PAGE analysis of the heteroduplexes formed between PCR-amplified 16S rRNA genes: estimation of sequence similarity and rDNA complexity

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Analysis of the 16S rRNA genes retrieved directly from different environments has proven to be a powerful tool that has greatly expanded our knowledge of microbial diversity and phylogeny. It is shown here that sequence similarity between 80 and 100% among 16S rDNAs can be estimated by the electrophoretic migration of their heteroduplexes. This was measured by hybridization and electrophoresis in polyacrylamide gels of the product obtained after PCR amplification of almost the entire 16S rRNA gene from different bacterial species. These heteroduplexes were also observed after amplification of samples containing DNA from two or more bacterial species and a procedure was applied to identify reliably heteroduplexes among the amplification products. The electrophoretic migration of the heteroduplexes observed after PCR was used to detect the presence of 16S rDNAs with different sequences in DNA extracted from both a mixture of two bacterial species and samples containing a natural bacterial community.

Keywords: 16S rRNA, heteroduplex, phylogeny, diversity

INTRODUCTION

Our knowledge of microbial diversity has been greatly extended by comparative sequence analysis of genes performed directly on DNA extracted from different environments. This has proven to be a powerful tool for detecting the presence of a high diversity of micro-organisms, independent of their ability to grow in culture media (Amann *et al.*, 1995). Because of their universal distribution, high conservation and apparent absence of interspecific transfer, 16S rRNAs genes are commonly analysed (Amann *et al.*, 1995; Lane *et al.*, 1985; Ward *et al.*, 1992; Woese, 1987), usually by cloning and sequencing after PCR amplification. This is labour-intensive and so several other methods for estimation of sequence differences among PCR-amplified 16S rDNAs have been applied. These include analysis by RFLP patterns (Moyer *et al.*, 1994), denaturing gradient gel electrophoresis (Muyzer *et al.*, 1993) and single-strand conformation polymorphism

(Lee *et al.*, 1996). The heteroduplex mobility assay (HMA) has been shown to be a powerful technique to screen sequence dissimilarity between annealed strands (Delwart *et al.*, 1993), but it has not been extended to the comparison of bacterial 16S rDNA sequences.

HMA is based on the annealing of nucleic acid strands that are not fully complementary and therefore produce heteroduplexes with distortions of the double helix in regions where mismatched or unpaired nucleotides occur. The unpaired single-stranded regions form structures of different shapes (Sheffield *et al.*, 1993; Yap & McGee, 1994), which decrease the electrophoretic mobility of the hybrid. This reduction in mobility has been used to estimate sequence dissimilarity among heteroduplexes formed between amplification products of different genes, and has been applied to the detection of mutations (Mauser *et al.*, 1996; Olds *et al.*, 1993). Though mispairing seldom causes a noticeable reduction in mobility in conventional agarose gels, it has a drastic effect in polyacrylamide gels (Delwart *et al.*, 1993), allowing in many cases detection of single base differences (Ganguly *et al.*, 1993; White *et al.*, 1992).

Abbreviation: HMA, heteroduplex mobility assay.

HMA has recently been applied to distinguish species or genotypes of bacteria (Espejo & Romero, 1997) and viruses (Bachman, 1997; Chezzi, 1996; Delwart *et al.*, 1993; Kreis, 1997).

Formation of heteroduplexes also occurs during PCR amplification of partially homologous genes and the pattern obtained from a single species enables allelic polymorphism and mutations in heterozygous individuals to be detected (Bowen *et al.*, 1997; White *et al.*, 1992), and has also been applied to the genotyping of different species (Jensen & Hubner, 1996; Rubocki *et al.*, 1992; Tang *et al.*, 1995, 1996; Zimmerman *et al.*, 1995).

We show here that heteroduplex formation by amplified 16S rDNA provides a rapid and convenient method for assessing the 16S rDNA similarity of bacterial isolates and also for detecting bacterial diversity in a natural community.

METHODS

Bacterial strains. DNA was extracted (see below) from the following strains: *Staphylococcus xylosus* ATCC 29971, *Staphylococcus epidermidis* ATCC 14990, *Staphylococcus simulans* ATCC 27851, *Staphylococcus saprophyticus* ATCC 15305, *Corynebacterium pseudodiphtheriticum* ATCC 10700, *Corynebacterium pseudotuberculosis* ATCC 19410, *Aeromonas hydrophila* ATCC 7966, *Escherichia coli* K-12, *Pseudomonas stutzeri* ATCC 17588, *Haemophilus influenzae* ATCC 33391, *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus casei* ATCC 393, *Acinetobacter calcoaceticus* ATCC 19606, *Clostridium novyi* ATCC 19402 and *Micrococcus roseus* (now *Kocuria rosea*) ATCC 186.

