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DNA amplification fingerprinting of bacteria

Brant J. Bassam, Gustavo Caetano-Anollés, and Peter M. Gresshoff

Plant Molecular Genetics, Institute of Agriculture and Center for Legume Research, The University of Tennessee, PO Box 1071, Knoxville, TN 37901-1071, USA

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Summary. We have amplified short arbitrary stretches of total bacterial DNA to produce highly characteristic and complex DNA fingerprints. This DNA amplification fingerprinting (DAF) strategy involves enzymatic amplification of DNA directed by a single arbitrary oligonucleotide primer. Amplification produces a characteristic spectrum of products that is adequately resolved by polyacrylamide gel electrophoresis and visualized by silver staining. Although DAF is simple in concept, we found that amplification parameters must be within an optimal range for reproducibility. We establish a safe window for these parameters, which include magnesium, primer and enzyme concentration as well as cycle number. The refined procedure was used to distinguish between clinical isolates of *Streptococcus uberis*, *Klebsiella pneumoniae*, and *Escherichia coli*. The use of template DNA concentrations higher than 1 $ng \cdot \mu l^{-1}$ and high $MgCl_2$ levels was especially important for reproducibility when amplifying small bacterial genomes. We tested a truncated *Thermus aquaticus* DNA polymerase, the Stoffel fragment, and found it more tolerant of reaction conditions, more efficient in the amplification of short products, and able to produce more informative fingerprints when compared to the normal thermostable polymerase from which it was derived. Because DAF produces representative fingerprints quickly and reliably from bacteria regardless of prior genetic or biochemical knowledge, we anticipate the general use of this diagnostic tool for bacterial identification and taxonomy.

Introduction

Fast and accurate identification of bacterial isolates is becoming increasingly important for epidemiology, taxonomy, and ecological studies. Traditionally, identification involved slow and cumbersome methods based on phenotypic, microbiological, and biochemical characterization of traits such as antibiotic resistance, phage sensitivity, or isoenzyme comparison. Genotypic identification methods have also been used including restriction endonuclease fingerprinting (Denning et al. 1989; Murray et al. 1990), hybridization with strain-specific oligonucleotides (DeLong et al. 1989; Amann et al. 1990), and the polymerase chain reaction (PCR) (Mullis and Faloona 1987; Medlin et al. 1988; McCabe 1990; Smith and Selander 1990; Erlich et al. 1991). These methods are often slow or tend to be application specific.

A recent innovation is amplification fragment length polymorphism (AFLP) analyses by arbitrary primers. This strategy involves the enzymatic amplification of template DNA directed by one or more arbitrary oligonucleotide primers to produce a characteristic spectrum of products, a proportion of which can be polymorphic. The procedure is fast, independent of prior genetic and biochemical knowledge of the organism tested, and allows tailoring of the number of products and polymorphisms generated. Several variations of this technology have been developed (Welsh and McClelland 1990; Williams et al. 1990; Caetano-Anollés et al. 1991a), each having differences in DNA amplification conditions, the length of primers used, and the resolution of products obtained. The approach of Caetano-Anollés et al. (1991a) has several advantages. Termed DNA amplification fingerprinting (DAF), it uses the simplest and most relaxed amplification conditions, the shortest primers, and offers the highest resolution (Caetano-Anollés et al. 1991b).

In-vitro DNA amplification is a new development and its biochemistry is poorly understood. Current DNA amplification protocols strive to control specificity and reproducibility. In this work we have investigated and optimized many important parameters affecting the experimental reproducibility of DAF, an understanding of which is essential before widespread adoption of this technology. Here we demonstrate the ease and utility of DAF for the differentiation of clinical isolates of *Streptococcus uberis*, *Klebsiella pneumoniae*, and *Escherichia coli* from cows with clinical mastitis.

