromosomal DNA RFLP analysis *(43)*: strain
2.2.2. (Observable) restriction fragment number-reducing modifications of chromosomal DNA restriction analysis
2.2.2.1. Low-frequency RFLP analysis *(13–15,44–48)*: strain, (species), chromosome mapping (macrorestriction analysis, large fragment restriction analysis) [pulsed field gel electrophoresis (PFGE)][6]
2.2.2.2. High size restriction fragment analysis *(41)* (high-frequency RFLP analysis)
2.2.2.3. Low size restriction fragment analysis *(49,50)*
2.2.2.4. Selective restriction fragment hybridization (SRFH): strain, (species)
e.g., *rDNA*-SRFH[g] *(3,14,17,18,21,22,52,57–62,119)*: [ribotyping],[1,4] [rDNA restriction analysis]:[7] strain, (species)
e.g., *M13 bacteriophage repeat*-SRFH *(53)*: strain
e.g., *IS6110*-SRFH *(16)*: *Mycobacterium tuberculosis* strain
2.2.2.5. Selective restriction fragment amplification (SRFA): strain, (species)
Primer dependent SRFA *(64–66)*: [AFLP™][3,4,7,8]
Type IIS-restriction SRFA *(63,68)*: [ligation-mediated PCR][4,6,7]
Mixed linker *IS6110* SRFA *(67)*: [mixed-linker PCR][4,6]
Partial ligation *IS6110* SRFA *(69)*: [ligation-mediated PCR][4,6,7]
2.3. R+E: 2-D-DNA gel electrophoresis *(72)* (RFLP-DGGE)
2.4. A: Chromosomal DNA AFLP analysis[h]
2.4.1. Arbitrarily primed-PCR (AP-PCR) *(4–10,18,19,28,73–79,141)*: strain, (species) [DNA-amplified fingerprinting (DAF), PCR-mediated fingerprinting,...],[3,4] [AFLP],[3,4,8] [RAPD][8]
2.4.2. Interrepeat spacer-length polymorphism analysis
ERIC-interrepeat-PCR, REP-interrepeat-PCR *(81)*: strain, (species)
tRNA-interrepeat-PCR *(29,78,84)*: (strain), species

Table 1 *(continued)*

2.5. A+E: strain, (species)
AP-PCR and DGGE *(85,86)*
AP-PCR and SSCP analysis *(87)*

3. Gene (fragment) fingerprinting (chromosomal or plasmidal origin)
3.1. E:
3.1.1. *Gene*-denaturing gradient gel electrophoresis (DGGE) *(88–90)*: gene, species
3.1.2. *Gene*-SSCP-analysis *(27,89,93–96,142)*: gene, species
3.1.3. Dye intercalation mediated sequence-dependent electrophoresis *(97)*
3.2. R: *Gene* RFLP analysis:
3.2.1. Sequence dependent gene RFLP analysis
(partial) *rDNA*-RFLP analysis *(28,52,99–118)*, *histidine*-RFLP analysis *(112)*, *hsp65*-RFLP analysis *(120–122)*: species
complete *rDNA*-RFLP analysis *(12)* [long-PCR-ribotyping][1,4,7] *flagellin*-RFLP analysis *(124)*: strain
β-*lactamase*-RFLP analysis *(34)*: plasmid, gene
3.2.2. Single-strand conformation dependent RFLP-analysis
Cleavase fragment length polymorpism (CFLP™) analysis *(125b)*: strain, species, gene
3.3. R+E: *Gene*-RFLP-SSCP-analysis *(126,127)*: strain, species, gene [REF][3,7]
3.4. A: *Gene* AFLP analysis
3.4.1. Tandem repeat-length polymorphism analysis: strain, (species)
e.g., *Mycobacterium tuberculosis* direct repeat-length polymorphism analysis *(130)*
e.g., *Staphylococcus aureus* protein A associated tandem repeat-length polymorphism analysis *(128)*
Variable number tandem repeat (VNTR) analysis (eukaryote species: *131*, bacterial strains: *24*)
3.4.2. *Gene* length polymorphism analysis *(132)*: species
3.4.3. Repeat-length polymorphism analysis: *rRNA-spacer* length polymorphism-analysis[i] [PCR-ribotyping][1,4,7]
(11,62,135): strain
(25,26,134): species
3.4.4. Gene AP-PCR[i] *(117)*: strain
3.4.5. Dideoxyterminated SSCP analysis *(23,143)* [dideoxyfingerprinting][4]

Remarks:
[a]Levels ranking taxonomically higher than DNA-fingerprinting are given to situate the position of the DNA-fingerprinting techniques in the scheme proposed here.
[b]Refers to the procedure essential for revealing the polymorphism: E: electrophoresis, R: restriction, A: amplification.
[c]Where possible the neutral analysis can be replaced by the more informative designation "typing" or "identification."
[d]Indication of purpose(s) for which the technique is most frequently applied.
[e]Evidence has been presented that some prokaryotes may possess more than one chromosome *(15,40)*.
[f]Interference of plasmids cannot be excluded for most of the chromosomal DNA RFLP analysis techniques. However, the techniques aim at studying only the chromosome.
[g]*Italic*: usage of the name of the repeat or the gene that has been used; provides a more precise description of the technique.
[h]Interference of plasmids is unlikely for most chromosomal DNA AFLP analysis techniques, while reports for AP-PCR differ *(7,8)*.
[i]The molecular explanations for this technique are contradictory and/or uncertain (*see*, e.g., *136,140*).
Names:
( ): other possible designation.
{ }: technique not yet described, according to our knowledge.
[ ]: confusing and/or erroneous designations for the following reasons:
[1]The use of typing preferably is reserved for assignation of bacterial isolates to clonally related lineages (clones or strains) within a species.
[2]Genomic, when used for bacteria, is comprised of chromosomal and plasmid DNA. Mostly DNA-fingerprinting techniques aim at studying only chromosomal or only plasmid DNA polymorphism.
[3]Refers to a higher level of techniques.
[4]Is not a descriptive designation.
[5]Refers to a lower level of techniques.
[6]Refers to only a part of the technique, which, moreover, is not the sequence polymorphism revealing part.
[7]Refers to another technique or the same designation has been used to refer to another technique.
[8]Refers to a polymorphism or to DNA, not to a technique. Should be used in conjunction with analysis.

the obtained DNA-fragments, to make the RFLP or AFLP apparent and interpretable. In other cases, the polymorphism can be read directly after isolation of the DNA (e.g., plasmid content analysis [*see* Section 4.2.1.2.], or is revealed only by the use of special, sequence-dependent, electrophoresis techniques (*see* Section 5.2.3.2.). Therefore, DNA-fingerprinting techniques could be classified in an informative manner according to the part of the genome (i.e., chromosome, plasmid, or gene[fragment]) that is studied and according to the technique (i.e., restriction, amplification, electrophoresis, or combinations) that is used to reveal the polymorphism present in the part of the genome studied. The technique responsible for revealing the polymorphism then receives priority in naming the DNA-fingerprinting method.

4. Application of Classification Proposal to DNA-Fingerprinting Techniques

4.1. Introduction

As indicated above (Section 2.), DNA-fingerprinting techniques rarely aim at studying the complete genome, i.e., chromosome and plasmid(s) together. To the contrary, plasmid interference in chromosomal fingerprinting is generally avoided, and vice versa. A theoretical basis to do so is apparent, since plasmids can also be horizontally transferred (even between isolates belonging to different species), while chromosomes are transferred only between generations (vertically) and thus allow the study of clonal relatedness. Although exchange of parts of the chromosome is known, and has even been suggested to occur between gram-positives and gram-negatives *(31)*, it is, according to current knowledge, a rare event which barely interferes with the clonal concept. The value of plasmids as clonal markers is probably more limited to the study of short time-events, like nosocomial outbreaks. Also plasmid constitution itself is more variable than that of the chromosome. On the other hand, it may in some instances be more relevant to fingerprint plasmids than chromosomes, since plasmids are often the carriers of virulence or of antibiotic resistance mechanisms, like the extended spectrum β-lactamases *(32–35)*, and therefore may be the underlying cause of nosocomial problems.

We will try to indicate for which techniques plasmid or chromosomal contamination may be expected to interfere with the fingerprints obtained. Also, the applicability of each technique for typing, species identification, chromosome mapping, or taxonomic studies will be addressed. Finally, the designations in use will be discussed and, where necessary, more appropriate names will be suggested. Table 1 gives an overview of the proposed classification and of the designations of the different techniques.

4.2. Classification, Nomenclature, and Discussion of the Different Techniques

4.2.1. Plasmid DNA Fingerprinting

4.2.1.1. Plasmid DNA Isolation

Multiple plasmid isolation techniques have been described. Although chromosomal DNA contamination is difficult to avoid in the simple and rapid plasmid isolation techniques that are mostly applied for the purpose of plasmid DNA-fingerprinting, chromosomal DNA contamination rarely interferes with the clustering, since, upon electrophoresis, the chromosomal DNA is present as a single band with the same length for all isolates.

4.2.1.2. Polymorphism Revealed by Electrophoresis

Plasmid DNA content polymorphism analysis is based on the electrophoretic separation of the isolated plasmids. Enhanced sensitivity of plasmid detection has been reported by hybridization with a probe complementary to the $(GTG)_5$-repeat, which allowed visualization of *Shigella* plasmids not observable after agarose electrophoresis *(36)* (*see also* Section 4.2.2.3., item 3d).

Plasmid DNA content polymorphism analysis or plasmid profile analysis has been applied for typing purposes (e.g., *18,35,37*). However, the applicability for typing purposes may strongly differ from species to species, depending on the frequency with which plasmids are present in isolates, on the number of plasmids per organism, and on the degree of intraspecific variability of the plasmid content. The major drawback of using plasmid content analysis for assessing clonal relatedness is the variable plasmidal cell-loyalty,

which may differ strongly among plasmids, strains, and/or species. Also, plasmids of the same length can have completely different sequences and the same plasmid can have different electrophoretic mobility, depending on its configuration (sheared, relaxed, coiled, or supercoiled).

4.2.1.3. Polymorphism Revealed by Restriction

Plasmid DNA RFLP analysis has been applied to characterize the plasmids themselves and as a typing technique *(33,35,38,39)*.

4.2.1.4. Polymorphism Revealed by Amplification

AFLP analysis of plasmids, whereby the isolated complete plasmid(s) are used as the targets for amplification (e.g., by arbitrarily primed-PCR), has not yet been described, but is theoretically possible, although minor chromosomal contamination may interfere strongly *(8)*.

4.2.2. Chromosomal DNA Fingerprinting

4.2.2.1. Chromosomal DNA Isolation

A whole array of chromosomal DNA isolation techniques has been described. Purity from proteins of the isolated DNA is crucial for chromosomal DNA restriction analysis to avoid incomplete digestion. Simple DNA-extraction methods, like boiling, sonication, or protease digestion of cell suspensions, may suffice for amplification.

4.2.2.2. Polymorphism Revealed by Electrophoresis

Chromosomal DNA content polymorphism analysis by separating chromosomes of different length, e.g., by pulsed field gel electrophoresis, would at first sight not be useful for the study of prokaryotes, since these have only a single chromosome. Chromosomal DNA content analysis, however, has been applied to study a strain of *Burkholderia cepacia,* which appears to contain three replicons, each possessing rRNA-cistrons *(40)*. Other bacterial species with at least two chromosomes have been described *(15)*.

4.2.2.3. Polymorphism Revealed by Restriction

1. Introduction: Plasmid contamination interference. For chromosomal DNA restriction analysis plasmid DNA contamination, especially when derived from large plasmids, may interfere and add some restriction fragments to the pattern *(41)*. This also applies for DNA restriction with rare cutting enzymes, whereby plasmids may remain integral. This means that, strictly speaking, chromosomal DNA restriction analysis should actually be called genomic DNA restriction analysis. Since the aim of the technique is to study chromosomal DNA polymorphism, and since little is known about the impact of plasmid contamination on the resulting fingerprint, the designation "chromosomal DNA RFLP analysis" remains appropriate for this approach.
2. Chromosomal DNA restriction fragment length polymorphism (RFLP) analysis. DNA-restriction enzymes have been applied for the study of the bacterial chromosome, at least since 1979 *(42)*.

   Chromosomal DNA RFLP analysis is applicable for typing and is usually carried out with hexacutters (e.g., *43*). For a bacterial genome of 4×10^6 bp, theoretically, this will yield about 1000 restriction fragments. The interpretation of the patterns, mostly obtained after 0.7% agarose electrophoresis, is often difficult because of the large number of fragments.
3. Chromosomal DNA RFLP-fingerprint simplifying modifications.

