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Long PCR-Ribotyping of Nontypeable *Haemophilus influenzae*

H. C. SMITH-VAUGHAN,* K. S. SRIPRAKASH, J. D. MATHEWS, AND D. J. KEMP
Menzies School of Health Research, Darwin, Northern Territory, Australia

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PCR-ribotyping, a new typing method based on long PCR, has been developed for nontypeable *Haemophilus influenzae* (NTHi). Ribosomal operons of NTHi were amplified by long PCR and were found to be highly polymorphic for internal *Hae*III sites. The technique was applied to 49 isolates previously subjected to conventional ribotyping, and the two methods showed a high level of concordance for serial isolates from individual subjects. PCR-ribotyping provides a powerful new typing tool for strain characterization in epidemiological investigations of NTHi.

The advent of the PCR technique has opened avenues for development of new and inventive typing methods, such as use of randomly amplified polymorphic DNA (RAPD). Prior to this, phenotypic methods such as serotyping, multilocus enzyme electrophoresis, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins were available. Genotypic methods such as restriction fragment length polymorphism (RFLP) analysis also proved to be useful, informative typing techniques. However, all had their limitations; the phenotypic methods were subject to variation and often had poor discriminatory capacities, while the conventional genotypic methods were time-consuming and tedious (13).

rRNA genes (rDNA) are ubiquitous in living organisms and have been studied extensively at the sequence level. The rates of evolution of different regions of these genes vary, providing sets of relatively conserved regions interspersed with more-variable regions (10). Moreover, variations within the 16S-23S-5S spacer regions have been reported and successfully used in typing (8). Consequently, the ribosomal operon is an attractive target for typing. RFLP analysis using rRNA (or cloned rDNA) as the probe (ribotyping) has been widely employed to type bacteria. Within a bacterial species, different isolates can often be distinguished by their ribotypes. A number of rRNA operons are usually present in a bacterial genome, and the polymorphisms giving rise to different ribotypes are most commonly located in the regions flanking these operons. However, as with other RFLP techniques, the procedure is time-consuming (13).

Nontypeable *Haemophilus influenzae* (NTHi) strains are important human pathogens associated with otitis media and respiratory infection. NTHi strains are extremely heterogeneous but difficult to study; newer techniques (3, 11) will allow the epidemiological behavior of NTHi to be studied in detail. In an earlier study (15), nasopharyngeal isolates of NTHi collected over a period of time from three Aboriginal infants with otitis media were ribotyped to investigate the dynamics of carriage. Ribotyping was discriminating but time-consuming because of the need for Southern blotting and probing. A typing technique that had a high level of discrimination, reproducibility, speed, and ease of performance was required.

Recently, a new method, long PCR (2, 4), has improved base pair fidelity and has made possible the amplification by PCR of much larger fragments of DNA than was previously possible. This is achieved by replacing *Taq* DNA polymerase with the combination of a high level of a thermostable DNA polymerase lacking 3′-5′ exonuclease activity (e.g., Klentaq [2]) and a very low level of a thermostable DNA polymerase exhibiting 3′-5′ exonuclease activity (e.g., *Pfu* DNA polymerase [2]).

We show here that long PCR can amplify approximately 6-kb rDNA operons of NTHi and that restriction fragments of the rDNA can readily be detected by agarose gel electrophoresis followed by ethidium bromide staining. The amplified ribosomal operons of NTHi were found to be highly polymorphic for internal *Hae*III sites, clearly discriminating between unrelated isolates of NTHi. This PCR-ribotyping scheme has proved to be as informative a system for typing NTHi as conventional ribotyping but is far less laborious.

MATERIALS AND METHODS

Bacterial strains. The NTHi strains were nasopharyngeal isolates collected originally as part of a longitudinal study of Aboriginal infants with otitis media in the Northern Territory of Australia (12). NTHi isolates were cultured on 7% chocolate agar and were identified by their requirements for X and V factors and by their lack of reaction with antisera against capsular antigens (Phadebact; KaroBio Diagnostics AB).