Correspondence to: G. Caetano-Anollés

Table 1. Bacterial isolates from cows with clinical mastitis

Isolate	Cow number	Isolation date	Udder quadrant
Escherichia coli J4	242	May 28, 1986	Left rear
E. coli J21	536	August 13, 1986	Left rear
E. coli J23	542	August 18, 1987	Left rear
E. coli J25	T545	July 2, 1986	Left front
Klebsiella pneumoniae J7	308	July 1, 1986	Right rear
K. pneumoniae J9	330	May 23, 1986	Left front
K. pneumoniae J14	368	June 12, 1986	Left front
K. pneumoniae J24	969	July 17, 1986	Right front

Materials and methods

Bacterial strains. Strains were provided by S. P. Oliver and B. Jayarao, Department of Animal Science, The University of Tennessee, Knoxville. *E. coli* strain Smith 92, *K. pneumoniae* strain Smith 20, and *S. uberis* ATCC27958 (type I) were used as reference. Other strains were isolated from individual udder quadrants of mastitis-infected cows. Table 1 lists the *E. coli* and *K. pneumoniae* isolates.

DNA isolation. Total DNA was isolated using a phenol-chloroform extraction method. A large bacterial colony grown on 5% sheep blood agar was resuspended for 10 min in a cold solution of 25% sucrose, 0.05 M TRIS-HCl, 0.05 M Na_2 ethylenediaminetetraacetate (EDTA) and 2 mg/ml lysozyme, pH 8.0. The suspension was then incubated at 37° C for 30 min with 1% sodium dodecyl sulfate and 0.2 mg/ml of ribonuclease A (Sigma, St. Louis, Mo., USA). The suspension was incubated a further 60 min at 37° C with 1 mg/ml of proteinase K (Sigma) then at 55° C overnight. Cell debris was centrifuged from the mixture and the supernatant was phenol:chloroform (1:1) extracted twice, chloroform extracted, and total DNA precipitated at −20° C in ethanol. DNA was resuspended in water.

DNA amplification. Amplification was generally done in a total volume of 25 µl with about 1 ng/µl of template DNA, 0.3 µM primer, 0.3 units/µl of AmpliTaq Stoffel fragment or 0.1 units/µl of normal AmpliTaq DNA polymerase each from *Thermus aquaticus* (Perkin-Elmer/Cetus, Norwalk, Conn., USA), in a reaction containing 200 µM of each deoxynucleoside triphosphate (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J., USA), 6 mM $MgCl_2$, 10 mM TRIS-HCl (pH 8.3), and 10 mM KCl for the Stoffel fragment or 50 mM KCl for the normal enzyme. The reaction mix was overlaid with two drops of mineral oil and amplified in an Ericomp thermocycler (Ericomp, San Diego, Calif.) connected to a refrigerated water bath for 35 two-step cycles of 1 s at 96° C and 1 s at 30° C. The heating and cooling rates of the thermocycler were 23°/min and 14°/min respectively. One complete cycle took about 8 min. The sample temperature was continuously monitored with a thermal probe. Oligonucleotide primers synthesized with >99% efficiency were used unpurified and behaved consistently from batch to batch.

DNA electrophoresis and silver staining. DNA amplification fragments were separated by polyacrylamide gel electrophoresis as previously described (Caetano-Anollés et al. 1991a; Bassam et al. 1991). Usually 4 µl of the amplification reaction was loaded with 4 µl of a loading buffer (5 M urea and 0.02% xylene cyanole FF). Electrophoresis was at 100 V, usually until the dye front was about 1 cm from the end of the gel. DNA was visualized using a fast and sensitive silver staining procedure (Bassam et al. 1991) that detects 1 pg DNA/mm^2 band cross-section. Polyester-backed gels were preserved for permanent record by soaking in 50% ethanol for 10 min and drying at room temperature.

Results

DAF relies on non-stringent reaction conditions for the amplification of arbitrary target sites. Thus, DAF is conceptually and mechanistically distinct from the PCR and assumptions derived from the PCR need not be extended to DAF. This is true both for the purposes of specifying amplification reaction conditions and for understanding the basic mechanisms involved. Using a defined strain of *S. uberis* as our model organism and the octamer GTAACGCC as an example amplification primer, we first optimized amplification parameters with both a DNA polymerase from *T. aquaticus* (Amplitaq) and its truncated derivative, the so-called Stoffel fragment. The parameters examined here condition the chemical environment and the extent of the amplification reaction and are crucial for the generation of reproducible fingerprints. Following an iterative process of analysis, the data presented were obtained by keeping fixed parameters within an optimal range.