   Several modifications have been developed of which the main purpose is to reduce the number of (observable) restriction fragments obtained after chromosomal DNA restriction. This allows us to consider all of these techniques, which are very different at first sight, as chromosomal DNA RFLP analysis techniques.
   a. Low frequency RFLP analysis. Restriction with rare cutting enzymes (e.g., *Sma*I) yields less restriction fragments than obtained after restriction with hexacutters. Special care must be taken during chromosome isolation to avoid shearing of the chromosome. Subsequent separation of the fragments needs electrophoresis techniques like pulsed-field electrophoresis, which can handle these large fragments *(44)*. Therefore, this approach is often named "pulsed field gel electrophoresis (PFGE)" (*see* Section 1.4.). More descriptive designations that have been used are, e.g., "macrorestriction analysis" *(45)*,

"usage of low-frequency cleavage restriction endonucleases for DNA analysis" *(46)*, and "large DNA restriction fragment polymorphism analysis" *(47)*.

Typing is the most important application of this widely used approach (e.g., *14,45–47*), and the technique has proven to be valuable in chromosome mapping *(15)* and chromosome size determination *(48)*.

Low frequency RFLP analysis reduces the actual number of restriction fragments, while all modifications discussed below reduce the number of observable restriction fragments.

b. High-size RFLP analysis. By using a tetracutter like *Hae*III, Jaworski et al. *(41)* obtained more interpretable fingerprints, because of the limited number of high-MW restriction fragments that could be more clearly separated than the higher number of high-MW fragments that are usually obtained after restriction with hexacutters (*see* Section 4.2.2.3., item 2). High-frequency RFLP analysis would be another valid description of this approach.
c. Low-size RFLP analysis. Another approach, enabling the reduction of the number of observable restriction fragments, consists of the separation of restriction fragments obtained with hexacutters and observing only the lower molecular size fragments after separation on a high resolution polyacrylamide gel and visualization by silver staining *(49)* or by (radioactive) end-labeling of the restriction fragments *(50)*. This last approach was reported to offer several advantages, including high reproducibility, identical intensity of all fragments, flexibility (depending on restriction enzyme choice), and high signal-to-noise ratio. Furthermore, low-size RFLP analysis was reported to be more discriminatory than (already discriminatory) low frequency RFLP analysis for typing of *Enterobacter cloacae (49)*.
d. Selective restriction fragment hybridization (SRFH). The number of observable restriction fragments can be reduced by hybridization with a probe complementary to a repeat element, which will be present on only some of the restriction fragments (Fig. 1). Subsequent visualization of the hybrids will highlight only those restriction fragments which carry (a part of) such a repeat.

A well-known and successful application is ribotyping *(3)*, which makes use of the rDNA, of which several copies are present in the chromosome of most bacterial species *(15,51)*. For example, use of the rDNA for SRFH will yield at least seven bands for *Escherichia coli* isolates, reflecting those seven chromosomal DNA-restriction fragments with (most probably) different lengths where the rRNA-cistrons happen to be located. Roughly, one more fragment will be generated for each restriction site of the chromosomal DNA, which happens to lie within a rRNA-cistron, causing that part of the cistron will be present on two neighboring restriction fragments (with, most probably, different lengths). According to the principle of this technique as explained here, it is clear that this is not 'restriction analysis of the rDNA' (*see* Section 1.3., Table 2). This is also apparent from the predominantly large size of the observed fragments in rDNA-SRFH fingerprints. However, it should be noted that by using a high-frequency restriction enzyme (like *Hae*III), Salzano et al. *(52)* obtained the same clustering of *Streptococcus thermophilus* isolates as obtained with amplified rDNA-restriction analysis (*see* Section 4.2.3.3.).

Other repeats have been used for SRFH. Use of the ubiquitously present M13 bacteriophage repeat *(53)* is applicable for SRFH-based fingerprinting of most organisms. Insertion sequence repeats are also often used, but these are mostly applicable for one or a few species only (e.g., IS6110-SRFH typing *[16]* or major polymorphic tandem repeat [MPTR]-SRFH typing *[54]* of *Mycobacterium tuberculosis* and BOX-SRFH typing for *Streptococcus pneumoniae [55]*). Finally, random trimer repeats have been used as the probe *(36,56)*.

These techniques are applicable mostly for typing, although species identification is possible with rDNA-SRFH *(17,22,57,58)*,

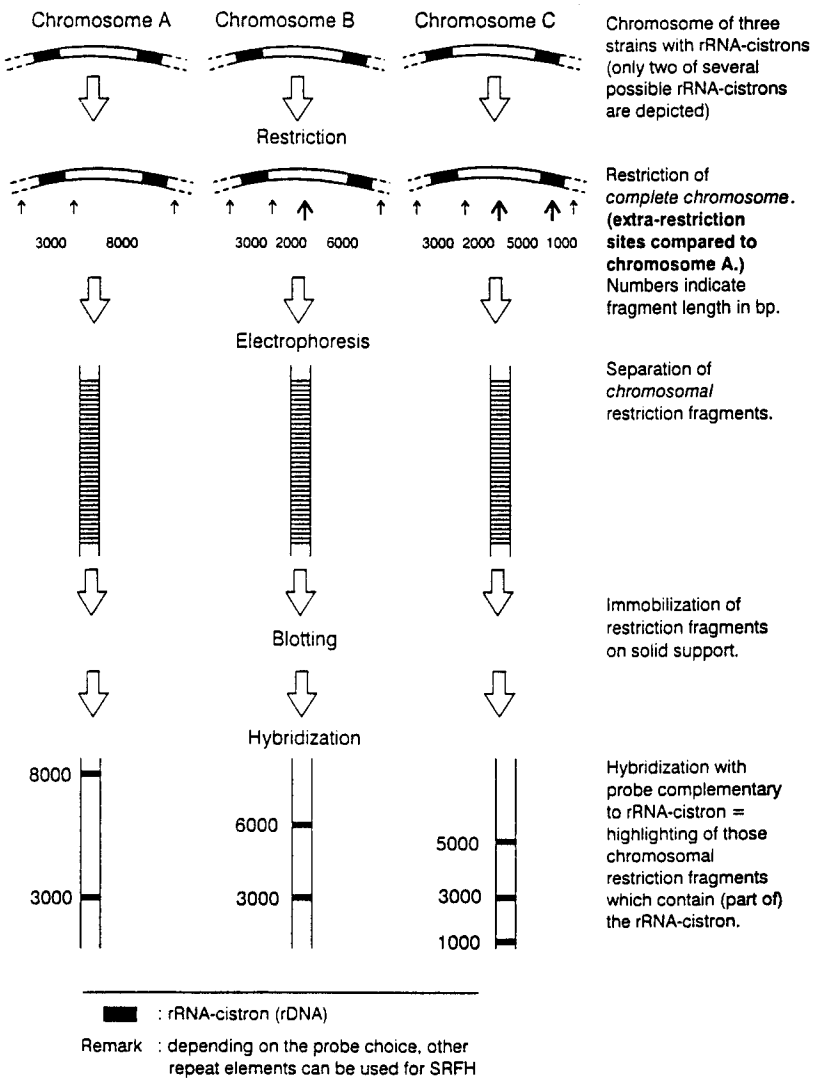

Fig. 1. rDNA-selective restriction fragment hybridization (SRFH).

and rDNA-SRFH has been applied for taxonomic studies (e.g., *59–61*) and for the study of the 16S-23S rRNA-spacer *(61,62)*. Low frequency restriction analysis with subsequent hybridization with a rDNA complementary probe has also been applied for species identification *(14)*. Plasmids do not interfere, e.g., for rDNA-SRFH, but interference might occur in case that probes, complementary to universal repeats, like that of M13-bacteriophage *(53)* or like the $(GTG)_5$-repeat *(36)* (*see also* Section 4.2.1.2.), are used, since these repeats can also be present on plasmids.

e. Selective restriction fragment amplification (SRFA). A number of ingenious methods (derived from biotechnology applications of PCR, e.g., anchor PCR) have been developed and enable reduction of the number of observable chromosomal restriction fragments through selective amplification. All SRFA approaches have in common that the chromosomal DNA restriction fragments are first ligated to adapters *(63–66)*, also named linkers *(67)* or indexers *(68)*. These are double-stranded oligonucleotides with an overhang complementary to all or some, depending on the approach, of the restric-

Table 2
Designations Used for Some of the DNA-Fingerprinting Techniques that Make Use of the rRNA-Cistron (rDNA) for Various Reasons

Descriptive designation	Part of the genome studied	Polymorphism studied	Basic principle of the technique	Confusing, erroneous, or noninformative designations of the technique
rDNA-selective restriction fragment hybridization (SRFH)	Chromosome	RFLP	Restriction digestion of the chromosome is combined with hybridization of a probe complementary to the rRNA-cistron. The rDNA is used here because it is a repeat which enables to highlight only those chromosomal DNA restriction fragments which contain the repeat.	Ribotyping, rRNA-gene restriction analysis
rRNA-spacer length polymorphism analysis	rRNA-spacer	AFLP	Amplification of the 16S-23S rRNA-spacer yields fragments of different length, since the spacer length may vary between cistrons of a single cell. The rDNA is used here because it is a repeat with variable length of the internal spacer.	PCR-ribotyping
rDNA restriction analysis	rRNA-cistron	RFLP	(Various parts of) the rDNA are isolated by amplification and sequence polymorphism within these rDNA-regions is studied by restriction analysis. The rDNA is used here because of its intrinsic sequence information.	Long PCR-ribotyping, ribosomal DNA-fingerprinting

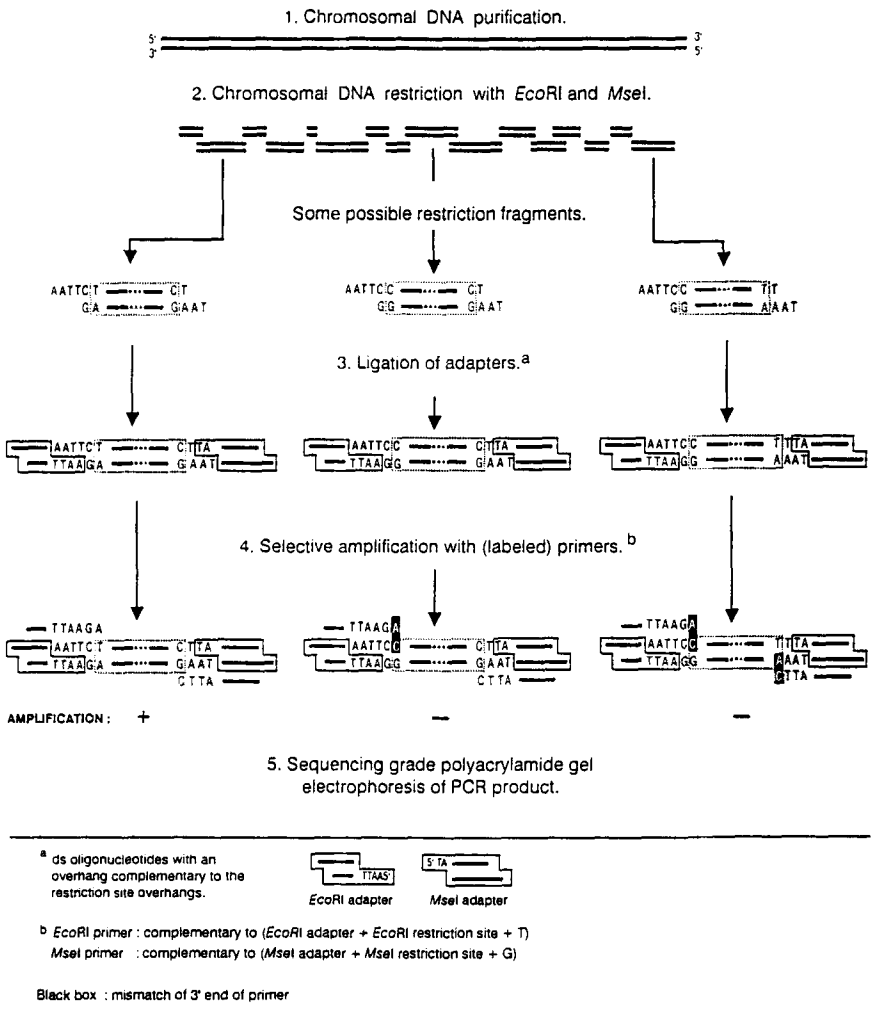

Fig. 2. Primer dependent selective restriction fragment amplification (SRFA). Modified after Lin and Kuo *(66)*.

tion site overhang(s). Ligation is necessary to enable amplification of only a few of the restriction fragments. SRFA-methods are a nice example of a hybrid technique, between amplification and restriction, whereby restriction is the sequence polymorphism revealing technique; amplification, instead of hybridization, as in SRFH, is only used to highlight a limited number of these fragments.