DNA preparation. Total chromosomal DNA was extracted by a modification of the method described by Pitcher et al. (14). Bacterial colonies from an overnight culture were suspended in 200 μl of 10 mM Tris-HCl–1 mM EDTA (pH 8) (TE) and pelleted by centrifugation. The pelleted cells were washed twice in this manner. Lysozyme was added to 200 μl of the resuspended pellet at 100 μg/ml, and the mixture was incubated at 4°C for 15 min. This was followed by the addition of RNase A at 150 μg/ml and a 30-min incubation. To this, proteinase K (100 μg/ml) was added, the mixture was incubated at 65°C for 2 h, and 500 μl of 5 M guanidinium thiocyanate–100 mM EDTA–0.5% (vol/vol) Sarkosyl was added. This mixture was placed on ice, and 250 μl of 7.5 M ammonium acetate was added. After extraction once with chloroform-isoamyl alcohol, the DNA was precipitated with isopropanol (0.54 volume). Following centrifugation, the pellet was washed three times with 70% ethanol and resuspended in TE.

DNA polymerases. Cloned *Pfu* DNA polymerase was provided by Stratagene at 2.5 U/μl (1 U is defined as the amount of enzyme required to incorporate 10 nmol of dTTP in 30 min at 72°C). Klentaq 1 was supplied by Ab Peptides (St. Louis, Mo.) at 30 U/μl. The mixture of 15 μl of Klentaq 1 and 1 μl of *Pfu* DNA polymerase is designated Klentaq LA-16.

DNA primers. Primers were supplied by DNA Express (Colorado State University). The 16S primers 16S-H (GGT ATT GAG GAA GGT TGA TGT GTT AAT AGC ACA TC) and 16S-G (TTG CTT CTT TGC TGA CGA GTG GCG GAC GGG TGA GT) correspond to positions 441 to 475 and 84 to 118 of the *H. influenzae* 16S rRNA sequence, respectively (GenBank accession no. M35019). The 5S primer (CAT TAC AGC GTT TCA CTT CTG AGT TCG GTA TGG TC) was the complement of positions 67 to 33 of the *Haemophilus aegypticus* 5S rRNA sequence (GenBank accession no. X05519).

Long PCR amplification. Approximately 270 ng of DNA was amplified in a 50-μl reaction mixture consisting of 5 μl of 10× PC2 buffer (2) supplied by Ab Peptides, 5 μl of 2 mM deoxynucleoside triphosphates, 0.5 μl of 16S-H or 16S-G primer and 0.5 μl of 5S primer (20 pmol/μl) (2), 37.7 μl of H_2O, and 0.3 μl of Klentaq LA-16. Each mixture was overlaid with 50 μl of liquid paraffin and

* Corresponding author. Mailing address: P.O. Box 41096, Casuarina, Northern Territory, 0811, Australia. Phone: 61 89 228 196. Fax: 61 89 275 187. Electronic mail address: heidi@menzies.su.edu.au.

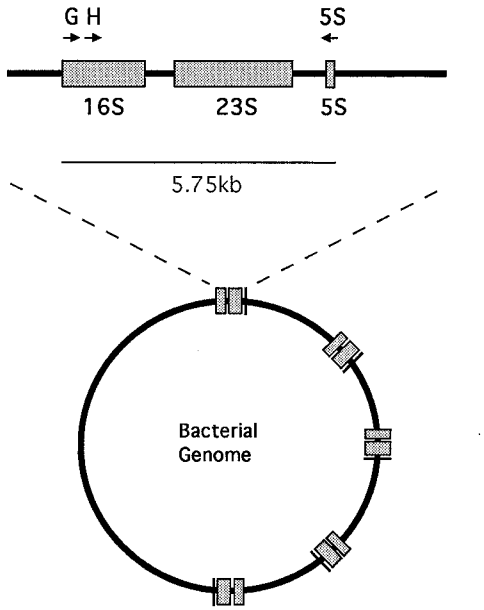

FIG. 1. Structure and genomic organization of ribosomal operons. An rRNA operon is shown at the top, and the positions of oligonucleotides used are indicated. A representation of a bacterial genome containing five rRNA operons is shown below.

placed in a thermal cycler (Corbett Research). Twenty-five cycles of denaturation at 94°C for 10 s and annealing and extension at 68°C for 8 min were done.