To simplify the description and discussion of results, we classify amplification products into primary, secondary and tertiary categories by visual inspection. Primary products are those most efficiently amplified, appearing as the strong bands in a gel. Secondary products are those which, while usually clear in the gel, are of intermediate intensity. Tertiary products are barely visible under normal gel-running conditions unless more concentrated amplification products are analyzed. To better check experimental consistency, all samples from optimization experiments were electrophoresed undiluted so that secondary and even tertiary products could be more clearly visualized.

Surprisingly, fingerprints obtained with the Soffel fragment and the Amplitaq enzyme were quite different. This observation was true for the wide variety of reaction parameters tested (see Figs. 1–4). Generally, the Stoffel fragment produced clearer fingerprints with a higher proportion of stronger primary products.

Magnesium concentration

Initially, we determined the effect of $MgCl_2$ concentration on the number, consistency and distribution of DAF products. We tested magnesium concentrations over a range of 0 to 10 mM with both enzymes (Fig. 1). More than 1 mM magnesium was required for amplifica-

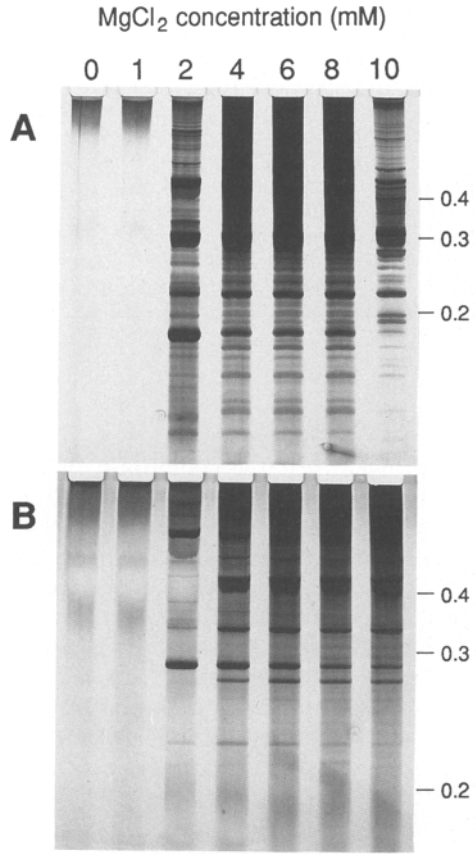

Fig. 1A, B. Effect of $MgCl_2$ concentration. Total DNA from *Streptococcus uberis* strain ATCC27958 was amplified using primer GTAACGCC under standard conditions (see Materials and methods) with $MgCl_2$ concentrations ranging between 0 and 10 mM (as shown). Similar amplifications were done using the Stoffel fragment (**A**) and Amplitaq (**B**) DNA polymerase enzymes. Each lane shows 4 µl of undiluted amplification products. Sizes shown are in kb

tion. Generally, the Stoffel fragment produced a broader distribution of amplification products, each in greater amounts. This was especially evident with smaller products (less than 300 bp). Both polymerases produced consistent fingerprints with 4–8 mM magnesium, but the Stoffel enzyme was inhibited at concentrations higher than about 8 mM. On the basis of these results, we consider 6 mM magnesium to be optimal for both enzymes.

Enzyme activity

The Stoffel fragment produced consistent amplification products over a wider enzyme concentration range when compared to the Amplitaq enzyme. Between 0.2 and 0.4 units/µl of the Stoffel fragment produced clear and consistent results, whereas only 0.075 and 0.1 units/µl of Amplitaq was suitable (Fig. 2). Although the Stoffel fragment had a wider and more useful range of activity, it has a lower processivity, requiring at least twice as much per assay than the Amplitaq enzyme (based on the manufacturers definition of units of enzyme activity). "Ladder bands" were found to occur when Amplitaq concentrations were higher than about 0.1 units/µl of reaction, an artifact absent in Stoffel fragment reactions. Fingerprints were not affected by batch-to-batch enzyme variation.