"Ligation mediated PCR" *(63,68)* makes use of type IIS-restriction enzymes, which have the special characteristic that they produce ambiguous 5' 4 bp overhang restriction sites. Whatever adapter with a 3' 4 bp overhang is used, only a limited number of restriction fragments will possess an overhang complementary to that of the adapter and thus can be ligated. Since the primers used are complementary to the adapter only, only these restriction fragments will be amplified.

A technique that has been designated "AFLP"™ *(64,66)*, makes use of normal restriction enzymes. Here, all restriction fragments are ligated to the added adapters, but amplification of a limited number of fragments occurs, since the primers used not only are complementary to the adapter but also to part of the restriction fragment (Fig. 2). By combining hexacutters and tetracutters for restriction and by varying the number of extra base pairs of the primer

required to be complementary on the restriction fragment, the number of restriction fragments amplified can be controlled.

Two SRFA techniques, even more ingenious than the other SRFA approaches, have been described simultaneously, are applicable for typing of *M. tuberculosis,* and use the IS6110 repeat. In mixed linker PCR *(67),* the adapters used contain uracil in one strand-whereof mixed linker: one strand with T, one strand with U. After ligation-of all of the restriction fragments-the restriction fragments are treated with uracil N glycosylase (UNG), which removes the uracil-containing part of the ligated mixed linkers. Consequently, when only primers complementary to the linker region are used, no amplification would occur, since only single-stranded DNA is present at the primer region after UNG-treatment. Since one of both primers is, however, complementary to the *M. tuberculosis* IS6110 repeat, those restriction fragments containing (part of) the IS6110 repeat will be amplified in a first round. This amplification round will reconstitute the region to which the linker primer is complementary and further amplification will occur, while fragments not containing the repeat are not amplified. This allows fingerprinting of *M. tuberculosis* strains, since the restriction fragments containing the repeat will vary in length. By the use of a high-frequency restriction enzyme (*Hha*I), the fingerprints obtained were almost identical to those obtained with IS6110-SRFH and clustering of the isolates was identical *(67).* Correspondingly, the use of a rDNA-specific primer in this approach might result in rDNA-SRFH fingerprints comparable to those obtained after rDNA-SRFH fingerprints (no publications known).

The other SRFA technique applicable for typing of *M. tuberculosis* makes use of a single adapter with a *Bam*HI overhang (complementary to the *Bgl*I digest overhangs on the chromosomal restriction fragments). Since the adapter is not phosphorylated, only ligation of the 5'end of the fragment will occur, the longer linker strand will not be ligated, and no amplification results when a primer only complementary to the longer strand of the linker is used. Only IS6110 containing restriction fragments will be amplified, since the linker primer is also complementary to a region at the edge of the repeat (facing outwards). The second primer used is complementary to the other repeat edge (facing outwards), so that, for each restriction fragment containing an IS6110 repeat, the flanking regions between the repeat and the nearest *Bgl*I restriction site will be amplified *(69).* Again, no other restriction fragments will be amplified.

The range of organisms for which mixed-linker SRFA *(67)* and partial-ligation SRFA *(69)* are applicable for typing depends on the choice of the repeat to which the specific primer is complementary; IIS-restriction SRFA *(63)* and primer-dependent SRFA *(64)* are universally applicable. It should be noted that the name "AFLP"™ *(64,66)* is quite confusing, since AFLP refers to a polymorphism and thus is not a valid technique designation, since most importantly it is actually a RFLP analysis technique, and since designations like "AFLP" preferably are reserved to refer to the complete collection of AFLP-techniques.

SRFA techniques apparently yield highly reproducible results, possess high discriminatory power, and, thanks to the amplification step, can start from a few colonies only.

4.2.2.4. Polymorphism Revealed by Restriction and Electrophoresis

2-D-DNA electrophoresis *(70,71; see also* Section 5.4.2.) is a very powerful approach for separating DNA (restriction) fragments. It consists of size-separating electrophoresis in one direction, followed by sequence-dependent denaturing gradient gel electrophoresis (*see* Section 4.2.3.2., item 1) in the perpendicular direction. It has been described for the analysis of the *E. coli* chromosome after *Eco*RI restriction *(72).* In this specific case of 2-D-DNA electrophoresis application, both restriction and electrophoresis contribute in revealing the polymorphism. Therefore, RFLP-DGGE is a possible designation for this application of 2-D-DNA electrophoresis.

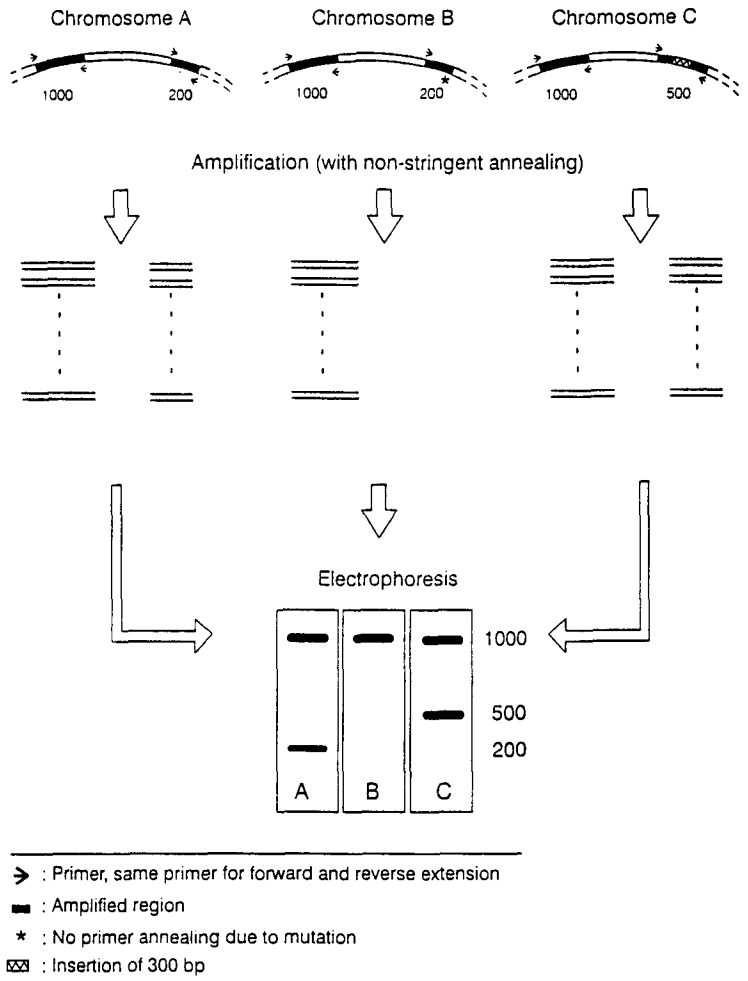

Fig. 3. Arbitrarily primed PCR (AP-PCR).

4.2.2.5. Polymorphism Revealed by Amplification

AFLP analysis of the chromosome is possible in different ways. Although only minor parts of the chromosome are amplified, chromosomal AFLP analysis techniques make use of the complete chromosome to produce fingerprints.

1. Arbitrarily primed-PCR (AP-PCR). AP-PCR is a very successful DNA-fingerprinting technique which has been described independently and almost simultaneously under different names: AP-PCR *(4)*, RAPD *(5)* and DAF *(6)* (*see also* Section 1.2.). Usually, one short primer is used at low annealing temperature. Because of the high probability that such a short primer at nonstringent annealing conditions will anneal at multiple sites of both strands of the chromosome and the plasmid(s), several events will occur whereby pairs of these annealing sites are located on opposite strands with facing 3' ends and at distances that allow amplification of the region between the primer annealing sites (Fig. 3). This will result in the amplification of multiple fragments of different length, which yields a DNA-fingerprint immediately after electrophoresis. Currently, fragments have a length of maximum 2000–3000 bp, but with the newer polymerases, amplification of stretches of more than 10 kbp may become possible (*see*, e.g., *12*).

The different names basically describe variations on a theme and there seems no need for a collective name like "multiple arbitrary amplicon profiling" (MAAP) *(9)*. Note that AP-PCR has

been erroneously named "arbitrary primer-PCR" *(7,8)*, although it is the priming which is arbitrary, while the primer can be complementary to a known sequence (e.g., the M13 bacteriophage repeat *[8,73]* or the ERICII repeat *[74]*) or complementary to, e.g., a random trimer-repeat (microsatellite-PCR *[73,75]*). Primers can be degenerate by containing inosine (ERICII repeat *[74]*, M13 bacteriophage repeat *[8]* or by being a mixture of primers differing in one nucleotide position *[76]*).

Although it has been shown on several occasions that AP-PCR is a rapid, simple, and reliable method for typing of isolates of most microorganisms, e.g., in nosocomial outbreaks (e.g., *18,74*), the major criticism is the limited interlaboratory reproducibility, as has been illustrated by some studies *(74,77)*.

The use of different dilutions of the target DNA has been shown to enhance the reproducibility of the fingerprints *(8,76,78)*. Also, the use of nonrandom primers that are complementary to known sequences has been claimed to enhance reproducibility as a consequence of the possibility to use higher annealing stringency *(73)*. However, careful analysis of microsatellite PCR suggests that the majority of bands created by this approach are generated by mismatch priming in a way similar to other AP-PCR techniques *(75)*. The results of reports studying the influence of plasmids differ strongly *(7,8)*.

Most applications of AP-PCR are concerned with typing, but taxonomic studies have been carried out as well *(28,79)*. AP-PCR has also been applied to develop species-specific probes *(19)*.

2. Interrepeat spacer-length polymorphism analysis. Other techniques that allow to study AFLP of the complete chromosome rely once more on the presence of repeat elements in bacterial genomes. Although only minor parts of the chromosome are amplified (as in AP-PCR), these techniques rely on the complete chromosome to produce fingerprints, and therefore they are chromosomal DNA-AFLP analysis techniques (like AP-PCR). Primers can be designed to anneal at conserved regions of repeats and to be directed outwardly, so that they do not allow amplification of the repeat itself, but of the regions between repeats (interrepeat spacer regions) (Fig. 4). Since the interrepeat spacer region length can differ between successive repeats, amplification again immediately yields a mixture of DNA fragments with variable length, which results in a fingerprint after electrophoresis. Depending on the kind of repeat chosen, the obtained fingerprints will be predominantly species-specific (as for tRNA-interrepeat-PCR *[80]*) or strain-specific (as for ERIC and REP-interrepeat-PCR *[81]* or for IS6110-interrepeat-PCR *[82]*). The use of different repeats in a single analysis has been reported as well *(83)*. tRNA-interrepeat-PCR has been shown to be applicable for identification of at least some of the *Staphylococcus* species *(29,78)* and for the identification of most of the *Acinetobacter* species *(84)*.

4.2.2.6. Polymorphism Revealed by Amplification and Electrophoresis

AP-PCR has been combined with DGGE *(85,86)* and with SSCP-analysis *(87)* to enhance the discriminatory power of the technique.

4.2.3. Gene Fingerprinting

4.2.3.1. Introduction

With the advent of nucleic acid amplification techniques, the possibility has arisen of studying the polymorphism present in one gene or in part of a gene only, irrespective of whether this gene is plasmid or chromosomally encoded. Indeed, amplification can be used to reveal the polymorphism (AFLP-analysis), but it is often applied only to purify or isolate a gene, whereafter other techniques reveal the polymorphism present. The polymorphism is not necessarily revealed by the amplification and only becomes apparent after, e.g., restriction. Thus, the use of amplification in fingerprinting does not necessarily mean that we deal with AFLP analysis (*see also* Section 4.2.2.3., item 3e).