DNA extraction and amplification. DNA was extracted and purified for amplification as previously described (Espejo & Romero, 1997). In short, the method comprises lysis of the bacteria with SDS, treatment with ribonuclease A and proteinase K, subsequent extraction with phenol/chloroform and dialysis against TE buffer (0.01 M Tris, 0.001 M EDTA, pH 7.5). The amount of DNA was estimated visually by comparison of the ethidium-bromide-stained bands with appropriate standards after electrophoresis in 1% agarose gels. PCR amplification was performed as described by DeLong (1992) with primers Eubac27F and 1492R in a Hybaid Omm-E thermal cycler, with the following modifications: the pH was 9.0, Triton X-100 was added to a final concentration of 0.1% (v/v) and the concentration of DNA template was 0.1 ng μl^{-1} . The different mixtures of DNA from *S. simulans*, *S. epidermidis*, *A. hydrophila* and *L. acidophilus* were amplified at a concentration of 0.03 ng μl^{-1} each, whilst DNA extracted from bioleached mineral was added at 0.003 ng μl^{-1} . Thermal cycling was as follows: a first cycle at 95 °C for 3 min, 58 °C for 6 min and 72 °C for 1.5 min; a second cycle at 95 °C for 1.5 min, 58 °C for 3 min and 72 °C for 1.5 min; 30 cycles at 95 °C, 58 °C and 72 °C for 1.5 min each; and a final cycle at 95 °C for 1.5 min, 58 °C for 1.5 min and 72 °C for 5 min. When amplification was performed with different numbers of cycles, the decrease was in the 30 cycle component of the above.

Heteroduplex analysis. Denaturation and renaturation of mixed amplified 16S rDNA from different bacterial species was performed as follows: pairs of amplified 16S rDNAs were mixed at a concentration of about 5 ng μl^{-1} each in

renaturation buffer [0.1 M NaCl, 10 mM Tris/HCl (pH 7.8), 2 mM EDTA] (Delwart *et al.*, 1993) diluted 1:2 in distilled water, denatured at 98 °C for 7 min and subsequently renatured at 60 °C for 40 min. Denaturation and renaturation of the amplification products from the samples containing more than one DNA species were performed directly in the same buffer and reagents used for the amplification after addition of EDTA to a final concentration of 4 mM, at the temperature and times described above. When other conditions were used they are specified in the text. Electrophoresis was performed in either 7% or 3.5% (w/v) polyacrylamide gels (7 cm long $\times$ 8 cm wide $\times$ 0.15 cm thick) with Tris/borate buffer (Sambrook *et al.*, 1989), at 250 V for 2.5 h. DNA was visualized by staining with silver nitrate (Espejo & Escanilla, 1993) and the amount of DNA in each band was calculated after scanning with Molecular Analyst/PC (Bio-Rad), using the volume analysis tool. Sequence similarities among the 16S rDNA amplified regions were calculated using sequences obtained from the Ribosomal Data Project (Van de Peer *et al.*, 1994) aligned with the program SSEARCH (Pearson, 1991; Smith & Waterman, 1981).

RESULTS

Electrophoretic mobility of 16S rDNA heteroduplexes in polyacrylamide gels and sequence similarity

To explore the suitability of the HMA method for analysis of 16S rDNAs, we first determined the decrease in the mobility of the heteroduplex formed between amplified 16S rDNAs with different sequence similarities. Amplified 16S rDNAs from different species were mixed in similar concentrations and then denatured and renatured as described above. Homoduplexes and heteroduplexes were subsequently resolved by electrophoresis in 7% polyacrylamide gels and the migration of the heteroduplex relative to that of the homoduplex determined. This was roughly proportional to the percentage similarity between the amplified regions of the 16S rDNAs of the bacteria to a level of about 80% (Fig. 1). Heteroduplexes with similarities below 80% were usually less conspicuous, probably due to formation in lower amounts because of lower annealing. They were, however, better observed in 3.5% polyacrylamide gels (Fig. 2). Heteroduplexes were usually resolved into two bands that probably correspond to the hybrids formed by each, plus and minus complementary strands of amplified 16S rDNAs. Although the extent of dissimilarity in hybrid pairs is identical, the single-stranded regions in each hybrid may form distinct structural conformations which decrease the mobility to different extents (Jensen & Straus, 1993). The difference in migration between these hybrids is a good indication of the variability that can be observed among heteroduplexes with the same percentage similarity. The difference in relative migration between these heteroduplex pairs can be as large as 0.12, which according to the approximate relationship shown in Figs 1 and 2 would correspond to a similarity difference of about 2%. These results demonstrate that the determination of the electrophoretic mobility of the heteroduplex formed by the amplified 16S rDNA from an unknown bacterial strain with those from other bacterial

