Fingerprinting genomes using PCR with arbitrary primers*

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ABSTRACT

Simple and reproducible fingerprints of complex genomes can be generated using single arbitrarily chosen primers and the polymerase chain reaction (PCR). No prior sequence information is required. The method, arbitrarily primed PCR (AP-PCR), involves two cycles of low stringency amplification followed by PCR at higher stringency. We show that strains can be distinguished by comparing polymorphisms in genomic fingerprints. The generality of the method is demonstrated by application to twenty four strains from five species of *Staphylococcus*, eleven strains of *Streptococcus pyogenes* and three varieties of *Oryza sativa* (rice).

INTRODUCTION

It is important in epidemiology and ecology to be able to identify bacterial species and strains accurately. Rapid identification and classification of bacteria is normally carried out by morphology, nutritional requirements, antibiotic resistance, isoenzyme comparisons, phage sensitivity (1,2,3,4) and, more recently, DNA based methods, particularly rRNA sequences (5), strain-specific fluorescent oligonucleotides (6,7) and the polymerase chain reaction (8,9,10). Each of these methods has specific applications and advantages. We demonstrate here a complementary method that is simple and fast, and can be applied to any species for which DNA can be prepared. This method, which we call Arbitrarily Primed Polymerase Chain Reaction (AP-PCR) 'fingerprinting' has the further merit of requiring little knowledge of the biochemistry or molecular biology of the species being studied.

As a first application, and to demonstrate the ease and utility of this method, we tested strains from five species of *Staphylococcus*, the relationships for which had been determined by DNA-DNA hybridization (11,12,13,14), eleven strains of the human pathogen *Streptococcus pyogenes*, and three varieties of rice, an agriculturally important plant.

METHODS

Strains

S. aureus, ISP-8, isolated from human. ATCC 8432, from bird. ATCC 15564, from human. ATCC 6538, from human. Sau3A, from human. ATCC 12600, from human.

S. cohnii, JL 143, from human. CM 89, from human. SS 521, from Squirrel monkey.

Staphylococcus haemolyticus, C 12J2, isolated from Mangabey. PAY 9F2, from Chimpanzee. AW 263, from human. MID 563, from Mouse lemur. ATCC 29970, from human.

S. hominis, ATCC 27844, from human. ATCC 27846, from human. Ful, questionable origin.

S. warneri, CPB 10E2, isolated from Cercopithecus. GAD 473, from Bush-baby. MCY 3E6, from Rhesus monkey. PBNZP 4D3, from Langur. LED 355, from Lemur.

All *Staphylococcus* strains were kindly provided by W.E. Kloos of North Carolina State Univ. except ISP-8 from Peter Pattee, Iowa State, Ful, and those from the American Type Culture Collection.

Streptococcus pyogenes. DNAs from the human pathogenic strains D471, TI/195/2, 40RS15, 52RS15, 47RS15, 55RS15, 1/E9, T28/51/4, K58Hg, SM6, UAB 092 and *Enterococcus faecalis*, OGI X, were all kindly supplied by Susan Hollingshead (Univ. of Alabama, Birmingham, AL).

Oryza sativa, (Rice). Genomic DNAs from ssp. *indica* IR54, ssp. *japonica* Calsorse 76 and *O. sativa* cv. 'Lemont' were kindly provided by Thomas Hodges (Purdue Univ., West Lafayette, IN) and Timothy Croughan (Louisiana State U., Rice Expt. Station, Crowley, LA).

Staphylococcus genomic DNA preparation

Staphylococcus strains were grown overnight at 37°C in 2–5 ml of brain heart infusion media. The cells were pelleted, resuspended in 0.2 ml of 1× TE. with 0.2 mg/ml lysostaphin and incubated at 37°C for one hour. 0.2 ml proteinase K solution (0.5 mg/ml proteinase K, 1% sarkosyl, 200 mM EDTA, 1 mM calcium chloride) was added to each, followed by digestion at 50°C for 1 hr. The cleared lysates were extracted with phenol, then chloroform, then ethanol precipitated. The DNA was dissolved in TE, and the final concentration was estimated by agarose gel electrophoresis and ethidium staining.