Primer concentration

We tested various concentrations of primer in DAF reactions. Higher primer concentration increased the yield of the amplification without altering fingerprint consistency (Fig. 3). Almost no amplification products were produced with less than 0.3 µM primer with either the Stoffel fragment or Amplitaq enzyme. The amount of products increased with concentration up to about 3 µM primer. Optimal amplification profiles were obtained with primer concentrations between 3 and more than 9 µM in Stoffel fragment reactions. Amplitaq reactions were optimal over a much narrower concentration range of only 3 to 4.2 µM. Ladder bands appeared in Amplitaq reactions with primer concentrations greater than 4.2 µM.

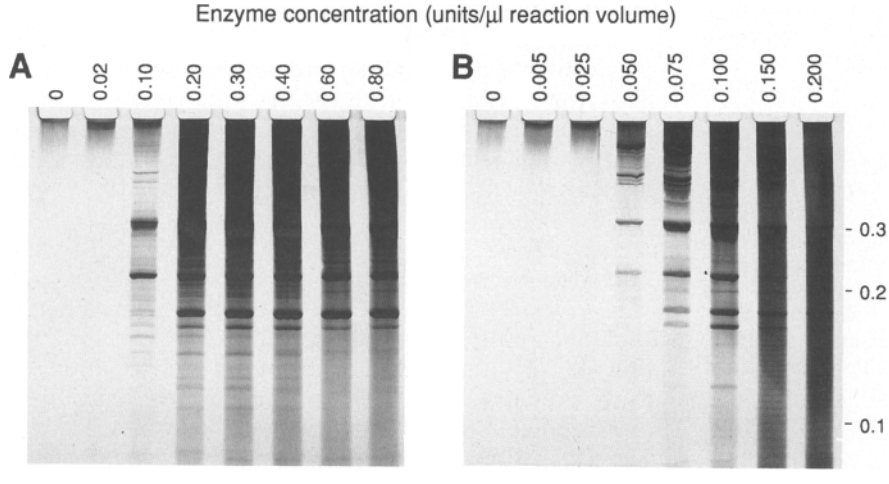

Fig. 2A, B. Effect of DNA polymerase concentration. Total DNA from *S. uberis* strain ATCC27958 was amplified using primer GTAACGCC under standard conditions (see Materials and methods). Amplifications were done using the Stoffel fragment (**A**) and Amplitaq (**B**) enzymes over a range of enzyme concentrations. Each lane shows 4 µl of undiluted amplification products. Sizes shown are in kb

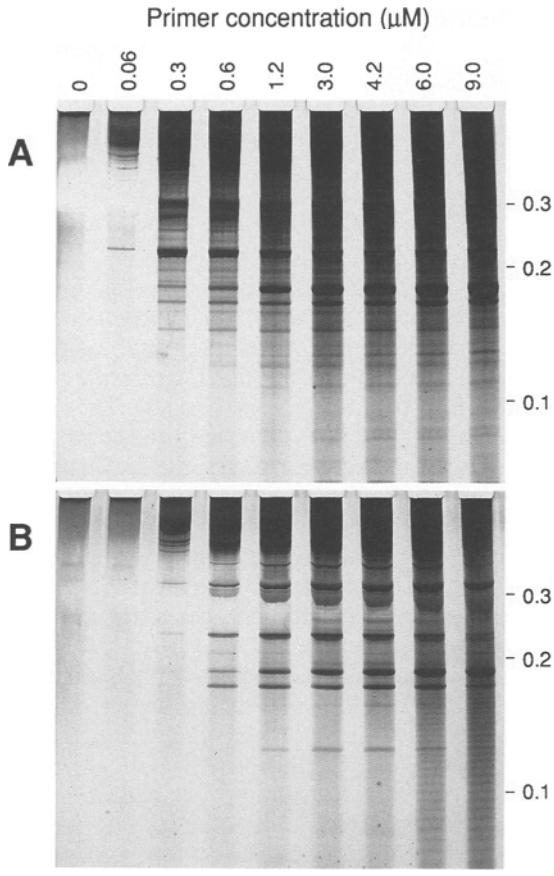

Fig. 3A, B. Effect of primer concentration. Total DNA from *S. uberis* strain ATCC27958 was amplified as described in Materials and methods using primer GTAACGCC. Amplifications were done using both the Stoffel fragment **(A)** and Amplitaq **(B)** enzymes over a range of primer concentrations (as shown). Each lane shows 4 μl of undiluted amplification products. Sizes shown are in kb