4.2.3.2. Polymorphism Revealed by Electrophoresis

Some DNA-fingerprinting techniques rely on special electrophoresis techniques to reveal the polymorphism (*see also* Section 4.2.2.2.). Mostly, genes have a constant length for all isolates within a given taxon. After amplification of such a gene,

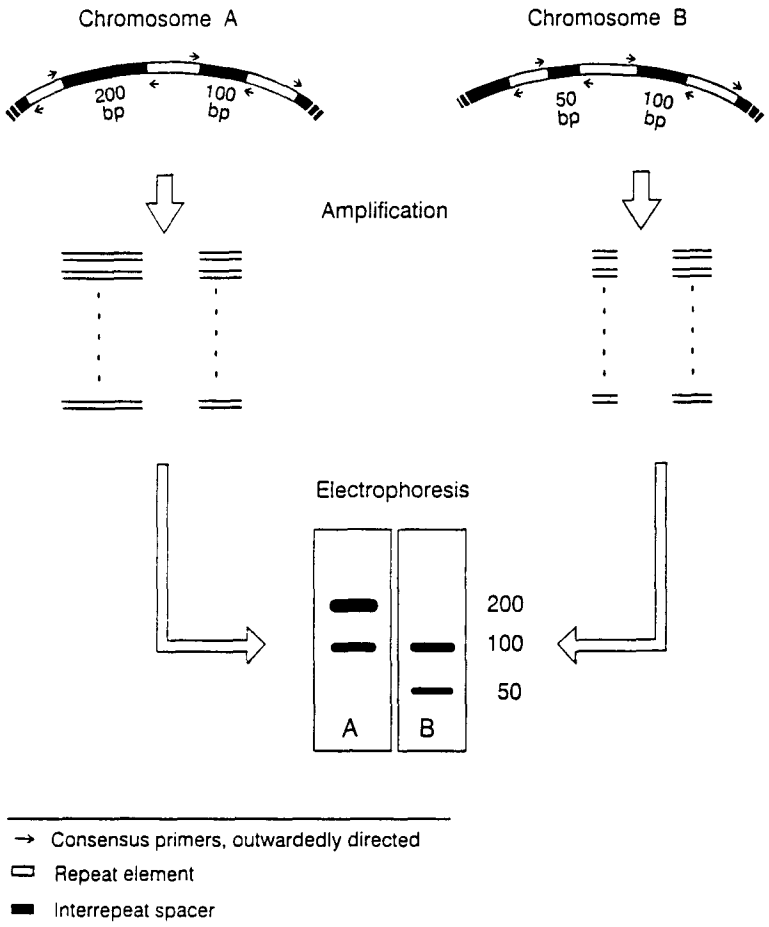

Fig. 4. Interrepeat spacer length polymorphism analysis.

the product on nondenaturing nongradient electrophoresis will appear as a fragment of the same length for all isolates studied. Some special electrophoresis conditions, however, allow for sequence dependent migration and separation of the DNA-fragments.

1. Denaturing gradient gel electrophoresis (DGGE). When applying a denaturing gradient onto the gel (by gradually changing temperature or formamide and urea content of the gel), the double-stranded amplification products will denature gradually domainwise *(88,89)*. This will influence electrophoresis migration speed. Primers can be synthesized to have extra GC-rich regions to provide the amplified products with flanking GC-clamps, which ensure that complete denaturation into single-stranded DNA is avoided, thus enhancing the discriminatory power of gene-DGGE. In the case of DGGE, no polymorphism is observable between the DNA fragments at the start of the electrophoresis. Here it is the denaturation gradient applied during electrophoresis itself that is elementary to make possible sequence polymorphism apparent. No polymorphism was observable between the PCR-products from different isolates before electrophoresis.

DGGE is most widely applied for mutation analysis of eukaryote genes, but has also been described for the characterization of bacterial populations. By amplifying the 16S rDNA with universal primers, this gene is amplified for the most abundantly present bacterial species (to an extent of at least 1% of the total population) in a mixture *(90)*. After, e.g., agarose gel electro-

phoresis, which separates fragments of different sizes this would be scored as a single fragment, but application of DGGE, which allows separation of fragments with equal size but different sequence, allows the number of predominant species present in the mixed bacterial population under study to be determined (e.g., 90).

2. Single-strand conformational polymorphism analysis (SSCP analysis). SSCP analysis detects sequence differences between different alleles by the sequence-dependent differential intramolecular folding of ssDNA under nondenaturing electrophoresis conditions, altering the migration speed of the molecules *(27,89)*. SSCP analysis is usually most efficient for short DNA-fragments up to 400 bp (compared to DGGE: 150–1200 bp). The discriminatory power and reproducibility of SSCP analysis depend strongly on the length of the fragment studied, the position of the mutation in the gene studied, and the test conditions. Therefore, several different conditions are usually required to detect all possible mutations *(23,27,91,92)*. Multiple modifications to enhance the mutation detection efficiency and reproducibility of SSCP analysis have been described. For example, Kasuga et al. *(93)* showed how magnetic streptavidin bead purification of ssDNAs (resulting in depletion of the complementary strand) enhanced the discriminatory power of SSCP. RNA-SSCP has been reported to have several advantages over DNA-SSCP *(89,90)*, like high yield (which enables visualization with ethidium bromide) and a possibly larger repertoire of secondary structure because of more stable duplices formed by ssRNA. A first step consists of amplifying part of the gene studied with the use of primers of which one contains a T7-polymerase promotor sequence and the other a SP6-polymerase promotor sequence. Transcription of the obtained PCR-product with one or both polymerases allows separate study the RNA-single strands.

   SSCP analysis of rDNA has been described as a tool that enables identification of cultured bacteria *(94)* and *Fungi (95)* to the species level and SSCP-analysis has been shown to enable the detection of rifampicin resistant mutations in the *rpo*B-gene of *M. tuberculosis (96)*.

3. Dye intercalation-mediated sequence-dependent electrophoresis. Wawer et al. *(97)* described another possibility for sequence-dependent electrophoresis by the application of the dye bisbenzimide/polyethylene glycol (PEG 6000) added to agarose gels. Bisbenzimide preferentially binds to A+T sequence motifs, and consequently A+T-rich fragments are retarded during electrophoresis by the long PEG chains linked to the bisbenzimide. Sequence polymorphism in PCR-amplified DNA fragments of up to 1440 bp could be studied by this method.

4.2.3.3. Polymorphism Revealed by Restriction (Gene RFLP Analysis)

Restriction of amplified genes (Fig. 5) can be carried out on genes with intraspecific variability (mostly protein encoding genes) for typing purposes or on genes like the rDNA, which are more conserved and carry species-specific information (identification) *(51,98)*. Also, plasmid encoded genes (e.g., TEM β-lactamases *[34]*) can be studied.

1. Gene RFLP analysis for identification. Application of restriction analysis of the amplified rDNA for the identification of microorganisms to the species level has been described independently *(99–104)*. RFLP analysis of the amplified rDNA provides a shortcut to rDNA sequencing and yields species-specific information applicable for identification or taxonomic studies. Different parts of the rDNA have been used: the 16S rDNA *(28,100,103,105–114)*, the 16S-23S spacer *(115–117)*, the 16S + spacer + part of the 23S *(52,104)*, or the 23S + spacer + part of the 5S rDNA *(99)*. rDNA-RFLP analysis has also been applied in the description of a new species *(61)* and RFLP analysis of the amplified small-subunit rRNA-gene has been used for the identification of eukaryote species *(102,118)*. The most obvious name for this approach would be "ribosomal DNA restriction analysis," but, as indicated above (*see* Sections 1.1., 1.3., 4.2.2.3., item 3d), this name is already widely used for rDNA-SRFH *(e.g., 3,21,22,57–60,119)*.

   Several protein-encoding genes seem to contain regions with little intraspecific variability and they have been used for species identification: histidine (112), *hsp*65 for *Mycobacterium* spp.

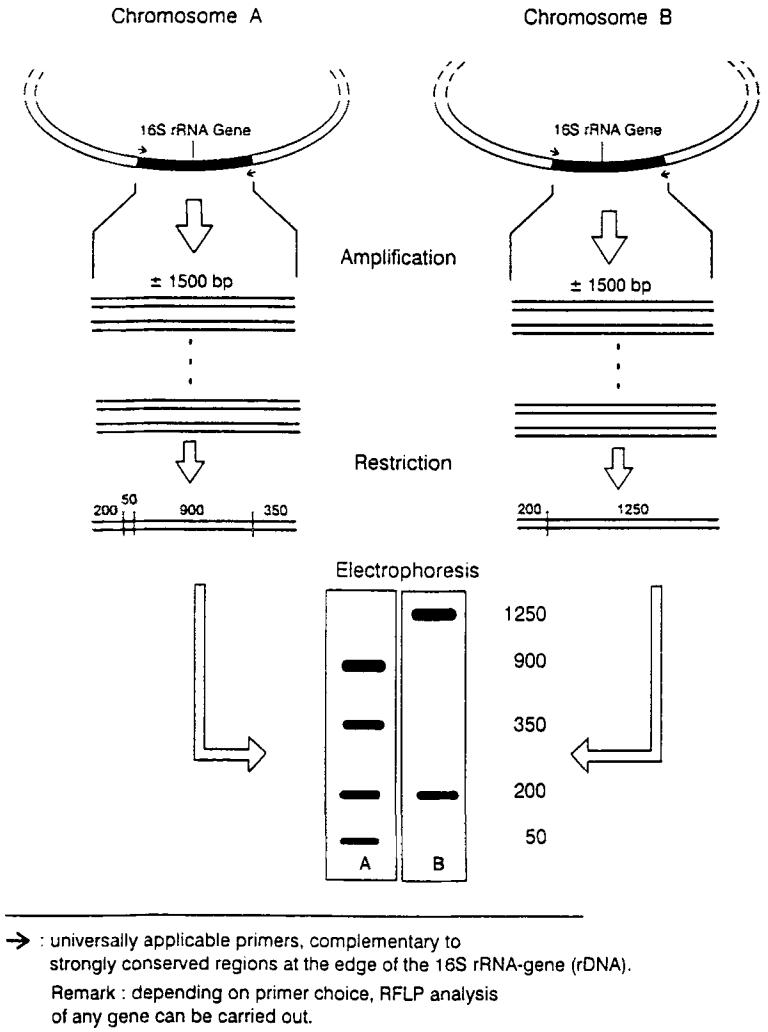

Fig. 5. 16S rDNA-RFLP analysis.

(120,121) and *Nocardia* spp. *(122)*, citrate synthase gene for identification of *Bartonella* species *(123)*. While one set of universal rDNA-primers can be used for the identification of most bacterial species, identification by restriction analysis of protein-encoding genes will mostly be limited to a few genera only.

2. Gene RFLP analysis for typing. Usually, protein-encoding genes represent more intraspecific variability and RFLP analysis of these genes has been used for strain differentiation within species (e.g., *124*). With the advent of the possibility to amplify several kb it is even possible to perform RFLP analysis on DNA-stretches up to 7 kb *(125)*. Since long PCR also allows amplification of the complete rDNA, and since this will contain more intraspecific variability than, e.g., the 16S rDNA or the spacer alone, rDNA-RFLP analysis has also been described for typing purposes (long PCR ribotyping *[12]*).
3. A novel gene-RFLP approach is possible by using a special restriction enzyme, cleavase™ (Boehringer Mannheim), of which cleavage activity is not sequence dependent but single strand conformation dependent. After amplification, the double-stranded amplicon is denatured and single strands fold in a sequence dependent manner (*see* Section 4.2.3.2., item 2). Since the restriction activity of cleavase is

sensitive for these altered conformations, the resulting restriction profile will change as well. Cleavase fragment length polymorphism (CLFP) analysis *(125b)*, which could be described as single-strand conformation dependent RFLP analysis, has been claimed to be as sensitive as sequence determination in assessing mutations (98% detection) and can be applied on DNA-fragments of up to 2.0 kbp *(125b)*.

4.2.3.4. Polymorphism Revealed by Restriction and Electrophoresis

Gene-RFLP-SSCP analysis: An approach which combines restriction of the amplified product and SSCP analysis of the restriction fragments *(126)*, and which has also been named "restriction endonuclease fingerprinting (REF)" *(127)*, enables longer fragments to be screened than possible with SSCP analysis alone. Amplification is used to isolate a 1 kb stretch of a gene; restriction is used to study RFLP analysis and to reduce the length of this fragment to about 150 bp per fragment; these fragments can then simultaneously be subjected to SSCP analysis. The designation "REF" is somewhat confusing, since it can also be used for RFLP analysis techniques in general.

4.2.3.5. Polymorphism Revealed by Amplification

1. Introduction. AFLP analysis of only parts of the chromosome is also possible. AFLP analysis of a gene seems to imply that a gene is reamplified after having been isolated through amplification. Although this approach has recently been described *(117)*, some approaches are possible where primer choice reveals length polymorphism at the level of genes.
2. Tandem repeat number polymorphism analysis. The observation that some repeats occur in tandem, whereby the number of tandem repeats varies according to the strain, has led to the development of PCR applications for the purpose of typing, whereby the tandem repeat region is amplified. Using primers that flank this region, one obtains an amplification product of variable length, according to the strain (Fig. 6) *(128–130)*. In bacteria, mostly only a single tandem repeat region is present and variable number tandem repeat (VNTR) analysis, whereby different tandem repeat regions occur, is not possible, in opposition to most eukaryotes. However, Goh et al. *(24)* showed the presence of at least two coagulase-genes in some *S. aureus* isolates, whereby each gene contained a tandem repeat region of possibly different length. Riley et al. *(131)* have shown that VNTR analysis is possible for unicellular eukaryotes. The T17-tandem repeat region appeared to be present only in unicellular eukaryotes and yielded species-specific patterns. VNTR analysis could be considered as a chromosomal AFLP-analysis technique.
3. Gene-length polymorphism analysis. Jordan *(132)* has shown that the use of a single pair of primers for amplification of the chitin synthase gene from four *Candida* species yielded amplification fragments with different species-specific length. This finding was applied to allow for simultaneous detection of the presence of these four species (which account for more than 90% of neonatal *Candida* infections) in clinical samples with a single primer pair, as opposed to the use of several primer pairs as in multiplex PCR, which is also applied to simultaneously detect different pathogens in a clinical sample (e.g., *133*).
4. Repeat-length polymorphism analysis. The spacer between the 16S and 23S rRNA-genes varies in length according to the species. Also, for some species, the multiple alleles of the rRNA-cistron (e.g., 7 for *E. coli [15]* contain spacers that can have different length, depending on the cistron. Consequently, amplification of this spacer yields a DNA fingerprint composed of fragments with different lengths (Fig. 7). However, the results obtained by rRNA-spacer length polymorphism analysis are rather confusing, since some workers report the amplification of only one or a few fragments that yields species-specific fingerprints *(25,26,134)*, while others obtain a large number of fragments that can be used to differentiate between strains of a single species *(11,62,135)*.