Primers

The primer Kpn-R (CCAAGTCGACATGGCACRTGTATA-CATAYGTAAC), and the pBS reverse sequencing primer (GG-AAACAGCTATGACCATGA), designed for other purposes and chosen arbitrarily for these experiments, were obtained from Genosys, Houston, TX.

*Publication of this paper was delayed by the authors to allow simultaneous publication with a paper submitted later by another group. *Nucleic Acids Research* regrets that due to administrative errors the other paper, by Williams *et al.*, was published on pages 6531–6535 of issue 22. Both sets of authors agree that the two papers should be considered as published simultaneously and should be referred to together.

AP-PCR amplification

10 μl reactions were prepared using 0.025 U Taq pol and 1× buffer (Stratagene) adjusted to 4 mM with $MgCl_2$, 0.2 mM of each dNTP, 10 μM Kpn-R primer and DNA at various concentrations as indicated in the figure legends. A high Mg^{2+} concentration was selected to enhance the stability of primer/template interactions. The reaction was overlaid with oil and cycled through the following temperature profile: 94°C for 5 min. to denature, 40°C for 5 min. for low stringency annealing of primer and 72°C for 5 min. for extension for two cycles. This temperature profile was followed by ten high stringency cycles: 94°C for 1 min., 60°C for 1 min. and 72°C for 2 min. for 10 cycles. At the end of this reaction, 90 μl of a solution containing 2.25 U Taq pol in 1× buffer, 0.2 mM dNTPs and 50 μCi α-[^{32}P] dCTP was added and the high stringency cycles were continued for an additional 20 or 30 rounds. This protocol was designed to allow for high primer concentration during the low stringency steps.

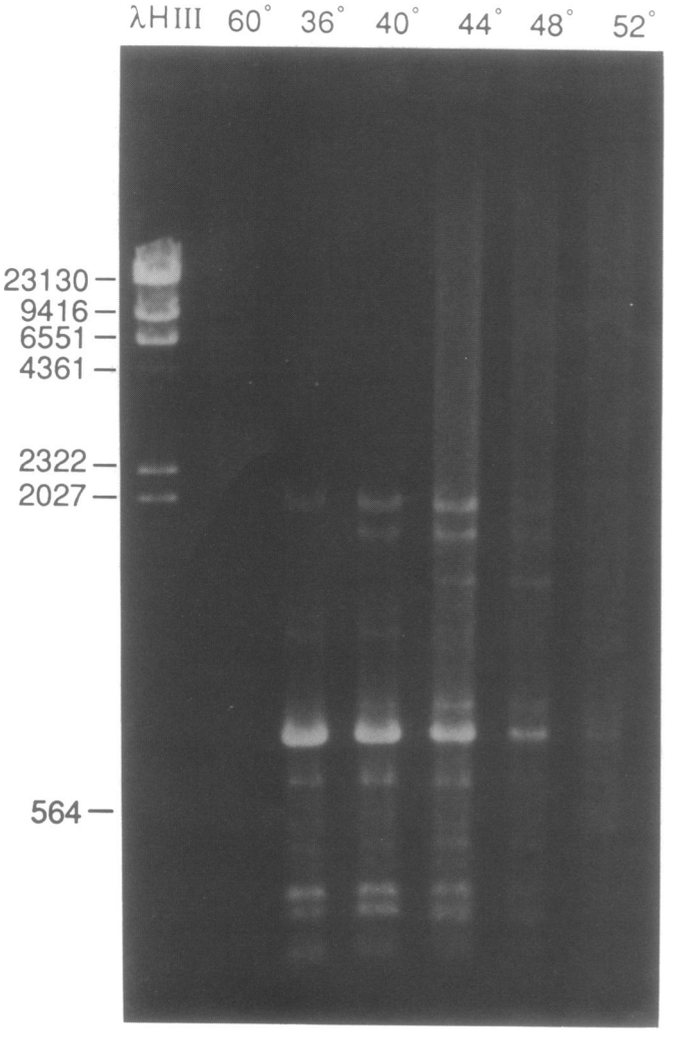

Figure 1. Variation of temperature in first two steps: Discrete AP-PCR products are produced over a wide range of temperatures. PCR was preformed using 1 ng of *S. aureus* ISP8 DNA with the Kpn-R primer and the standard protocol (methods) except the two initial low temperature steps were varied. DNA was electrophoresed in 1% agarose 1× TBE gel and visualized by ethidium staining. The low stringency annealing temperature was 36° (lane 2), 40° (lane 3), 44° (lane 4), 48° (lane 5) and 52° (lane 6). In lane 1, the low stringency step was 60° which is typical for conventional PCR. Lane M is *Hin*dIII-digested bacteriophage lambda DNA.