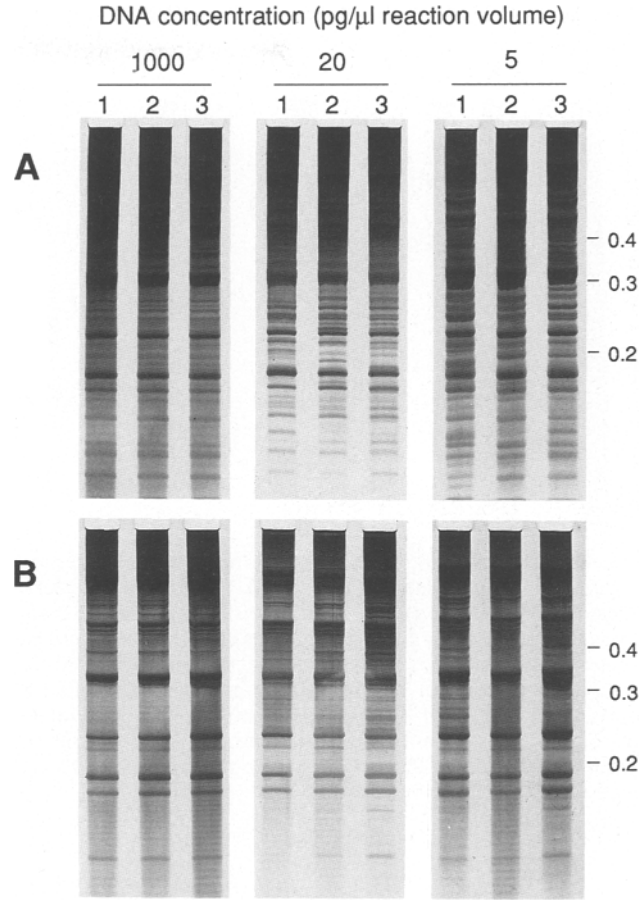

Fig. 4A, B. Effect of DNA template concentration on experimental consistency. Total DNA from *S. uberis* strain ATCC27958 was amplified as described in Materials and methods using primer GTAACGCC. Amplifications were done using both the Stoffel fragment **(A)** and Amplitaq **(B)** enzymes. Different concentrations of the DNA template were used in amplification reactions (as shown). Three replicates were compared (labelled **1, 2, 3**) for each dilution level tested. Inconsistent profiles occurred when less than 1000 pg/μl of template was used. All lanes show 4 μl of undiluted amplification products. Sizes shown are in kb

Thermal cycling

Since the majority of DAF products are less than about 500 bp, a two-step cycling between the melting temperature (96° C) and a non-stringent primer annealing temperature (30° C) was sufficient for amplification. DAF profiles did not improve if an extension step at 72° C was used (data not shown). The Amplitaq enzyme required more cycles to produce the same amount of product compared to the Stoffel fragment at optimal enzyme concentrations (data not shown). Consistent products amplified with high yield were produced after 30 cycles with the Stoffel enzyme and 35 cycles with the AmpliTaq enzyme. Cycle numbers as high as 50 can be used with each enzyme without affecting fingerprint quality. However, few products are generated after 30–35 cycles due to enzyme limitation.

Template concentration

Fingerprints can be produced from very small amounts of template DNA. Between 10 and 20 pg of template can be used (Caetano-Anollés et al. 1991a), and sometimes less than 1 pg is sufficient (data not shown). However, very low amounts of template are not adequate for bacterial fingerprinting analyses. We produced DAF profiles from replicate samples of our standard *S. uberis* template at various dilutions and showed that about 1 ng of template per μl of reaction mix was required for reliable results (Fig. 4). Fingerprint patterns produced by Amplitaq, while simpler, were more reliable at very low template concentrations. The inconsistent bands that occasionally appeared at low template concentrations were usually weaker secondary or tertiary products. Amplitaq also tended to produce ladder bands with *S. uberis* template where the Stoffel fragment did not.