Restriction enzyme digestion. To 15 μl of the resulting PCR product, 2 μl of buffer C (New England Biolabs) and 1 μl of *Hae*III (12 U/μl; New England Biolabs) were added, and the mixture was incubated at 37°C for 2 h. Restriction fragments were separated by electrophoresis for 16 h at 1.1 V/cm on a 1.5% agarose gel in 1× TAE (4 mM Tris acetate–0.2 mM EDTA, pH 8.0) buffer containing 0.5 μg of ethidium bromide per ml and photographed on Polaroid 667 film by using a 300-nm transilluminator.

Conventional ribotyping. DNA was digested with *Xba*I, fractionated by electrophoresis on a 0.8% agarose gel in 1× TAE buffer for 16 h at 1.1 V/cm, and blotted onto Hybond N Plus (Amersham) in 0.4 M NaOH. The prehybridization mixture was 0.3 M NaCl–20 mM NaH_2PO_4–2 mM EDTA–1% SDS–0.5% nonfat skim milk powder–0.5 mg of herring sperm DNA per ml. The probe was prepared by random primer labelling with [^{32}P]dATP of a 7-kb insert from plasmid pKK3535 (1) bearing an *Escherichia coli* rRNA operon. Hybridization was done for 16 h in the solution described above at 65°C, and posthybridization washes were done at 65°C with 0.1% SDS–1×SSC (1×SSC is 0.15 M NaCl plus 15 mM trisodium citrate).

Ribotype nomenclature. Conventional ribotype numbers were assigned in the order in which we encountered the ribotypes. PCR-ribotypes were then assigned by examining the isolates by PCR in order of their conventional ribotypes, and each new PCR pattern encountered was assigned the next sequential PCR-ribotype number.

Information content and statistical analysis. The information gained from ribotyping and PCR-ribotyping was measured by using Shannon's information measure: $I = -\Sigma r_i \log_e (r_i/n)$, where r_i is the number of isolates typed in the ith category and where $\Sigma r_i = n = 49$ in this instance.

RESULTS

The 5S primer, which targeted the 5S region of the rRNA operon (Fig. 1), was designed to avoid stem regions of the secondary structure. The primer targeting the 16S-H ribosomal region of *H. influenzae* was chosen from a region of the 16S sequence which varies considerably from species to species (5).

A long PCR using an NTHi template and primers targeting the 5S and 16S-H regions generated an approximately 5.5-kb product (Fig. 2A, lane 8) that hybridized with an rDNA probe (Fig. 2B). Often, a less intense band of 1.8 kb that also hybridized with the probe was generated. The major product of the long PCR corresponds to that expected from amplification of single rRNA operons. The nature of the smaller band is not known, but the band may correspond to a truncated operon. The presence of an incomplete rDNA in mitochondrial DNA of the yeast *Kloeckera africana* has been reported (6). Only one