RESULTS

We observed that by using a single primer and two cycles of low stringency PCR followed by many cycles of high stringency PCR we were able to produce a discrete and reproducible set of products characteristic of genomes. We have found that between three and twenty products predominate from most bacterial and eukaryotic genomes. The fingerprints we have produced are reminiscent of those produced by *specific* priming at the *Alu* repeat in human genomic DNA (15), except that AP-PCR does not rely on high homology to abundant dispersed repeats.

Our rationale for the phenomenon of AP-PCR is as follows: At a sufficiently low temperature, primers can be expected to anneal to many sequences with a variety of mismatches. Some of these will be within a few hundred base pairs of each other and on opposite strands. Sequences between these positions will be PCR amplifiable. The extent to which sequences amplify will depend on the efficiency of priming at each pair of primer annealing sites and the efficiency of extension. At early cycles, those that prime most efficiently will predominate. At later times, those that amplify most efficiently will predominate.

We investigated the parameters that affected the production of reproducible, species- and strain-specific, AP-PCR fingerprints. The number, reproducibility and intensity of bands in a fingerprint should be a function of several parameters,

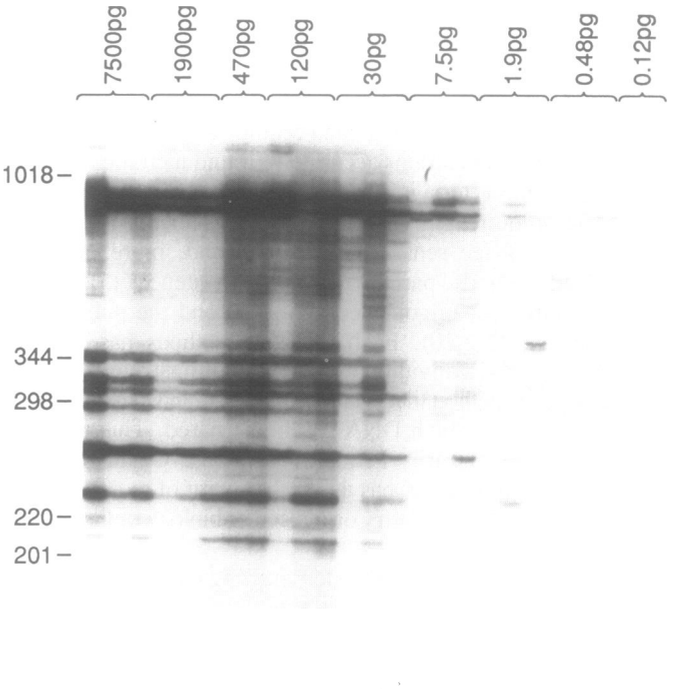

Figure 2. Template titration: The pattern of AP-PCR products depends on the template DNA concentration. AP-PCR was performed using *S. aureus* ISP8 DNA with the Kpn-R primer and the standard protocol (methods) except the template concentration was varied. Each series of nine different concentrations was from three independent dilutions (amount of template per reaction is shown above each set of three lanes. Size markers are the 1 kb ladder from BRL/Gibco. The DNA was separated on a 5% acrylamide, 50% urea 1× TBE gel and visualized by autoradiography.

including the concentration of salts, primer annealing temperature, template concentration, primer length and primer sequence.