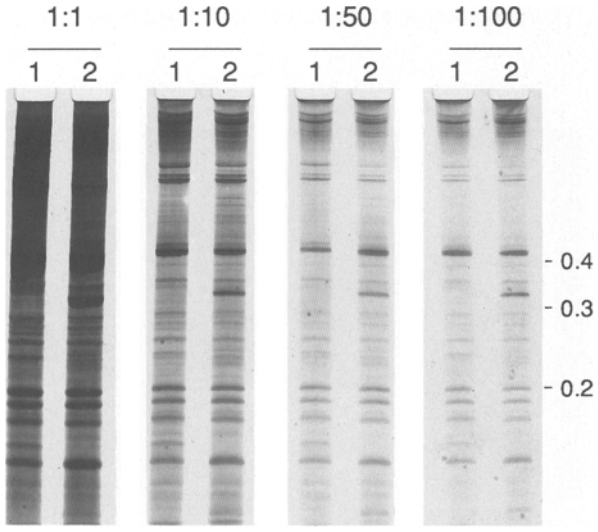

Fig. 5. Effect of dilution of amplification products on resolution of profiles in polyacrylamide gels. Total DNA from *S. uberis* strain ATCC27958 (*lane 1*) and the *S. uberis* clinical isolate 6 from the mastitis-infected cow L277 (*lane 2*) was amplified as described in Materials and methods with the decameric primer GTGACGTAGG using the Stoffel fragment DNA polymerase. Undiluted samples (1:1) consist of 4 µl of amplification products. Fingerprints of the two strains are best differentiated at dilution levels between 1:10 and 1:50. All lanes are from the same gel and were stained equally with silver. Sizes shown are in kb

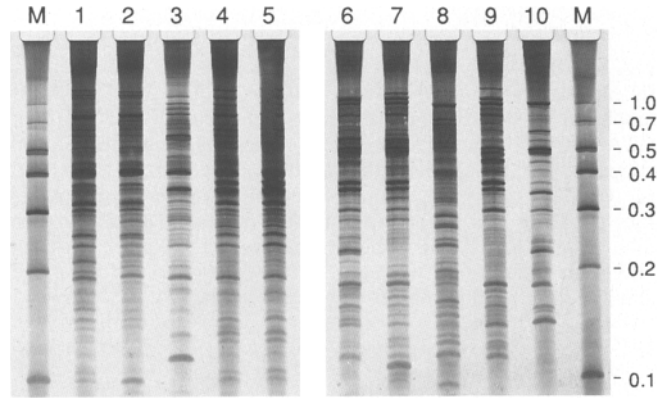

Fig. 6. DNA amplification fingerprints of *Escherichia coli* (*lanes 1–5*) and *Klebsiella pneumoniae* (*lanes 6–10*). Amplification was *lane 1,* Smith 92; *lane 2,* J21, *lane 3,* J25; *lane 4,* J4; *lane 5,* J23; *lane 6,* J7; *lane 7,* J9; *lane 8,* Smith 20; *lane 9,* J14; *lane 10,* J24. Total DNA from each strain was amplified using primers CGAGCTG (*lanes 1–5*) or GATCGCAG (*lanes 6–10*) as described in Materials and methods using the Stoffel fragment DNA polymerase. All lanes show 4 µl of amplification products that were diluted 1:10. Sizes shown are in kb as indicated by relative molecular mass markers (*lanes M*)

Appropriate visualization of DAF profiles

Very thin silver-stained polyacrylamide gels (less than 1 mm thickness) have the highest resolution but are easily overloaded with sample DNA. Thus, unless there is a need to examine the very weakest of DAF products (tertiary products) as we have here, we find it advantageous to run diluted DAF reaction products in a gel. This results in clearer, more precise fingerprints and minimizes the problems of gel saturation. Figure 5 illustrates how product dilution enhances resolution of polymorphisms between clinical isolates of *S. uberis*.