   A possible explanation for these reported differences, besides that of different primer choice, can come from the observation of Jensen and Straus *(136)* that some bands of fingerprints obtained after amplification of the

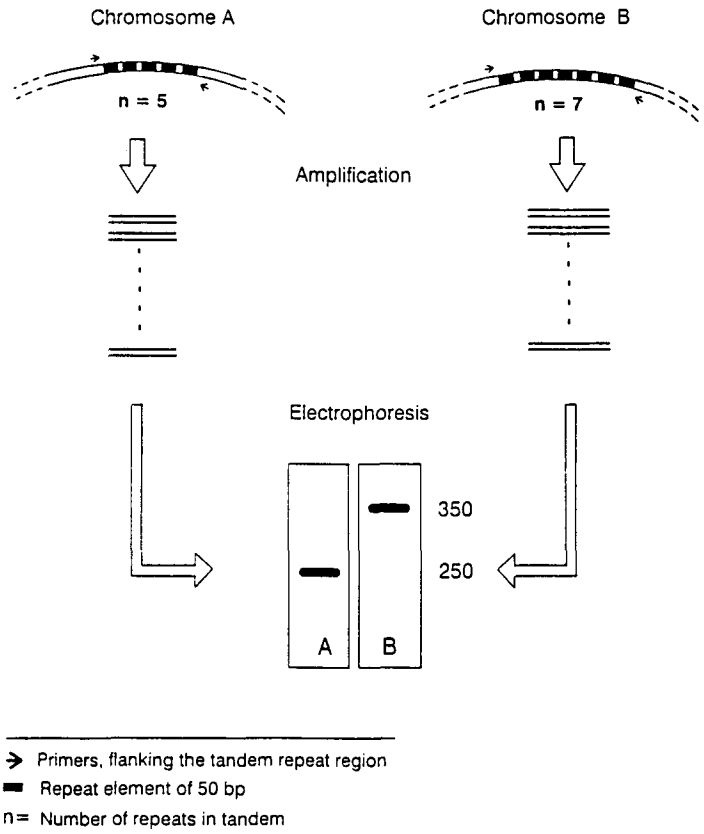

Fig. 6. Tandem repeat number polymorphism analysis.

rRNA spacer region are the result of heteroduplex ds DNA and of ss DNA formation during PCR. Heteroduplex formation was suggested to result from amplification procedures whereby some of the products carry homologous 3' and 5' ends (besides the primer region) flanking a variety of heterologous sequences. These conditions can be met in, e.g., rRNA spacer-length polymorphism analysis and AP-PCR. Heterologous DNA fragments with sufficiently long homologous flanking regions can then cross-hybridize. During the last PCR cycle, the probability of heteroduplex formation caused by cross-hybridization is maximal and these heteroduplices will be present in the final PCR-mixture, since no final denaturation is carried out. Upon electrophoresis, the heteroduplices with long single-stranded internal regions will be observed near the slots (as can be observed also after AP-PCR [unpublished data]); others with only a few mismatches will migrate within the range of the fully ds PCR-products. Heteroduplex formation during PCR has been reported by others (e.g., *137–139*). Ss DNA was also shown to be formed during the experiments of Jensen and Straus *(136)*. They suggested that asymmetric amplification, e.g., caused by asymmetric amplification efficiency of the primers, together with complex secondary structure of the rRNA spacer region (which enables formation of intramolecular stable conformations) could result in ss DNA formation. Ss DNA will migrate somewhat slower than ds DNA and thus will strongly influence the interpretation of the fingerprint. It was shown that the use of special highly stringent amplification conditions could avoid these artifacts *(26,134)*.

Thus the explanation of Kostman et al. *(11)* that their fingerprints result from multiple different lengths of the spacer regions of the rRNA-cistrons might be disputed, based on current knowledge about the organization of

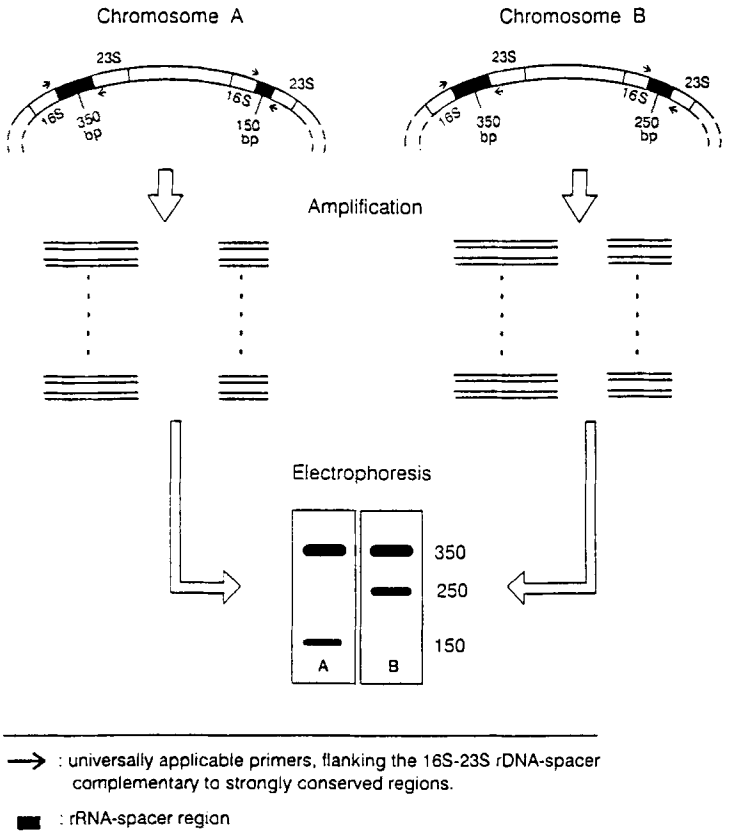

Fig. 7. rRNA-spacer length polymorphism analysis.

the rRNA-spacers. Rather, the fingerprints obtained may be caused by nonspecific amplification, which nevertheless may be reproducible, since the reason for aspecific amplification may be found in the structure of the region amplified. On the other hand, Gürtler *(62)* showed convincingly that for some *Clostridium difficile* isolates, up to 9 of the 16S-23S rRNA spacers may be of different length and we could confirm (data to be published) the findings of Hookey et al. *(25)* that simple species-specific rRNA spacer-length polymorphism fingerprints could be obtained using nonstringent amplification conditions with the primers of Jensen et al. *(26),* while the primers described by Kostman et al. *(11)* applied for, e.g., *S. aureus,* yielded complex fingerprints (unpublished data). Although the observations and hypotheses of Jensen and Straus *(136)* seem to supply a valid explanation for some phenomena, further theoretical work is needed to explain in more detail some of the contradictory findings.

5. Gene-AP-PCR. Recently, the rRNA-spacer region of *M. tuberculosis* was isolated by PCR and this product was used as the target for AP-PCR *(117)*. The authors reported that this technique enabled them to differentiate between strains of this species. Although the approach by itself is rather stimulating, the finding of sequence polymorphism suggested by these AP-PCR results is rather puzzling, since the sequence of the rRNA-spacer region (of which only one or two are present in mycobacteria) is well-conserved throughout the species *(140)*. Possibly some of the observations of Jensen and Straus *(136)* may apply here also to explain the multiple banding patterns obtained by Abed et al. *(117)*.

5. Discussion

5.1. Workload, Cost, Technicality

Chromosomal DNA RFLP-techniques require thorough purification of the DNA to avoid partial digestion. Moreover, most of the approaches to reduce the number of (observable) restriction fragments add additional steps, enlarging the workload.

AFLP-techniques are less demanding for DNA extraction and simple boiling of the cells is often sufficient to obtain interpretable AFLP fingerprints (e.g., *8,76*). AFLP techniques yield fingerprints immediately after PCR and electrophoresis.

5.2. Discriminatory Power

Discriminatory power of species identification techniques, like rRNA-spacer length polymorphism analysis *(26)*, tRNA-interrepeat-PCR *(80,82)*, 16S rDNA SSCP analysis *(94)*, and amplified rDNA-restriction analysis *(113,114)*, will have to be assessed in comparative studies. Some of the DNA-fingerprinting techniques for typing have been compared recently, and it was concluded that SRFH, low-frequency restriction analysis, and low-size restriction analysis were the most powerful approaches *(55)*. SRFA (not included in the above study) probably has the highest discriminatory power of RFLP-techniques. The ability of DGGE, SSCP-analysis, and dideoxy terminated SSCP-analysis (*see* Section 5.4.3.1.) to detect all mutations present in an amplified DNA-fragment differs strongly, according to the report. The discriminatory power of these approaches, however, is continuously being enhanced.

5.3. Reproducibility

Reproducibility of RFLP-techniques is generally accepted to be high. Problems with partial digestion may occur in chromosomal RFLP analysis and therefore these techniques require thorough DNA-purification and standardization of the amount of DNA that is being digested. Partial digestion is usually not a problem for gene-RFLP analysis.

A disadvantage of, e.g., AP-PCR is the often-reported limited reproducibility of the fingerprints *(75,77)*, which limits interrun and interlaboratory comparison. This limited reproducibility does not apply to all chromosomal AFLP techniques, since, e.g., tRNA interrepeat PCR is highly reproducible (unpublished data).

5.4. Ongoing and Future Technical Developments

5.4.1. Fluorescent Fragment Electrophoresis

Fluorescent fragment electrophoresis (FFE), whereby DNA fragments are labeled fluorescently (e.g., by incorporation of fluorescent primers during amplification), and which is carried out on denaturing polyacrylamide slab gels (automated sequencers [e.g., ABI Prism 377, Perkin-Elmer, Norwalk, CT; ALF-Express, Pharmacia Biotech, Uppsala, Sweden]) or in capillaries (ABI Prism 310, Perkin-Elmer), offers several advantages over the currently most-used electrophoresis techniques. Since the fragments are scored when passing an optical device and thus run off the gel, separation of low-size as well as high-size fragments is maximal. For electrophoresis techniques whereby the run has to be stopped in order to visualize the separated molecules, one has to choose between good separation of the high molecular size fragments and running off the gel of the low-size fragments, or vice versa. FFE also enables immediate digitalization of the fingerprints. Furthermore, capillary electrophoresis allows for automatic loading and circumvents the need of pouring gels. Several DNA- fingerprinting techniques reviewed here have already been carried out by using FFE *(20,84,141,142)*.

5.4.2. 2-D-DNA-Electrophoresis

In 2-D-DNA-electrophoresis, the polymorphism is revealed by size-dependent electrophoresis in one direction and by sequence-dependent DGGE in the perpendicular direction. Most applications are currently in human genomics for, e.g., diagnosis of heritable diseases, but the same technique seems to be well-suited for the study of genes and chromosomes of microorganisms *(70–72)*, and might be applied to enhance the discriminatory power of most DNA-fingerprinting techniques discussed here.

5.4.3. Sequence Determination

5.4.3.1. Dideoxy Terminated SSCP Analysis

A hybrid of dideoxy terminated sequencing and SSCP analysis has been designated "dideoxy-fingerprinting" *(23,143)*. Only one of the four sequencing reactions is carried out and the resulting fragments are subjected to SSCP analysis. Dideoxy terminated SSCP analysis was reported to have several advantages over gene SSCP analysis (*see* Section 4.2.3.2., item 2) and gene RFLP-SSCP analysis (*see* Section 4.2.3.4.). Strictly speaking, "dideoxyfingerprinting" could also refer to the well-established sequence determination by dideoxy termination.