Temperature

Initially, we determined the effect of temperature on AP-PCR using the genome of the bacteria *Staphylococcus* as a template. If the stringency is kept high (at least 60°C) throughout the thermo-cycling reaction and the primer is sufficiently different from any sequence in the template, no specific product will be observed (lane 1, Figure 1). Cycling at lower temperatures for two cycles (annealing at 35–50°C) followed by 40 cycles of standard PCR is sufficient to generate a pattern of bands which is characteristic of the species from which the template is derived (lanes 2–5, Figure 1). The pattern of bands changes only slightly as the temperature is raised, until, at some point, the temperature is too high for this set of matches to predominate (lane 6, Figure 1). We conclude that consistent AP-PCR can be achieved over a fairly broad range of temperatures. Other primers, ranging in size from 20 to 34 bases, give a different pattern but have similar temperature dependent characteristics (data not shown). However, the temperature that results in reproducible PCR products may need to be adjusted if very large genomes or much shorter or much longer primers are employed. In preliminary experiments we have recently performed with primers that have considerable sequence redundancy, very low temperatures for the initial two steps were not required, presumably because some of the sequences in the mixture inevitably anneal quite well to any complex genome (data not shown).

Template concentration

Figure 2 shows the effect of varying template concentration on AP-PCR patterns. *S. aureus* ISP8 genomic DNA was varied through nine different concentrations in the range (7.5 ng to 0.12 pg). When run on a high resolution polyacrylamide gel, the pattern generated by AP-PCR with the 34 base Kpn-R primer is very consistent for template concentrations between 7.5 ng and 30 pg. Below this level the PCR is more sporadic. However, the products produced are also almost all represented at higher template concentrations. In other experiments 10 pg has been the lower limit of reliable AP-PCR for genomes of this size (approx 3×10^6 bp) (16). Ten pg of template DNA equals approximately 3000 molecules. One interpretation of this data is that even the best priming events are quite inefficient and, at this low template concentration, the probability of initiating each AP-PCR event during each of the two low temperature cycles is about one in 55 (sq root of 3000). We expect that the efficiency of priming can be improved by altering our present protocol.

Intra-specific patterns of AP-PCR products in *S. aureus*

We compared the pattern of AP-PCR products from six *S. aureus* strains over a concentration range of 7.5 ng to 30 pg of genomic DNA (Figure 3). In this range the patterns are highly reproducible and also very similar between strains. The differences between strains should be diagnostic of specific strains and their relatedness. Each primer of similar length but of different sequence gives a different set of patterns, as expected since the template/primer interactions are different. Intra-specific variation in the pattern is observed for each primer (data not shown). Such data should be of value in the construction of phylogenetic trees.

Species specific AP-PCR products

Genomic DNA of strains from five different species of *Staphylococcus* were each amplified at two different DNA concentrations (Figure 4). In general, there is a species-specific pattern of PCR products, for instance, the *S. warneri* strains (lanes

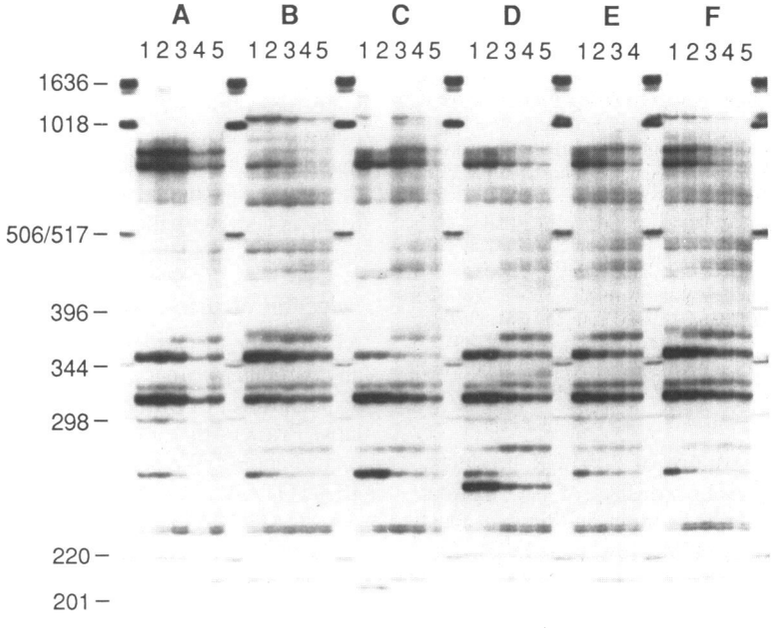

Figure 3. Genomic fingerprints of six strains of *Staphylococcus aureus*: AP-PCR patterns are similar between strains of the same species. Six strains of *S. aureus* were fingerprinted by AP-PCR with the Kpn-R primer and the standard protocol and five different amounts of template; 7.5 ng, 1.9 ng, 470 pg, 120 pg and 30 pg per reaction. The highest concentrations are shown on the left for each strain. Lanes 1, ISP8; lanes 2, ATCC 8432; lanes 3, ATCC 15564; lanes 4, ATCC 6538; lanes 5, *Sau*3A; lanes 6, ATCC 12600. The resulting amplified material was resolved by electrophoresis through 5% polyacrylamide containing 50% urea and 1× TBE and visualized by autoradiography. Size markers are the 1 kb ladder from BRL/Gibco.