Fingerprinting clinical isolates

Using optimized amplification conditions we examined several bacterial isolates of *E. coli* and *K. pneumoniae* from mammary secretions of cows with clinical mastitis. Complex fingerprint profiles were generated in all cases with a heptamer and octamer primer (Fig. 6). The total mass of amplification products obtained was comparable with that from similar experiments using decamers (data not shown). Of the five *E. coli* isolates examined, two produced indistinguishable fingerprint profiles (J4 and J23) despite being from different cows and isolated over a year apart. In contrast, all strains of *K. pneumoniae* produced unique and characteristic fingerprints.

We examined polymorphic and monomorphic bands between 0.1 and 0.3 kb (Fig. 6). Fingerprints from *E. coli* isolates had an average of 17.6 bands (ranging from 13 to 18) out of a total of 25 identified in this range. Fingerprints from *K. pneumoniae* isolates had an average of 14 bands (ranging from 10 to 16) out of a total of 27 bands identified. However, *E. coli* fingerprints were less heterogeneous than those of *K. pneumoniae* when examined with the chosen primers, as evidenced by the existence of a higher proportion of monomorphic bands. For example, nine bands were common to all *E. coli* isolates, six were common to four, three were common to three, three were common to two, and four were unique among isolates. In contrast, only two bands were common to all *K. pneumoniae* isolates, four were common to four, seven were common to three, eight were common to two, and six were unique among isolates. Thus, fingerprints generated with just one primer can distinguish clinical isolates as well as provide a measure of relatedness.

Discussion

The use of single arbitrary oligonucleotide primers to initiate amplification of discrete portions of a genome can be used to generate complex and characteristic fingerprints. Each amplification product can, at least, reflect single base pair changes in a particular primer-target site making DAF a powerful fingerprinting strategy, especially for organisms that are closely related. Here we show that DAF can be used as a fast and reliable tool for general bacterial characterization provided that the amplification conditions are optimized.

We produced bacterial fingerprints without day-to-day experimental variability, even at the highest level of resolution. This reliability was emphasized by the fact that separate DNA preparations from a single *E. coli* isolate and strains of *E. coli* from mammary secretions

of different cows isolated over a year apart produced identical DAF profiles down to the barely detectable tertiary products. Thus, amplification products should be useful as markers to identify individual bacterial clones in epidemiological studies. Complex DAF profiles render information that can measure both variation and relatedness between organisms. This is especially valuable when there is a need to study closely related and quickly evolving bacterial consortia. In a related study, DAF was used to sub-group different isolates of *S. uberis* (Jayarao et al. 1992). The data matched sub-group characterization using different physiological, biochemical, and molecular approaches (including restriction endonuclease fingerprinting and 16S ribosomal DNA analysis) but had higher resolution.

In order to use bands as markers for genotyping sample individuals, data must first be obtained from the population as a whole. The validity of uniquely identifying or classifying an individual depends on the frequency at which the characteristic markers are expressed within the population. This is especially crucial with restriction fragment length polymorphism and other types of AFLP analyses (Welsh and McClelland 1990; Williams et al. 1990) in which few markers are analyzed, but less so with DAF since many more marker bands are produced. For example, Welsh and McClelland (1990) used long primers to identify bacteria but were unable to find diagnostic intra-specific markers. DAF now pushes the strategy of using arbitrary primers to the resolution of bacteria within species. Here we show that DAF could readily distinguish and classify clinical isolates of *E. coli*, *K. pneumoniae* and *S. uberis* in single experiments with only one arbitrarily chosen primer.