Fig. 1. Electrophoretic migration of heteroduplexes between amplified 16S rDNAs with different percentage similarity in 7% polyacrylamide gels. Amplification products from different bacterial species were mixed, denatured, renatured and subsequently subjected to electrophoresis (see Methods). (a) Lanes 1–7 show the pattern observed with annealed, amplified 16S rDNAs from the following bacterial pairs: 1, *S. xyloso* with *S. epidermidis*; 2, *S. simulans* with *S. saprophyticus*; 3, *C. pseudodiphtheriticum* with *C. pseudotuberculosis*; 4, *A. hydrophila* with *E. coli*; 5, *A. hydrophila* with *P. stutzeri*; 6, *A. hydrophila* with *H. influenzae*; 7, *H. influenzae* with *S. simulans*. Lanes 8 and 9 show controls performed with amplified 16S rDNAs of *A. hydrophila* and *H. influenzae* (pattern after annealing shown in lane 6); lane 8 shows the pattern obtained when mixing both amplified 16S rDNAs after denaturation and renaturation, and lane 9 that obtained after denaturation without renaturation. (b) Relative migration, calculated as the distance of migration of the heteroduplex divided by that of the homoduplex, plotted against percentage sequence homology. Data include those obtained in (a) and in additional experiments. When reciprocal heteroduplexes were resolved, both relative migrations were included in the graph joined by a vertical line.

species is a useful and simple tool to estimate 16S rDNA similarity values larger than 80%. The possible origin of the band observed immediately above the 16S rDNA homoduplex is discussed below.

Fig. 2. Electrophoretic migration of heteroduplexes between amplified 16S rDNAs with different percentage similarity in 3.5% polyacrylamide gels. Amplification products were mixed, denatured, renatured and subsequently subjected to electrophoresis. (a) Lanes 1–8 show the electrophoretic pattern observed with annealed 16S rDNAs from the following bacterial pairs: 1, *S. simulans* with *S. saprophyticus*; 2, *P. stutzeri* with *A. hydrophila*; 3, *S. simulans* with *H. influenzae*; 4, *S. saprophyticus* with *C. pseudotuberculosis*; 5, *L. acidophilus* with *A. hydrophila*; 6, *L. casei* with *A. calcoaceticus*; 7, *L. acidophilus* with *H. influenzae*; 8, *L. acidophilus* with *E. coli*. Lane 9 shows the pattern obtained when mixing the amplified 16S rDNAs shown in lane 8 but after independent denaturation and renaturation, and lane 10 that obtained with the same sample after denaturation without renaturation. (b) As described in Fig. 1.

Heteroduplex formation during PCR amplification of DNA samples that contain different 16S rDNAs

Amplification of 16S rDNA from DNA samples containing different bacterial species undoubtedly forms heteroduplexes, but their presence is usually undetected as the electrophoresis is performed in agarose gels. The heteroduplexes which develop probably originate from annealing of the single-stranded products between the denaturation and primer annealing steps of the thermal

Fig. 3. Analysis of the products obtained after increasing cycles of amplification of a mixture of DNA from *S. epidermidis* and *S. simulans*. The number of cycles is indicated above each lane. (a₁) Products in 1 µl amplified mixture; (a₂) products using adjusted volumes of sample from each cycle to allow comparison of similar amounts. The ratios of the amount of heteroduplex relative to the homoduplex are indicated below each lane. Subsequent electrophoresis separations were performed using adjusted sample volumes as in (a₂). (b) Products obtained after denaturation and renaturation of the samples shown in (a). (c) Products obtained after a single amplification cycle of samples in (a), performed after a 1/10 dilution in amplification mixture [five times the volume applied for electrophoresis in (a₂) was used to compensate for the dilution performed, assuming that the product was doubled after the amplification cycle]. (d) Products after denaturation and renaturation of samples shown in (c). Denaturation and renaturation were performed at 95 °C for 10 min and 70 °C for 100 min, respectively, in the same buffer and reagents used for the amplification.