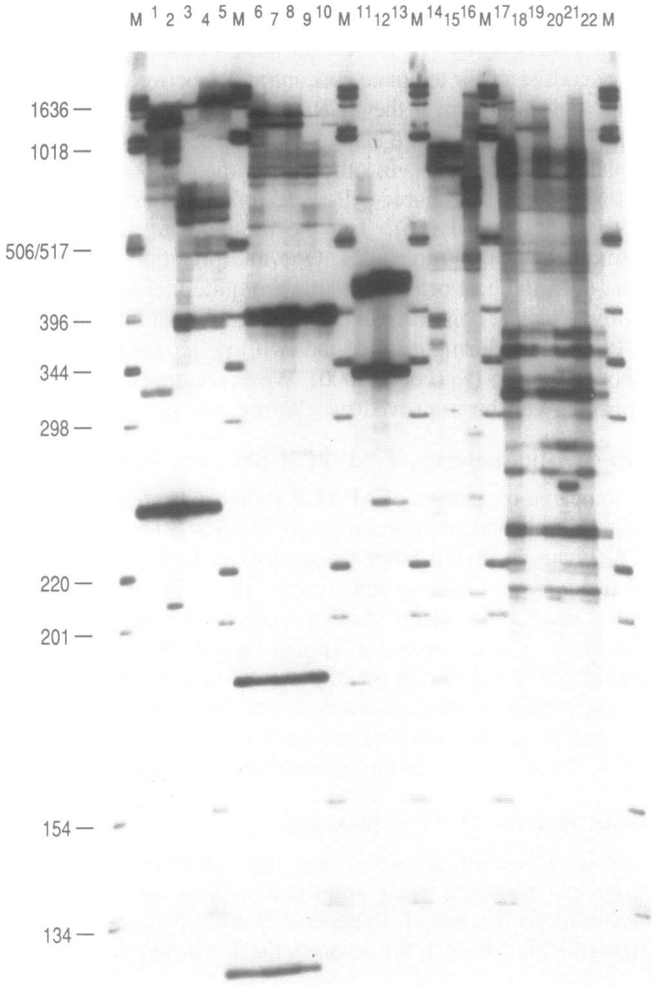

Figure 4. Genomic fingerprints of several strains from five species of *Staphylococcus*: AP-PCR patterns are characteristic of the species. Fingerprints of seventeen isolates representing four different species of *Staphylococcus* were fingerprinted by AP-PCR with the Kpn-R primer and the standard protocol with 16 ng of template per reaction. The resulting amplified material was resolved by electrophoresis through 5% polyacrylamide containing 50% urea and 1× TBE and visualized by autoradiography. Size markers are the 1 kb ladder from BRL/Gibco. The species fingerprinted were lane 1, *S. haemolyticus* CC 12J2; lane 2, PAY 9F2; lane 3, AW 263; lane 4, MID 563; lane 5, ATCC 29970; lane 6, *S. warneri* CPB 10E2; lane 7, GAD 473; lane 8, MCY 3E6; lane 9, PBNZP 4D3; lane 10, LED 355; lane 11, *S. hominis* ATCC 27844; lane 12, ATCC 27846; lane 13, Fu1; lane 14, *S. cohnii* JL 143; lane 15, CM 89; lane 16, SS 521; lane 17, *S. aureus* ISP-8; lane 18, ATCC 8432; lane 19, ATCC 15564; lane 20, ATCC 6538; lane 21, Sau 3A; lane 22, ATCC 12600. Size markers are the 1 kb ladder from BRL/Gibco.