The choice of DNA polymerase had significant effects upon the quality, reproducibility, and complexity of fingerprints. We found the Stoffel fragment was superior to the native Amplitaq enzyme as it was less affected by experimental variables such as primer and enzyme concentration and produced more informative profiles. Unlike the Stoffel fragment, Amplitaq produced ladder bands under certain circumstances, for example when primer and enzyme concentrations were high. We found the "rungs" of these ladders were separated by a repeated unit length of about 4 bp when an octamer primer was used. While ladders could result from primer-primer amplification events, they could result from "slippage of the polymerase" or from "out-of-register" annealing of repeat unit sequences at high product concentrations (Erlich et al. 1991). Since ladders were only observed with Amplitaq, native exonuclease activity could be involved in their formation. Our results suggest that by varying enzyme properties, such as exonuclease and polymerase activities, tolerance to primer mismatching, or thermostability, better enzymes could conceivably be produced.

DAF profiles are composed of bands of varying intensity that may result from products amplified to varying extents. Several explanations could account for this effect. For example, less efficiently amplified secondary or tertiary products could result from weaker mismatch annealing of the primer to one or both target sites defining a product. More intense products could also arise when amplified regions are present in higher copy number in the template, such as from multicopy elements like plasmids. Finally, in the initial rounds of amplification, template effects like steric hindrance of annealing and/or primer extension could affect product yield.

Calculating an expected number of amplification products is a complex undertaking. Theoretically, the number of perfect matches (N) of an oligonucleotide of length L to a single strand of a genome of complexity C having random sequence distribution is given by the equation $N=(1/4)^L \cdot C$ (Sambrook et al. 1989). The complexity of the *E. coli* genome in nucleotide pairs is about 4.0×10^6. Thus, the number of perfect matches to a heptamer primer is expected to be about 488. This would space annealing sites about 8.2 kb apart. However, sequence distribution is not random and in practice only a fraction of sites (about one tenth) occur within the 1-kb range suitable for amplification. Of these sites, only one quarter will anneal primers on opposite strands with 3′ ends facing each other, the only orientation of the four possible that sustains amplification. These considerations could reduce the number of bonafide products dramatically, to about ten.

Since the fingerprint complexity of small bacterial genomes compares to that of larger genomes like plants and mammals (Caetano-Anollés et al. 1992), amplification products could also arise from mismatching annealing events. Allowing single base mismatches at all positions in a heptamer would produce 21-fold more annealing sites (Sambrook et al. 1989) reducing their spacing to about 0.39 kb. However, Caetano-Anollés et al. (1992) showed that mismatches that sustain amplification are allowed only at the 5′ end of the primer. Thus, if only one mismatch is tolerated, the number of products will be only threefold higher than for perfect annealing. This is roughly the relationship that we find between primary and secondary products in our fingerprints (Fig. 6). It should be noted that a combination of perfect and mismatch annealing events could also yield products, perhaps of intermediate intensity. Depending upon the rarity of perfect priming sites in the template, primary products may originate from mismatch annealing events.

We found that a bacterial template concentration of at least 1 $ng \cdot \mu l^{-1}$ was crucial for experimental consistency. Inconsistent bands at lower concentrations were usually confined to secondary and tertiary products. During the first one or two cycles of amplification, target sites that amplify with low efficiency or are rare may not be stoichiometrically represented in the later rounds of amplification. Increasing template concentration and thus the number of target sites can compensate for this stochastic effect and avoid fingerprint inconsistencies. Higher template concentrations were not required when using animal or plant templates (Caetano-Anollés et al. 1991a) probably due to their higher complexity.

Appropriate primer concentration was also important for adequate amplification. When compared to other protocols (Williams et al. 1990), over ten times more primer (3 μM) was required to reveal all amplification products. These concentrations are also higher than

those normally used in the PCR. Several explanations may account for this requirement. For example, single primer amplification will produce products with terminal symmetry that can form hairpin structures (Caetano-Anollés et al. 1992) as well as concatemers linked by the symmetrical sequences. Increasing primer concentration will favor competition for annealing sites. Hairpin structures should be less stable the longer the amplification product and the shorter the primer, and thus the region of symmetry (Caetano-Anollés et al. 1992).

The optimized amplification conditions described here for fingerprinting bacteria work well with a variety of other templates of similar complexity (unpublished) and should therefore serve as a basis for general DAF protocols. Here we have shown that the interplay of several parameters affect the amplification reaction, a careful examination of which should precede any fingerprinting study of an untested set of closely related genomes.

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