cycle. The suitability of a method based on the appearance of heteroduplexes to assess bacterial diversity was tested with different samples consisting of synthetic

mixtures of two, three and four DNAs from different species and with a DNA sample extracted from bio-leached copper sulphides. The first sample examined was a mixture of equal amounts of DNA extracted from *S. epidermidis* and *S. simulans*, which show a similarity of 97.5% in the amplified region. Fig. 3 shows the appearance of the heteroduplexes and the results of subsequent tests that confirm their actual heteroduplex nature. Fig. 3(a₁) and (a₂) show the relative increase of heteroduplexes with increasing number of amplification cycles, probably due to higher annealing because of the increasing product concentration. Fig. 3(b) shows the increase in the relative amount of heteroduplexes when the amplification products were subjected to denaturation and subsequently to renaturation at 70 °C to avoid primer competition during annealing. Fig. 3(c) shows the resolution of the heteroduplexes into homoduplexes when the products were subjected to an additional amplification cycle after appropriate dilution to avoid their annealing, and Fig. 3(d) shows the recovery of the heteroduplexes from these homoduplexes after denaturation and renaturation.

The second sample examined was a mixture of equal amounts of DNA from three different bacterial species. The discrimination of heteroduplexes in the amplification product of mixtures of DNA from either *A. hydrophila* or *L. acidophilus* with the above staphylococci is shown in Fig. 4(a) and (b), respectively. Fig. 4(c) shows the heteroduplexes of the mixture of these four DNAs. The similarity between the amplified 16S rDNA regions of *A. hydrophila* and *L. acidophilus* with those of the staphylococci is about 77% and 84%, respectively, while the similarity between *A. hydrophila* and *L. acidophilus* is 76%. As indicated above, heteroduplexes which formed between strands sharing low similarity appeared weaker and were more difficult to distinguish, especially as some bands remained in the upper part of the gel after dilution and a single amplification cycle to resolve the heteroduplexes (lane 2). To avoid primer extension during renaturation at 60 °C, 4 mM EDTA was added in these experiments. Fig. 4 also shows the product obtained from a DNA extract from copper sulphides subjected to bioleaching. Analysis of the spacer regions between the 16S and 23S rRNA genes in this DNA had indicated that the bacteria in the original sample consisted of a community composed of a homogeneous population of *Thiobacillus thiooxidans*, and populations of *Thiobacillus ferrooxidans* and '*Leptospirillum ferrooxidans*', comprised of at least four and three different strains, respectively (Espejo & Romero, 1997). Fig. 4(d₁) shows the products obtained after annealing at 70 °C and electrophoresis in 7% polyacrylamide gel. Under these conditions, only the heteroduplexes expected between different strains of *T. thiooxidans* and *T. ferrooxidans*, with similarities between 97 and 100% in 16S rRNA (Goebel & Stackebrandt, 1994), were observed. Those expected between '*Leptospirillum ferrooxidans*' and the thio-bacilli, with similarities of about 74% (Goebel & Stackebrandt, 1994), could only be observed after

Fig. 4. Analysis of the amplification products obtained from samples containing DNA from different bacterial species. Lanes: 1, amplification products; 2, same products subjected to an additional amplification cycle after a 1/10 dilution; 3, products shown in lane 2 subjected to denaturation and renaturation. Gels show the amplification of a mixture of DNAs from (a) *S. epidermidis*, *S. simulans* and *A. hydrophila*; (b) *S. epidermidis*, *S. simulans* and *L. acidophilus*; and (c) *S. epidermidis*, *S. simulans*, *A. hydrophila* and *L. acidophilus*. Gels shown in (d₁) and (d₂) show the products from DNA extracted from bioleached copper ore. Lane 1* shows the products obtained after denaturation and renaturation of those shown in lane 1. Lane 4 shows the heteroduplex formed between amplified 16S rDNA of *T. ferrooxidans* DSM 583 and *T. thiooxidans* ATCC 19377. In (d₁), renaturation was performed at 70 °C as described in Fig. 3 and the electrophoresis was performed in 7% polyacrylamide gels. In (a), (b), (c) and (d₂), renaturation was performed at 60 °C as described in Methods and the electrophoresis in 3.5% polyacrylamide gels.

annealing at 60 °C and electrophoresis in 3.5% polyacrylamide gels (Fig. 4d₂).