6−10) give an almost identical pattern of AP-PCR products. However, the *S. haemolyticus* group consists of two distinct patterns that share only one AP-PCR product and have at least three prominent products that are not shared (lanes 1−5). The differences in products were dramatic and are presumably the result of a large amount of sequence difference between strains. The most likely possibility seems to be that the *S. haemolyticus* species has two distinct 'sub-species'. It is interesting to note that one 'sub-species' AP-PCR pattern is produced by *S. haemolyticus* strains (AW 263, ATCC 29970 and PAY 9F2) that live on the closely related primate species human and chimpanzee, the other strains of *S. haemolyticus* (MID 563 and CC 12J2) are from Mangabey and Lemur (Kloos, unpublished).

The utility of the method was further demonstrated when AP-PCR revealed the incorrect classification of a strain. Using AP-PCR, strains that are misassigned are very rapidly uncovered.

The AP-PCR products are species or sub-species specific and not conserved even between relatively closely related species, such as *S. haemolyticus*, *S. warneri*, and *S. hominis*. Nevertheless, a few AP-PCR products may be shared between species. For instance, the product at about 400 base pairs in *S. warneri* may be the same as in the *S. haemolyticus* strains PAY9F2, AW263 and ATCC29970, however, we cannot exclude the possibility of coincident migration.

It should be noted that occasionally a plasmid may contribute one of the prominent AP-PCR products. There are small plasmids in some of the *Staphylococcus* strains. The best way to avoid being confused by the lateral transfer of plasmids between unrelated strains is to perform separate AP-PCR reactions with a few different primers (data not shown). Small plasmids, because of their low sequence complexity, are extremely unlikely to contribute to the AP-PCR patterns of most arbitrary selected primers.

The AP-PCR method can, in principle, be applied to detect polymorphisms in a wide variety of organisms using a variety of different primers. To illustrate this, we examined several *Streptococcus* strains and a plant, rice, by amplification using the twenty base pBS reverse sequencing primer and genomic DNA. Figure 5 shows the polymorphic fingerprints that are produced for these organisms. For instance, *Streptococcus pyogenes* strains (genome size 2−3 megabases) have some AP-PCR products in common, whereas, others are shared by only some strains and yet others are unique to the strain. Consistent with our results in *Staphylococcus*, the one strain from another species, *E. faecalis*, (Figure 5, lane b11) shares no common PCR products with *S. pyogenes* strains. We are currently constructing a phylogenetic tree for *S. pyogenes* using data accumulated from AP-PCR with three separate primers (manuscript in prep.).

Rice, despite having a genome size of about 700−1000 megabases (17,18,19), gave a simple AP-PCR pattern (with a 48°C low stringency step) which included a mix of species-specific and strain-specific products. Strain specific polymorphisms can, in principle, be used as markers for genetic mapping. Experiments with an assortment of primers and with the maize and human (each about 3,000 megabases) genomes (data not shown) indicate that AP-PCR will work with most species and most primers.

Selection of primers

AP-PCR for species level identification of strains does not require any particular primer sequence. Investigators may wish to choose a standard set of primers so that data can be compared between labs. Primers that are already in extensive use seem to be the logical choice. We propose the 20 base pair sequencing primers, TTATGTAAAACGACGGCCAGT (Universal M13 −20), GGAAACAGCTATGACCATG, (M13 reverse sequencing), GTAATACGACTCACTATAG (T7), and GCAATTAACCCTCACTAAAG (T3). These primers have been very successful in our subsequent AP-PCR studies. These primers will also have other unrelated uses in most labs; for sequencing projects and for the specific PCR of clones as labelled probes for Southern blots.

Lysis by boiling

For bacteria, the data presented here involved the purification of DNA by cell wall lysis, proteinase K digestion and phenol