5.4.3.2. Nonchemical Sequence Determination

We should keep in mind that the ultimate goal of all of the DNA-fingerprinting techniques is to study sequence polymorphism, while avoiding laborious sequence determination. However, in the near future, completely new approaches to sequence determination may alter the situation thoroughly and sequencing might become a very feasible easy-to-perform technique. Two major routes may be prominent: hybridization of the DNA-fragment to be sequenced with oligonucleotide arrays fixed on a microchip *(144)*, and powerful microscopy, like, e.g., oscillating resonance magnetic imaging *(145)*. Study of the whole chromosome at once, as is possible with chromosomal DNA RFLP and AFLP analysis, probably will remain out of scope of these sequencing techniques, but gene-RFLP, SSCP, and DGGE analysis techniques and chemical sequencing itself might be replaced completely by these new sequencing possibilities.

5.5. Conclusion

Several approaches to determine sequence polymorphism in microorganisms in an indirect way and combinations of these approaches are currently available. We have tried to illustrate how careless designation of DNA-fingerprinting techniques may lead to confusion about the nature of these techniques. Also, appropriate designation becomes more and more important in order to facilitate software-aided literature retrieval, and because new techniques and combinations of techniques are constantly being described. Finally, it appears that new electrophoresis techniques and sequence determination techniques will vastly enhance our possibilities of studying sequence polymorphism in the near future.

Acknowledgment

The author wants to thank Christian De Boever for layout of the figures.

References

1. Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., and Arnheim, N. (1985) Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230,** 1350–1354.
2. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239,** 487–491.
3. Grimont, F. and Grimont, P. A. D. (1986) Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. *Ann. Inst. Pasteur/Microbiol.* **137B,** 165–175.
4. Welsh, J. and McClelland, M. (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucl. Acids Res.* **18,** 7213–7218.
5. Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., and Tingey, S. V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucl. Acids Res.* **18,** 6531–6535.
6. Caetano-Anollés, G., Bassam, B. J., and Gresshoff, P. M. (1991) DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. *Bio/Technology* **9,** 553–557.
7. Brikun, I., Suziedelis, K., and Berg, D. E. (1994) DNA sequence divergence among derivatives of *Escherichia coli* K-12 detected by arbitrary primer PCR (random amplified polymorphic DNA) fingerprinting. *J. Bacteriol.* **176,** 1673–1682.
8. Elaichouni, A., van Emmelo, J., Claeys, G., Verschraegen, G., Verhelst, R., and Vaneechoutte, M. (1994) Study of the influence of plasmids on the arbitrary primer polymerase chain reaction fingerprint of *Escherichia coli* strains. *FEMS Microbiol. Lett.* **115,** 335–340.
9. Caetano-Anollés, G. (1993) Amplifying DNA with arbitrary oligonucleotide primers. *PCR Methods Applications* **3,** 85–94.
10. van Belkum, A. and Meis, J. (1994) Polymerase chain reaction-mediated genotyping in microbial epidemiology. *Clin. Infect. Dis.* **18,** 1017,1018.

11. Kostman, J. R., Edlind, T. D., LiPuma, J. J., and Stull, T. L. (1992) Molecular epidemiology of *Pseudomonas cepacia* determined by polymerase chain reaction ribotyping. *J. Clin. Microbiol.* **30,** 2084–2087.
12. Smith-Vaughan, H. C., Sriprakash, K. S., Mathews, J. D., and Kemp, D. J. (1995) Long PCR-ribotyping of nontypeable *Haemophilus influenzae*. *J. Clin. Microbiol.* **33,** 1192–1195.
13. Arbeit, R. D., Arthur, M., Dunn, R., Kim., C., Selander, R. K., and Goldstein, R. (1990) Resolution of recent evolutionary divergence among *Escherichia coli* from related lineages: the application of pulsed field electrophoresis to molecular epidemiology. *J. Infect. Dis.* **161,** 230–235.
14. Gundisch, C., Kirchhof, G., Baur, M., Bode, W., and Hartmann, A. (1993) Identification of *Azospirillum* species by RFLP and pulsed-field gel electrophoresis. *Microb. Releases* **2,** 41–45.
15. Cole, S. T. and St. Girons, I. (1994) Bacterial genomics. *FEMS Microbiol. Rev.* **14,** 139–160.
16. van Embden, J. D. A., Cave, M. D., Crawford, J. T., Dale, J. W., Eisenach, K. D., Gicquel, B., Hermans, P., Martin, C., McAdam, R., Shinnick, T. M., and Small, P. M. (1993) Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *J. Clin. Microbiol.* **31,** 406–409.
17. Gerner-Smidt, P. (1992) Ribotyping of *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex. *J. Clin. Microbiol.* **30,** 2680–2685.
18. Grattard, F., Pozzetto, B., Berthelot, P., Rayet, I., Ros, A., Lauras, B., and Gaudin, O. G. (1994) Arbitrarily primed PCR, ribotyping, and plasmid pattern analysis applied to investigation of a nosocomial outbreak due to *Enterobacter cloacae* in a neonatal intensive care unit. *J. Clin. Microbiol.* **32,** 596–602.
19. Giesendorf, B. A. J., van Belkum, A., Koeken, A., Stegeman, H., Henkens, M. H. C., van der Plas, J., Goossens, H., Niesters, H. G. M., and Quint, W. G. V. (1993) Development of species-specific probes for *Campylobacter jejuni, Campylobacter coli,* and *Campylobacter lari* by polymerase chain reaction fingerprinting. *J. Clin. Microbiol.* **31,** 1541–1546.
20. Martin, F., Vairelles, D., and Henrion, B. (1993) Automated ribosomal DNA-fingerprinting by capillary electrophoresis of PCR products. *Anal. Biochem.* **214,** 182–189.
21. Blumberg, H. M., Rimland, D., Kiehlbauch, J. A., Terry, P. M., and Wachsmuth, I. K. (1992) Epidemiologic typing of *Staphylococcus aureus* by DNA restriction fragment length polymorphisms of rRNA genes: elucidation of the clonal nature of a group of bacteriophage-nontypeable, ciprofloxacin-resistant, methicillin-susceptible *S. aureus* isolates. *J. Clin. Microbiol.* **30,** 362–369.
22. Domenech, P., Menendez, M. C., and Garcia, M. J. (1994) Restriction fragment length polymorphisms of 16S rRNA genes in the differentiation of fast-growing mycobacterial species. *FEMS Microbiol. Lett.* **116,** 19–24.
23. Ellison, J., Squires, G., Crutchfield, C., and Goldman, D. (1994) Detection of mutations and polymorphisms using fluorescence-based dideoxy fingerprinting (F-ddF). *BioTechniques* **17,** 742–753.
24. Goh, S.-H., Byrne, S. K., Zhang, J. L., and Chow, A. W. (1992) Molecular typing of *Staphylococcus aureus* on the basis of coagulase gene polymorphisms. *J. Clin. Microbiol.* **30,** 1642–1645.
25. Hookey, J. V., Birtles, R. J., and Saunders, N. A. (1995) Intergenic 16S rRNA gene (rDNA)-23S rDNA sequence length polymorphisms in members of the family *Legionellaceae*. *J. Clin. Microbiol.* **33,** 2377–2381.
26. Jensen, M. A., Webster, J. A., and Straus, N. (1993) Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Appl. Environ. Microbiol.* **59,** 945–952.
27. Orita, M., Suzuki, Y., Sekiya, T., and Hayashi, K. (1989) Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* **5,** 874–879.
28. Ralph, D., McClelland, M., Welsh, J., Baranton, G., and Perolat, P. (1993) *Leptospira* species categorized by arbitrarily primed polymerase chain reaction (PCR) and by mapped restriction polymorphisms in PCR-amplified rRNA genes. *J. Bacteriol.* **175,** 973–981.
29. Welsh, J. and McClelland, M. (1992) PCR-amplified length polymorphisms in tRNA intergenic spacers for categorizing *Staphylococci. Mol. Microbiol.* **6,** 1673–1680.
30. Wong, K. K. and McClelland, M. (1994) Stress-inducible gene of *Salmonella typhimurium* identified by arbitrarily primed PCR of RNA. *Proc. Natl. Acad. Sci. USA* **91,** 639–643.
31. Davies, J. (1994) Inactivation of antibiotics and the dissemination of resistance genes. *Science* **264,** 375–382.
32. Bradford, P. A., Cherubin, C. E., Idemyor, V., Rasmussen, B. A., and Bush, K. (1994) Multiply resistant *Klebsiella pneumoniae* strains from two Chicago hospitals: identification of the extended-spectrum TEM-12 and TEM-10 ceftazidime-hydrolyzing beta-lactamases in a single isolate. *Antimicrob. Agents Chemother.* **38,** 761–766.
33. Mariotte, S., Nordmann, P., and Nicolas, M. H. (1994) Extended-spectrum beta-lactamase in *Proteus mirabilis*. *J. Antimicrob. Chemother.* **33,** 925–935.
34. Scriver, S. R., Walmsley, S. L., Kau, C. L., Hoban, D. J., Brunton, J., McGeer, A., Moore, T. C., Witwicki, E., Group, C. H. S., and Low, D. E. (1994) Determination of antimicrobial susceptibilities of Canadian isolates of *Haemophilus influenzae* and characterization of their beta- lactamases. *Antimicrob. Agents Chemother.* **38,** 1678–1680.
35. Vatopoulos, A. C., Mainas, E., Balis, E., Threlfall, E. J., Kanelopoulou, M., Kalapothaki, V., Malamou-Lada, H.,

and Legakis, N. J. (1994) Molecular epidemiology of ampicillin-resistant clinical isolates of *Salmonella enteritidis*. *J. Clin. Microbiol.* **32,** 1322–1325.
36. Doll, L., Moshitch, S., and Frankel, G. (1993) Poly-$(GTG)_5$-associated profiles of *Salmonella* and *Shigella* genomic DNA. *Res. Microbiol.* **144,** 17–24.
37. Seifert, H., Schulze, A., Baginski, R., and Pulverer, G. (1994) Plasmid DNA fingerprinting of *Acinetobacter* species other than *Acinetobacter baumannii*. *J. Clin. Microbiol.* **32,** 82–86.
38. Grinsted, J. and Bennett, P. M. (1988) Analysis of plasmid DNA with restriction endonucleases. *Methods Microbiol.* **21,** 143–153.
39. Hartstein, A. I., Phelps, C. L., Kwok, R. Y. Y., and Mulligan, M. E. (1995) In vivo stability and discriminatory power of methicillin-resistant *Staphylococcus aureus* typing by restriction endonuclease analysis of plasmid DNA compared with those of other molecular methods. *J. Clin. Microbiol.* **33,** 2022–2026.
40. Cheng, H.-P. and Lessie, T. G. (1994) Multiple replicons constituting the genome of *Pseudomonas cepacia* 17616. *J. Bacteriol.* **176,** 4034–4042.
41. Jaworski, M. D., Ward, A. C. S, Hunter, D. L., and Wesley, I. V. (1993) Use of DNA analysis of *Pasteurella haemolytica* biotype T isolates to monitor transmission in bighorn sheep *(Ovis canadensis canadensis)*. *J. Clin. Microbiol.* **31,** 831–835.
42. Southern, E. M. (1979) Analysis of restriction-fragment patterns from complex deoxyribonucleic acid species. *Biochem. Soc. Symp.* **44,** 37–41.
43. Chen, C.-K. C., Sunday, G. J., Zambon, J. J., and Wilson, M. E. (1990) Restriction endonuclease analysis of *Eikenella corrodens*. *J. Clin. Microbiol.* **28,** 1265–1270.
44. Schwarz, D. C. and Cantor, C. R. (1984) Separation of yeast chromosome-sized DNAs by pulsed-field gel electrophoresis. *Cell* **37,** 67–75.
45. Struelens, M. J., Deplano, A., Godard, C., Maes, N., and Serruys, E. (1992) Epidemiologic typing and delineation of genetic relatedness of methicillin-resistant *Staphylococcus aureus* by macrorestriction analysis of genomic DNA by using pulsed-field gel electrophoresis. *J. Clin. Microbiol.* **30,** 2599–2605.
46. Allardet-Servent, A., Bouziges, N., Carles-Nurit, M.-J., Bourg, G., Gouby, A., and Ramuz, M. (1989) Use of low-frequency-cleavage restriction endonucleases for DNA analysis in epidemiological investigations of nosocomial bacterial infections. *J. Clin. Microbiol.* **27,** 2057–2061.
47. Mazurek, G. H., Hartman, S., Zhang, Y., Brown, B. A., Hector, J. S. R., Murphy, D., and Wallace, R. J., Jr. (1993) Large DNA restriction fragment polymorphism in the *Mycobacterium avium-M. intracellulare* complex: a potential epidemiologic tool. *J. Clin. Microbiol.* **31,** 390–394.
48. Bergthorsson, U. and Ochman, H. (1995) Heterogeneity of genome sizes among natural isolates of *Escherichia coli*. *J. Bacteriol.* **177,** 5784–5789.
49. Haertl, R. and Bandlow, G. (1993) Epidemiological fingerprinting of *Enterobacter cloacae* by small-fragment restriction endonuclease analysis and pulsed-field gel electrophoresis of genomic restriction fragments. *J. Clin. Microbiol.* **31,** 128–133.
50. van Steenbergen, T. J. M., Colloms, S. D., Hermans, P. W. M., de Graaff, J., and Plasterk, R. H. A. (1995) Genomic DNA fingerprinting by restriction fragment end labeling. *Proc. Natl. Acad. Sci. USA* **92,** 5572–5576.
51. Neefs, J.-M., Van de Peer, Y., Hendriks, L., and De Wachter, R. (1990) Compilation of small ribosomal subunit RNA sequences. *Nucl. Acids Res.* **18,** r2237–r2317.
52. Salzano, G., Moschetti, G., Villani, F., Pepe, O., Mauriello, G., and Coppola, S. (1994) Genotyping of *Streptococcus thermophilus* evidenced by restriction analysis of ribosomal DNA. *Res. Microbiol.* **145,** 651–658.
53. Ryskov, A. P., Jincharadze, A. G., Prosnyak, M. I., Ivanov, P. L., and Limborska, S. A. (1988) M13 phage DNA as a universal marker for DNA fingerprinting of animals, plants and microorganisms. *FEBS Lett.* **233,** 388–392.
54. Hermans, P. W. M., van Soolingen, D., and van Embden, J. D. A. (1992) Characterization of a major polymorphic tandem repeat in *Mycobacterium tuberculosis* and its potential use in the epidemiology of *Mycobacterium kansasii* and *Mycobacterium gordonae*. *J. Bacteriol.* **174,** 4157–4165.
55. Hermans, P. W. M., Sluijter, M., Hoogenboezem, T., Heersma, H., van Belkum, A., and de Groot, R. (1995) Comparative study of five different DNA fingerprint techniques for molecular typing of *Streptococcus pneumoniae* strains. *J. Clin. Microbiol.* **33,** 1606–1612.
56. Wiid, I. J. F., Werely, C., Beyers, N., Donald, P., and van Helden, P. D. (1994) Oligonucleotide $(GTG)_5$ as a marker for *Mycobacterium tuberculosis* strain identification. *J. Clin. Microbiol.* **32,** 1318–1321.
57. de Carvalho, A. F., Gautier, M., and Grimont, F. (1994) Identification of dairy *Propionibacterium* species by rRNA gene restriction patterns. *Res. Microbiol.* **145,** 667–676.
58. Picard-Pasquier, N., Ouagued, M., Picard, B., Goullet, P., and Krishnamoorthy, R. (1989) A simple, sensitive method of analyzing bacterial ribosomal DNA polymorphism. *Electrophoresis* **10,** 186–189.
59. Liebl, W., Ehrmann, M., Ludwig, W., and Schleifer, K. H. (1991) Transfer of *Brevibacterium divaricatum* DSM 20297^T, *"Brevibacterium flavum"* DSM 20411, *"Brevibacterium lactofermentum"* DSM 20412 and DSM 1412, and *Corynebacterium lilium* DSM 20137^T to *Corynebacterium glutamicum* and their distinction by rRNA gene restriction patterns. *Int. J. Syst. Bacteriol.* **41,** 255–260.
60. Martinetti Lucchini, G. and Altwegg, M. (1992) rRNA gene restriction patterns as taxonomic tools for the genus *Aeromonas*. *Int. J. Syst. Bacteriol.* **42,** 384–389.