DISCUSSION

The results presented here demonstrate that 16S rDNA sequence similarities between 80 and 100% can be estimated by the relative electrophoretic migration of the heteroduplexes in polyacrylamide gels. Thus, comparison of the migration of the heteroduplexes formed between amplified 16S rDNA from an unknown bacterium and properly selected bacterial species is potentially a useful taxonomic tool. It can be performed in a short time and requires only nanograms of DNA. Generally, 1 µl of the amplification product is sufficient for each hybridization and the product can be readily compared with 20 other amplified 16S rDNAs in a single day. Its usefulness has been shown by the extensive application of the method developed by Delwart *et al.* (1993) in the genetic classification of HIV viruses (Bachmann *et al.*, 1994; Novitsky *et al.*, 1996). Heteroduplex formation could be also applied to explore the presence of 16S rDNAs related to any particular species, following a procedure similar to that previously described with 16–23S rDNA spacers (Espejo & Romero, 1997). In this case labelled amplified 16S rDNA from a particular species, at low concentration to avoid self-annealing, could be hybridized to amplified 16S rDNA from the sample. The presence of labelled homoduplex will show the existence of that particular species, while

that of heteroduplexes will show the presence of species phylogenetically related according to the migration of the heteroduplexes. Moreover, the results also indicate that heteroduplex formation during PCR amplification of DNA extracted from bacterial communities can be useful for preliminary assessments of the number of 16S rDNAs differing in nucleotide sequence in the sample. Interpretation of the results obtained with simple communities containing two species is straightforward. However, interpretation of the heteroduplex pattern from more complex communities, which usually contain four or more different rRNA genes, is difficult because of the large number of potential heteroduplexes. Also, heteroduplexes between 16S rDNAs in lower proportion or/and with lower similarity can be undetected. True heteroduplexes could be distinguished from other possible hybridization products because they disappear after dilution and a single cycle of amplification, and reappear after denaturation and renaturation of this product. Interestingly, the band observed immediately above the 16S rDNA homoduplex after amplification of DNA from pure cultures behaved as a heteroduplex when examined for these properties. Its presence could be due to sequence polymorphism among the 16S rDNAs, even in pure cultures. As recently suggested, micro-heterogeneity within the rRNA operons of a single species could be a common phenomenon in nature (Nübel *et al.*, 1996; Wang *et al.*, 1997).

Heteroduplexes are probably formed during the tem-

perature decrease from the denaturation to the annealing step of the amplification cycle. They were observed only after the product reached a concentration of about 10^{-5} nucleotide-mol l^{-1} (Fig. 3). At this concentration, assuming a half complete renaturation ($C_0t_{1/2}$) value of 0.003 (nucleotide-mol s) l^{-1} (1400/3500 times the $C_0t_{1/2}$ of bacteriophage MS2 DNA) (Marmur *et al.*, 1963; Wetmur & Davidson, 1968) and that annealing nucleation sites can be formed between 85 and 70 °C, about 20% of the product would renature during the approximately 60 s that elapses in our thermal cycler between these two temperatures. The increment in the heteroduplex/homoduplex ratio (He/Ho) observed with increasing number of thermal cycles (Fig. 3) deserves a detailed discussion. According to the theory of annealing or reassociation kinetics (Britten & Davidson, 1976; Wetmur & Davidson, 1968), He/Ho should be a function of the ratio between the relative concentrations of heterologous DNAs and the annealing constants for heteroduplex and homoduplex formation, and should be independent of the total DNA concentration. However, during PCR amplification, homoduplex is formed by both annealing of the single-stranded DNA and primer extension, and in this situation He/Ho would be equal to the amount of heteroduplex formed by annealing divided by the amount of homoduplex formed by annealing plus that formed by primer extension. Since annealing and hence heteroduplex formation will increase with product concentration, He/Ho should increase with augmenting cycles, as observed. Formation of homoduplex by primer extension would also explain the large decrease in He/Ho after denaturation and renaturation of the product that was subjected to dilution and a further single amplification cycle (Fig. 3d₁). Since in this case active enzyme and reagents were still available and EDTA was not added, a considerable proportion of the homoduplex could have been formed by primer extension during the 100 min annealing time, although this was performed at 70 °C. To avoid primer extension during reannealing at 60 °C, EDTA was added to the reaction buffer (Fig. 4). Heteroduplex formation may have serious consequences when denaturing gradient gel electrophoresis is employed to estimate diversity. The heteroduplexes formed during amplification of heterogeneous samples may generate additional bands that would lead to a serious overestimation of diversity. Heteroduplex formation should be considered to explain the bias caused by template annealing in the amplification of mixtures of 16S rDNA genes by PCR. This has been observed and studied by Suzuki & Giovannoni (1996) but they assumed that each template reanneals only to the homologous complement and so does not inhibit the priming reaction of the other template. As shown here, the extent of annealing between heterologous 16S rDNAs is large and this would alter the conclusions of the above authors. Further quantitative studies on heteroduplex formation are needed to understand the complex product formation kinetics during PCR amplification of a mixture with partially homologous target regions.

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