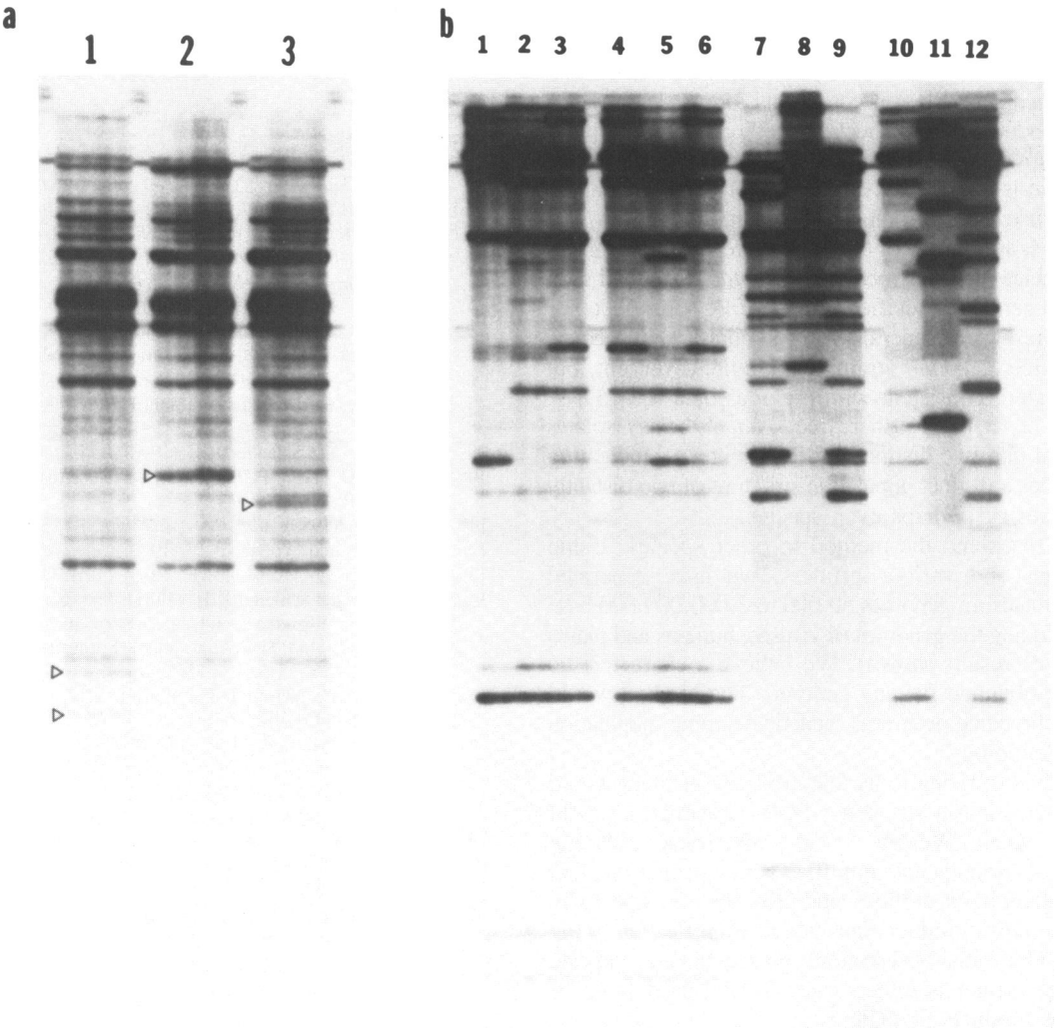

Figure 5. Genomic fingerprints of rice and *Streptococcus* strains: AP-PCR patterns can be generated for any species. Fingerprints of three inbred strains of rice and eleven strains of *Streptococcus* were fingerprinted by AP-PCR with the standard protocol and the pBS reverse sequencing primer. The low stringency annealing step was performed at 48°C for rice and at 40°C for *Streptococcus*. In the case of rice a serial dilution of 51 ng, 12 ng, 3 ng and 0.8 ng of genomic template DNA is presented for each strain. In the case of *Streptococcus*, 19 ng and 5 ng of genomic template were used. The resulting amplified material was resolved by electrophoresis through 5% polyacrylamide containing 50% urea and 1× TBE and visualized by autoradiography. Size markers are the 1 kb ladder from BRL/Gibco. The strains fingerprinted were: Lanes a1, *O. sativa* ssp. *indica* IR54; lanes a2, ssp. *japonica* Calsorse 76; lanes a3, Lemont. Lanes b1, *S. pyogenes* D471; lanes b2, TI/195/2; lanes b3, 40RS15; lanes b4, 52RS15; lanes b5, 47RS15; lanes b6, 55RS15; lanes b7, 1/E9; lanes b8 T28/51/4; lanes b9, K58 Hg; lanes b10, SM6, lanes b11, *E. faecalis* OGI X; lanes b12, *S. pyogenes* UAB 092. Size markers are the 1 kb ladder from BRL/Gibco.

extraction. We have found that equivalent data can be generated by a much simpler DNA preparation in which the cells from a colony on a petri dish are simply boiled, the debris pelleted and the supernatant diluted for AP-PCR (data not shown). This allows the time from petri dish to strain identification to be reduced to 36 hours.