61. Jannes, G., Vaneechoutte, M., Lannoo, M., Gillis, M., Van Canneyt, M., Van Damme, P., Kersters, K., Verschraegen, G., Van Heuverswyn, H., and Rossau, R. (1993) Polyphasic taxonomy leading to the proposal of *Moraxella canis* sp. nov. for a group of *Moraxella catarrhalis*-like strains. *Int. J. Syst. Bacteriol.* **43,** 438–449.
62. Gürtler, V. (1993) Typing of *Clostridium difficile* strains by PCR-amplification of variable length 16S-23S rDNA spacer regions. *J. Gen. Microbiol.* **139,** 3089–3097.
63. Smith, D. R. (1992) Ligation-mediated PCR of restriction fragments from large DNA molecules. *PCR Methods Applications* **2,** 21–27.
64. Zabeau, M. (1993) Selective restriction fragment amplification: a general method for DNA fingerprinting. European Patent Application 0 534 858 A1.
65. Huys, G., Coopman, R., Vancanneyt, M., Kersters, I., Verstraete, W., Kersters, K., and Janssen, P. (1993) High resolution differentiation of aeromonads. *Med. Microbiol. Lett.* **2,** 248–255.
66. Lin, J.-J. and Kuo, J. (1995) AFLP™: a novel PCR-based assay for plant and bacterial DNA fingerprinting. *Focus* **17,** 52–56.
67. Haas, W. H., Butler, W. R., Woodley, C. L., and Crawford, J. T. (1993) Mixed-linker polymerase chain reaction: a new method for rapid fingerprinting of isolates of the *Mycobacterium tuberculosis* complex. *J. Clin. Microbiol.* **31,** 1293–1298.
68. Unrau, P. and Deugau, K. V. (1994) Non-cloning amplification of specific DNA fragments from whole genomic DNA digests using DNA "indexers." *Gene* **145,** 163–169.
69. Palittapongarnpim, P., Chomyc, S., Fanning, A., and Kunimoto, D. (1993) DNA fingerprinting of *Mycobacterium tuberculosis* isolates by ligation-mediated polymerase chain reaction. *Nucl. Acids Res.* **21,** 761,762.
70. Vijg, J. (1995a) Detecting individual genetic variation. Parallel DNA analysis by 2-D DNA electrophoresis may provide a cost-effective way to look at sequence variation. *Bio/Technology* **13,** 137–139.
71. Vijg, J. (1995b) Two-dimensional DNA typing. A cost-effective way of analyzing complex mixtures of DNA fragments for sequence variations. *Mol. Biotechnol.* **4,** 275–295.
72. Fischer, S. G. and Lerman, L. S. (1979) Length-independent separation of DNA restriction fragments in two-dimensional gel electrophoresis. *Cell* **16,** 191–200.
73. Meyer, W., Mitchell, T. G., Freedman, E. Z., and Vilgalys, R. (1993) Hybridization probe for conventional DNA fingerprinting used as single primers in the polymerase chain reaction to distinguish strains of *Cryptococcus neoformans*. *J. Clin. Microbiol.* **31,** 2274–2280.
74. van Belkum, A., Kluytmans, J., van Leeuwen, W., Bax, R., Quint, W., Peters, E., Fluit, A., Vanderbroucke-Grauls, C., van den Brule, A., Koeleman, H., Melchers, W., Meis, J., Elaichouni, A., Vaneechoutte, M., Moonens, F., Maes, N., Struelens, M., Tenover, F., and Verburgh, H. (1995) Multi-center evaluation of arbitrarily primed PCR for typing of *Staphylococcus aureus* strains. *J. Clin. Microbiol.* **33,** 1537–1547.
75. Weising, K., Atkinson, R. G., and Gardner, R. C. (1995) Genomic fingerprinting by microsatellite-primed PCR: a critical evaluation. *PCR Methods Applications* **4,** 249–255.
76. Sakallah, S. A., Lanning, R. W., and Cooper, D. L. (1995) DNA fingerprinting of crude bacterial lysates using degenerate RAPD primers. *PCR Methods Applications* **4,** 265–268.
77. Penner, G. A., Bush, A., Wise, R., Kim, W., Domier, L., Kasha, K., Laroche, A., Scoles, G., Molnar, S. J., and Fedak, G. (1993) Reproducibility of random amplified polymorphic DNA (RAPD) analysis among laboratories. *PCR Methods Applications* **2,** 341–345.
78. McClelland, M. and Welsh, J. (1994) DNA fingerprinting by arbitrarily primed PCR. *PCR Methods Applications* **4,** S59–S65.
79. Welsh, J., Pretzman, C., Postic, D., Saint Girons, I., Baranton, G., and McClelland, M. (1992) Genomic fingerprinting by arbitrarily primed polymerase chain reaction resolves *Borrelia burgdorferi* into three distinct phyletic groups. *Int. J. Syst. Bacteriol.* **42,** 370–377.
80. Welsh, J. and McClelland, M. (1991) Genomic fingerprints produced by PCR with consensus tRNA gene primers. *Nucl. Acids Res.* **19,** 861–866.
81. Versalovic, J., Koeuth, T., and Lupski, J. R. (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucl. Acids Res.* **19,** 6823–6831.
82. Ross, B. C. and Dwyer, B. (1993) Rapid, simple method for typing isolates of *Mycobacterium tuberculosis* by using the polymerase chain reaction. *J. Clin. Microbiol.* **31,** 329–334.
83. Friedman, C. R., Stoeckle, M. Y., Johnson, W. D., Jr., and Riley, L. W. (1995) Double-repetitive-element PCR method for subtyping *Mycobacterium tuberculosis* clinical isolates. *J. Clin. Microbiol.* **33,** 1383,1384.
84. Wiedmann-Al-Ahmad, M., Tichy, H.-V., and Schön, G. (1994) Characterization of *Acinetobacter* type strains and isolates obtained from wastewater treatment plants by PCR fingerprinting. *Appl. Environ. Microbiol.* **60,** 4066–4071.
85. Dweikat, I., Mackenzie, S., Levy, M., and Ohm, H. (1993) Pedigree assessment using RAPD-DGGE in cereal crop species. *Theor. Appl. Genet.* **85,** 497–505.
86. Penner, G. A. and Bezte, L. J. (1994) Increased detection of polymorphism among randomly amplified wheat DNA fragments using a modified temperature sweep gel electrophoresis (TSGE) technique. *Nucl. Acids Res.* **22,** 1780,1781.