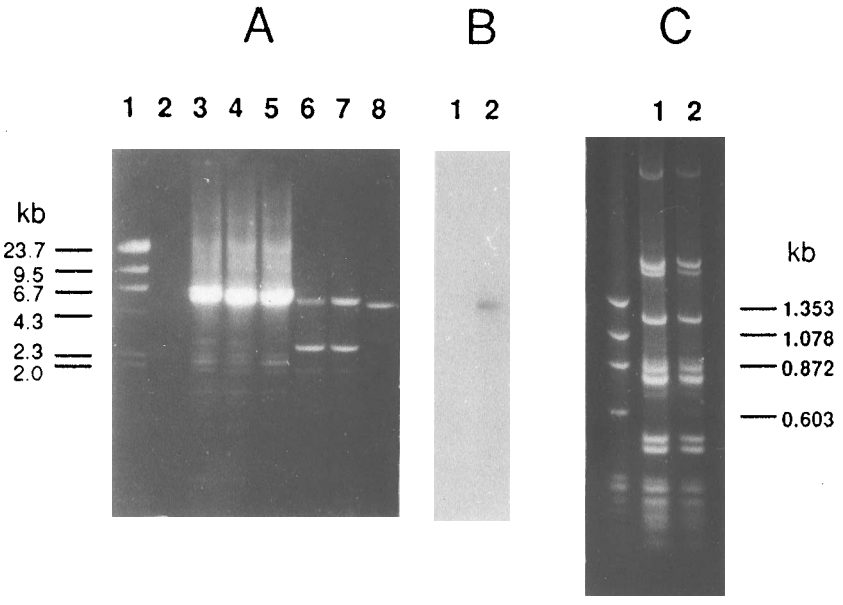

FIG. 2. Long PCR of ribosomal operons of NTHi and characterization of the products. (A) Lane 1, marker (*Hin*dIII fragments of λ DNA); lane 2, negative PCR control (no DNA); lanes 3 to 5, amplification products of PCR-ribotypes 3, 8, and 4 with the 16S-G primer; lanes 6 and 7, products of two ribotype 15 (PCR-ribotype 14) isolates with the 16S-G primer (long PCR of ribotype 15 was unsuccessful when the 16S-H primer was used); lane 8, amplification product of PCR-ribotype 3 with the 16S-H primer. (B) Autoradiograph after hybridization with an rDNA probe. Lane 1, negative PCR control; lane 2, amplification product with the 16S-H primer. (C) *Hae*III restriction fragments from products of long PCR amplifications of PCR-ribotype 8 using 16S-H (lane 1) and 16S-G (lane 2) primers. Markers (*Hae*III fragments of Φ×174 DNA) are shown in the leftmost lane.