DISCUSSION

The classification of bacterial species or strains is an area of active research in population biology and medicine. Recent progress has included the use of oligonucleotides for the direct staining of cells using fluorescent probes to species or strain specific genes (eg 6,7) and the Polymerase Chain Reaction (PCR) (8). This latter method may revolutionize epidemiology and population biology. Specific PCR primer pairs can be used to identify genes characteristic of a particular species or even strain. PCR also obviates the need for cloning in order to compare the sequences of genes from related organisms (eg 10), allowing the very rapid construction of DNA sequence based phylogenies. For epidemiological purposes, specific primers to informative pathogenic features can be used in conjunction with PCR to assign identity.

While fluorescent probes and PCR are both very powerful methods, conventional primers for PCR and oligos for *in situ* hybridization require sequence information from the relevant genes and must be custom built for each location within the genome of a species or strain. We have developed an alternative method, arbitrarily primed PCR (AP-PCR), which is very rapid and simple and generates a fingerprint of PCR products. AP-PCR does not require a particular set of primers. Instead, this method uses primers chosen without regard to the sequence of the genome to be fingerprinted. Thus, AP-PCR requires no prior knowledge of the molecular biology of the organisms to be investigated. Each primer gives a different pattern of AP-PCR products, each with the potential of detecting polymorphisms

between strains. Thus, the data produced allows the differentiation of even closely related strains of the same species. In this respect it is similar to isozyme studies (3,4).

We have defined a window of conditions for AP-PCR which results in a reproducible and effective method for preliminary identification of any strain. This method may be very useful when the particular aim is to survey a large number of individuals in a population, for epidemiological or population studies, for example. As a first application of the method we compared strains from various species of *Staphylococcus*, *Streptococcus*, and rice. We were able to generate specific patterns of PCR products for each strain and these were generally quite similar within species. However, the three *S. cohnii* strains were quite divergent, but recognizably related, consistent with previous data from DNA renaturation (11,12,13,14). Further, five *S. haemolyticus* isolates could be grouped into two distinct AP-PCR patterns, indicating that this species has a distinct population structure of two distantly related sub-species or perhaps even species.

It is possible to extend the method to other species. Using similar conditions and various primers, we have generated discrete fingerprints from genomes 50,000 to 3,000,000,000 base pairs in size, including the genomes of viruses, humans and plants (including the rice results shown). We believe a characteristic pattern could be obtained for any genome. The method would then be useful in breeding programs, genetic mapping, population genetics or epidemiology.

It was initially surprising to us that arbitrary primers would give discrete patterns in low stringency PCR. However, it should be noted that when Wesley et al., dissected polytene chromosomes of drosophila and amplified with a primer that had a string of redundant bases at the 3′ end (20), they unexpectedly observed discrete DNA products rather than amplification of the whole fragment. Our initial observations, presented here, indicate that a degenerate primer is unnecessary for the production of multiple discrete products in PCR.

In the Wesley et al. study (20), PCR products could be removed from the gel and used to hybridize back to genomic digests, clones or chromosomes. Since polymorphic AP-PCR bands can be used as probes, a link between the genetic and physical map can be envisioned.

For reproducible AP-PCR, many hundreds of template molecules were required under our present conditions. We are investigating conditions that may allow more efficient priming. In future experiments we would like to define conditions that generate common patterns between closely related species by making the AP-PCR less sensitive to sequence divergence. Conversely, we are attempting to develop conditions that will increase the ability of AP-PCR to detect differences in DNA sequence and, thereby, distinguish strains within a species. We are currently experimenting with shorter oligonucleotides and different PCR conditions. A more variable AP-PCR pattern within a species would allow a more detailed picture of intra-specific population structure.

Williams et al. have independently shown that arbitrary primers, ten bases long, can be used to generate polymorphic genomic fingerprints by the polymerase chain reaction (21).

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