87. McClelland, M., Arensdorf, H., Cheng, R., and Welsh, J. (1994) Arbitrarily primed PCR fingerprints resolved on SSCP gels. *Nucl. Acids Res.* **22,** 1770,1771.
88. Myers, R. M., Maniatis, T., and Lerman, L. S. (1987) Detection and localization of single base changes by denaturing gradient gel electrophoresis. *Methods Enzymol.* **155,** 501–527.
89. Rolfs, A., Schuller, I., Finckh, U., and Weber-Rolfs, I. (1992) Detection of single base changes using PCR, in *PCR: Clinical Diagnostics and Research* (Rolfs, A., Schuller, I., Finckh, U., and Weber-Rolfs, I., eds.), Springer-Verlag, Berlin, Germany, pp. 149–167.
90. Muyzer, G., De Waal, E. C., and Uitterlinden, A. G. (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.
91. Liu, Q. and Sommer, S. S. (1994) Parameters affecting the sensitivities of dideoxy fingerprinting and SSCP. *PCR Methods Applications* **4,** 97–108.
92. Sarkar, G., Yoon, H.-S., and Sommer, S. S. (1992a) Screening for mutations by RNA single-strand conformation polymorphism (rSSCP): comparison with DNA-SSCP. *Nucl. Acids Res.* **20,** 871–878.
93. Kasuga, T., Cheng, J., and Mitchelson, K. R. (1995) Metastable single-strand DNA conformational polymorphism analysis results in enhanced polymorphism detection. *PCR Methods Applications* **4,** 227–233.
94. Widjojoadmotjo, M. N., Fluit, A. C., and Verhoef, J. (1994) Molecular identification of bacteria by fluorescence-based PCR-single-strand conformation polymorphism analysis of the 16S rRNA gene. *J. Clin. Microbiol.* **33,** 2601–2606.
95. Walsh, T. J., Francesconi, A., Kasai, M., and Chanock, S. J. (1995) PCR and single-strand conformational polymorphism for recognition of medically important opportunistic *Fungi. J. Clin. Microbiol.* **33,** 3216–3220.
96. Telenti, A., Imboden, P., Marchesi, F., Schmidheini, T., and Bodmer, T. (1993b) Direct, automated detection of rifampicin-resistant *Mycobacterium tuberculosis* by polymerase chain reaction and single-strand conformation polymorphism analysis. *Antimicrob. Agents Chemother.* **37,** 2054–2058.
97. Wawer, C., Rüggeberg, H., Meyer, G., and Muyzer, G. (1995) A simple and rapid electrophoresis method to detect sequence variation in PCR-amplified fragments. *Nucl. Acids Res.* **23,** 4928,4929.
98. Woese, C. R. (1987) Bacterial evolution. *Microbiol. Rev.* **51,** 221–271.
99. Vilgalys, R. and Hester, M. (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *J. Bacteriol.* **172,** 4238–4246.
100. Gürtler, V., Wilson, V. A., and Mayall, B. C. (1991) Classification of medically important clostridia using restriction endonuclease site differences of PCR-amplified 16S rDNA. *J. Gen. Microbiol.* **137,** 2673–2679.
101. Deng, S., Hiruki, C., Robertson, J. A., and Stemke, G. W. (1992) Detection by PCR and differentiation by restriction fragment length polymorphism of *Acholeplasma, Spiroplasma, Mycoplasma,* and *Ureaplasma,* based upon 16S rRNA genes. *PCR Methods Applications* **1,** 202–204.
102. Henrion, B., Le Tacon, F., and Martin, F. (1992) Rapid identification of genetic variation of ectomycorrhizal *Fungi* by amplification of ribosomal RNA genes. *New Phytologist* **122,** 289–298.
103. Jayarao, B. M., Doré, J. J. E., Jr., Baumbach, G. A., Matthews, K. R., and Oliver, S. P. (1991) Differentiation of *Streptococcus uberis* from *Streptococcus parauberis* by polymerase chain reaction and restriction fragment length polymorphism analysis of 16S ribosomal DNA. *J. Clin. Microbiol.* **29,** 2774–2778.
104. Vaneechoutte, M., Rossau, R., De Vos, P., Gillis, M., Janssens, D., Paepe, N., De Rouck, A., Fiers, T., Claeys, G., and Kersters, K. (1992) Rapid identification of bacteria of the *Comamonadaceae* with amplified ribosomal DNA-restriction analysis (ARDRA). *FEMS Microbiol. Lett.* **93,** 227–234.
105. Hughes, M. S., Skuce, R. A., Beck, L.-A., and Neill, S. D. (1993) Identification of *Mycobacteria* from animals by restriction enzyme analysis and direct DNA cycle sequencing of polymerase chain reaction-amplified 16S rRNA gene sequences. *J. Clin. Microbiol.* **31,** 3216–3222.
106. Jayarao, B. M., Doré, J. J. E., and Oliver, S. P. (1992) Restriction fragment length polymorphism analysis of 16S ribosomal DNA of *Streptococcus* and *Enterococcus* species of bovine origin. *J. Clin. Microbiol.* **30,** 2235–2240.
107. Vaneechoutte, M., de Beenhouwer, H., Claeys, G., Verschraegen, G., De Rouck, A., Paepe, N., Elaichouni, A., and Portaels, F. (1993) Identification of *Mycobacterium* species by using amplified rDNA-restriction analysis. *J. Clin. Microbiol.* **31,** 2061–2065 (*J. Clin. Microbiol.* **31,** 3355).
108. Avaniss-Aghajani, E., Jones, K., Chapman, D., and Brunk, C. (1994) A molecular technique for identification of bacteria using small subunit ribosomal RNA sequences. *BioTechniques* **17,** 144–149.
109. Bascunana, C. R., Mattsson, J. G., Bölske, G., and Johansson, K.-E. (1994) Characterization of the 16S rRNA genes from *Mycoplasma* sp. strain F38 and development of an identification system based on PCR. *J. Bacteriol.* **176,** 2577–2586.
110. Carlotti, A. and Funke, G. (1994) Rapid distinction of *Brevibacterium* species by restriction analysis of rDNA generated by polymerase chain reaction. *Syst. Appl. Microbiol.* **17,** 380–386.
111. Laguerre, G., Allard, M.-R., Revoy, F., and Amarger, N. (1994) Rapid identification of rhizobia by restric-

tion fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. *Appl. Environ. Microbiol.* **60,** 56–63.

112. Grifoni, A., Bazzicalupo, M., Di Serio, C., Fancelli, S., and Fani, R. (1995) Identification of Azospirillum strains by restriction fragment length polymorphism of the 16S rDNA and of the histidine operon. *FEMS Microbiol. Lett.* **127,** 85–91.
113. Vaneechoutte, M., Dijkshoorn, L., Tjernberg, I., Elaichouni, A., De Vos, P., Claeys, G., and Verschraegen, G. (1995a) Identification of *Acinetobacter* genomic species by amplified ribosomal DNA restriction analysis. *J. Clin. Microbiol.* **33,** 11–15.
114. Vaneechoutte, M., Riegel, P., de Briel, D., Monteil, H., Verschraegen, G., De Rouck, A., and Claeys, G. (1995b) Evaluation of the applicability of amplified rDNA-restriction analysis of species of the genus *Corynebacterium. Res. Microbiol.* **146,** 633–641.
115. Harasawa, R., Mizusawa, H., Nozawa, K., Nakagawa, T., Asada, K., and Kato, I. (1993) Detection and tentative identification of dominant *Mycoplasma* species in cell cultures by restriction analysis of the 16S-23S rRNA intergenic spacer regions. *Res. Microbiol.* **144,** 489–493.
116. Dolzani, L., Tonin, E., Lagatolla, C., Prandin, L., and Monti-Bragadin, C. (1995) Identification of *Acinetobacter* isolates in the *A. calcoaceticus-A. baumannii* complex by restriction analysis of the 16S-23S rRNA intergenic spacer sequences. *J. Clin. Microbiol.* **33,** 1108–1113.
117. Abed, Y., Davin-Regli, A., Bollet, C., and De Micco, P. (1995). Efficient discrimination of *Mycobacterium tuberculosis* strains by 16S-23S spacer region-based random amplified polymorphic DNA analysis. *J. Clin. Microbiol.* **33,** 1418–1420.
118. Fedorko, D. P., Nelson, N. A., and Cartwright, C. P. (1995) Identification of microsporidia in stool specimens by using PCR and restriction endonucleases. *J. Clin. Microbiol.* **33,** 1739–1741.
119. Kanaujia, G. V., Katoch, V. M., Shivannavar, C. T., Sharma, V. D., and Patil, M. A. (1991) Rapid characterization of *Mycobacterium fortuitum-chelonei* complex by restriction fragment length polymorphism of ribosomal RNA genes. *FEMS Microbiol. Lett.* **77,** 205–208.
120. Telenti, A., Marchesi, F., Balz, M., Bally, F., Böttger, E. C., and Bodmer, T. (1993a) Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *J. Clin. Microbiol.* **31,** 175–178.
121. Steingrube, V. A., Gibson, J. L., Brown, B. A., Zhang, Y., Wilson, R. W., Rajagopalan, M., and Wallace, R. J., Jr. (1995a) PCR amplification and restriction endonuclease analysis of a 65- Kilodalton heat shock protein gene sequence for taxonomic separation of rapidly growing mycobacteria. *J. Clin. Microbiol.* **33,** 149–153.
122. Steingrube, V. A., Brown, B. A., Gibson, J. L., Wilson, R. W., Brown, J., Blacklock, Z., Jost, K., Locke, S., Ulrich, R. F., and Wallace, R. J., Jr., (1995b) DNA amplification and restriction endonuclease analysis for differentiation of 12 species and taxa of *Nocardia,* including recognition of four new taxa within the *Nocardia asteroides* complex. *J. Clin. Microbiol.* **33,** 3096–3101.
123. Norman, A. F., Regnery, R., Jameson, P., Greene, C., and Krause, D. C. (1995) Differentiation of *Bartonella*-like isolates at the species level by PCR-restriction fragment length polymorphism in the citrate synthase gene. *J. Clin. Microbiol.* **33,** 1798–1803.
124. Clayton, C. L., Kleanthous, H., Morgan, D. D., Puckey, L., and Tabaqchali, S. (1993) Rapid fingerprinting of *Helicobacter pylori* by polymerase chain reaction and restriction fragment length polymorphism analysis. *J. Clin. Microbiol.* **31,** 1420–1425.
125. Gardiner, D., Hartas, J., Currie, B., Mathews, J. D., Kemp, D. J., and Sriprakash, K. S. (1995) Vir typing: a long-PCR typing method for group A streptococci. *PCR Methods Applications* **4,** 288–293.

125b. Lyamicheva, N., Heisler, L., Brow, M. A., and Olive, D. M. (1996) Analysis of bacterial genotypes, drug resistance loci, and p53 genes using cleavase fragment length polymorphism analysis. *Biochemica (Boehringer Mannheim)* **3,** 33,34.

126. Iwahana, H., Yoshimoto, K., and Itakura, M. (1992) Detection of point mutations by SSCP of PCR-amplified DNA after endonuclease digestion. *BioTechniques* **12,** 64–66.
127. Liu, Q. and Sommer, S. S. (1995) Restriction endonuclease fingerprinting (REF): a sensitive method for screening mutations in long, contiguous segments of DNA. *BioTechniques* **18,** 470– 477.
128. Frénay, H. M. E., Theelen, J. P. G., Schouls, L. M., Vandenbroucke-Grauls, C. M. J. E., Verhoef, J., van Leeuwen, W. J., and Mooi, F. R. (1994) Discrimination of epidemic and nonepidemic methicillin-resistant *Staphylococcus aureus* strains on the basis of protein A gene polymorphism. *J. Clin. Microbiol.* **32,** 846,847.
129. Goyal, M., Young, D., Zhang, Y., Jenkins, P. A., and Shaw, R. J. (1994) PCR amplification of variable sequence upstream of *kat*G gene to subdivide strains of *Mycobacterium tuberculosis* complex. *J. Clin. Microbiol.* **32,** 3070,3071.
130. Hermans, P. W. M., van Soolingen, D., Bik, E. M., de Haas, P. E. W., Dale, J. W., and van Embden, J. D. A. (1991) Insertion element IS987 from *Mycobacterium bovis* BCG is located in a hot-spot integration region for insertion elements in *Mycobacterium tuberculosis* complex strains. *Infect. Immun.* **59,** 2695–2705.
131. Riley, D. E., Samadpour, M., and Krieger, J. N. (1991) Detection of variable DNA repeats in diverse eukaryotic microorganisms by a single set of poly-

merase chain reaction primers. *J. Clin. Microbiol.* **29,** 2746–2751.
132. Jordan, J. A. (1994) PCR identification of four medically important *Candida* species by using a single primer pair. *J. Clin. Microbiol.* **32,** 2962–2967.
133. Wilton, S. and Cousins, D. (1992) Detection and identification of multiple mycobacterial pathogens by DNA amplification in a single tube. *PCR Methods Applications* **1,** 269–273.
134. Leys, E. J., Griffen, A. L., Strong, S. J., and Fuerst, P. A. (1994) Detection and strain identification of *Actinobacillus actinomycetemcomitans* by nested PCR. *J. Clin. Microbiol.* **32,** 1288–1294.
135. Cartwright, C. P., Stock, F., Beekmann, S. E., Williams, E. C., and Gill, V. J. (1995) PCR amplification of rRNA intergenic spacer regions as a method for epidemiological typing of *Clostridium difficile. J. Clin. Microbiol.* **33,** 184–187.
136. Jensen, M. A. and Straus, N. (1993) Effect of PCR conditions on the formation of heteroduplex and single-stranded DNA products in the amplification of bacterial ribosomal DNA spacer regions. *PCR Methods Applications* **3,** 186–194.
137. Zorn, A. and Krieg, P. (1991) PCR analysis of alternative splicing pathways: identification of artifacts generated by heteroduplex formation. *BioTechniques* **11,** 181–183.
138. Ruano, G. and Kidd, K. K. (1992) Modeling of heteroduplex formation during PCR from mixtures of DNA templates. *PCR Methods Applications* **2,** 112–116.
139. Separovic, E. and Nadin-Davis, S. A. (1994) Secondary structure within PCR target sequences may facilitate heteroduplex production. *PCR Methods Applications* **3,** 248–251.
140. Frottingham, R. (1995) Discrimination of *Mycobacterium tuberculosis* strains by PCR. *J. Clin. Microbiol.* **33,** 2801,2802.
141. Grundmann, H., Schneider, Ch., Tichy, H. V., Simon, R., Klare, I., Hartung, D., and Daschner, F. D. (1995) Automated laser fluorescence analysis of randomly amplified polymorphic DNA: a rapid method for investigating nosocomial transmission of *Acinetobacter baumannii. J. Med. Microbiol.* **43,** 446–451.
142. Widjojoadmotjo, M. N., Fluit, A. C., and Verhoef, J. (1994) Molecular identification of bacteria by fluorescence-based PCR-single-strand conformation polymorphism analysis of the 16S rRNA gene. *J. Clin. Microbiol.* **33,** 2601–2606.
143. Sarkar, G., Yoon, H.-S., and Sommer, S. S. (1992b) Dideoxy fingerprinting (ddF): a rapid and efficient screen for the presence of mutations. *Genomics* **13,** 441–443.
144. Chetverin, A. B. and Kramer, F. R. (1994) Oligonucleotide arrays: new concepts and possibilities. *Bio/Technology* **12,** 1093–1099.
145. Kopelman, R. and Tan, W. (1993) Near-field optics: imaging single molecules. *Science* **262,** 1382–1384.