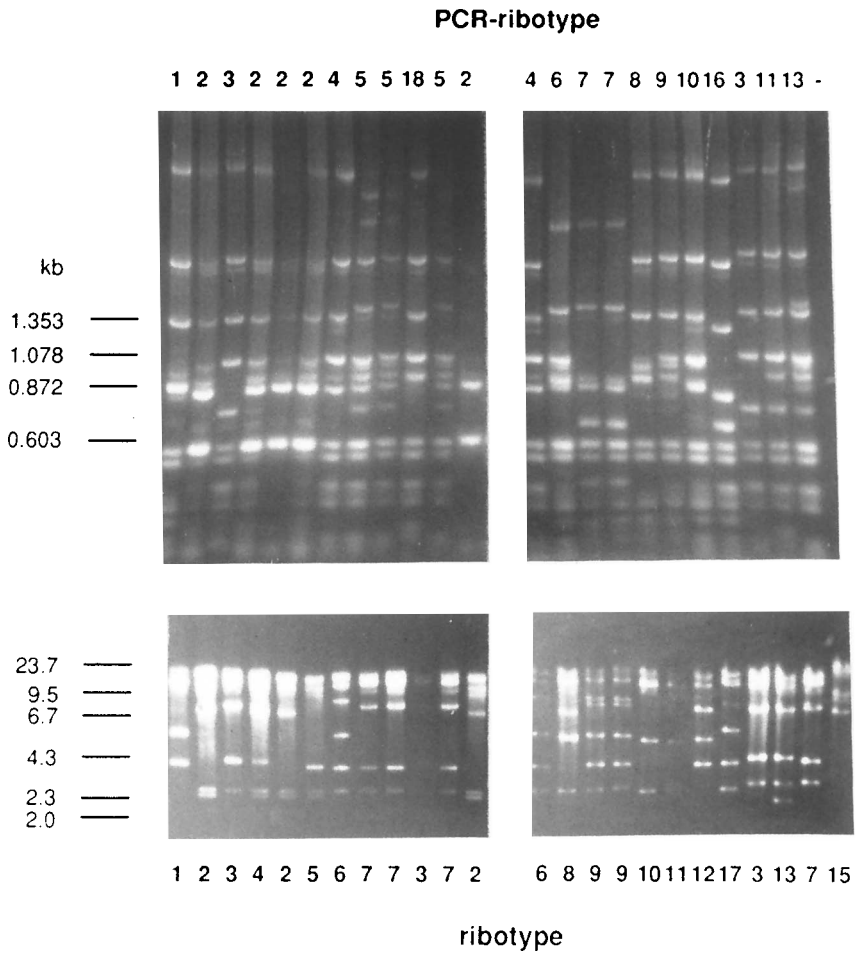

FIG. 3. PCR-ribotypes (top) and conventional ribotypes (bottom; a negative image of the autoradiographs is shown) of 24 different isolates of NTHi. The samples were loaded in the same order for both PCR-ribotyping and conventional ribotyping. Details of both methods are given in the text.

of the 49 clones tested (of conventional ribotype 15) failed to give a product when the 16S-H primer was used. However, the relative amounts of the two bands varied considerably in different experiments, and the long PCR did not generate an optimal level of product for routine use.

An alternative 16S primer (16S-G) was selected from a region of the sequence 357 bases upstream from the previous region. This portion of the 16S sequence was conserved with respect to other bacteria, such as *Streptococcus pyogenes* and *E. coli*, and was not located in a hairpin stem region.

Long PCR using the primer 16S-G amplified a slightly longer product (5.75 kb) from most NTHi clones (Fig. 2A) and in a far higher yield than those of the 5S and 16S-H primers. This reaction was highly reproducible in independent experiments, and the second, smaller band usually seen with the 16S-H primer was not observed in the majority of the clones. However, with three clones (of conventional ribotypes 2, 4, and 5), the product was predominantly and reproducibly a 1.8-kb molecule (data not shown). With isolates of conventional ribotype 15, when primer 16S-G was used, in addition to the 5.75-kb fragment a 2.4-kb fragment was observed (Fig. 2A, lanes 6 and 7).

Restriction enzyme digestion with *Hae*III generated clearly definable fragments. Restriction enzymes *Apo*I, *Bst*UI, *Hha*I, *Aci*I, *Dra*I, *Xba*I, *Sau*3AI, and *Alu*I were tested but not adopted for use because they had less discriminatory capacity (data not shown). A comparison of *Hae*III restriction fragments of the products from the two sets of primers is shown in Fig. 2C. As expected, the patterns generated are almost identical. The banding patterns are diverse between types and easily distinguishable. Most patterns have distinct markers taking the form of one or two intense bands, making pattern identification relatively simple. The variation in intensity suggests that several rRNA operons in the genome were amplified and that the intense fragments are due to restriction fragments common to most or all of these operons. This reasoning came about after similar experiments generated equimolar restriction fragments when a single operon in the genome was amplified (data not shown). The patterns shown are not due to partial digestion products. Isolates of PCR-ribotypes 2 and 8 were shown to be stable to an increased time of digestion (4 h) and increased enzyme levels (24 U of *Hae*III per reaction mixture) (data not shown).

PCR-ribotyping was applied to a collection of 49 NTHi clones derived from 34 nasopharyngeal swabs taken from three Aboriginal infants over a period of 9 months. The clones had previously been ribotyped by conventional methods using the restriction endonuclease *Xba*I (15), and 18 ribotypes had been found. A comparison of the *Xba*I ribotypes determined by conventional procedures for 24 of these clones representing 15 ribotypes and the PCR-ribotypes for the same batches of DNA from these isolates is shown in Fig. 3. The two methods gave

remarkably similar discrimination, with PCR-ribotyping splitting the isolates into 14 types. Three of the conventional ribotypes (2, 4, and 5) were the same PCR-ribotype (type 2). Interestingly, these three isolates gave only the 1.8-kb PCR product. Conventional ribotypes 3 and 7 each split into two PCR-ribotypes (types 3 and 18 and types 5 and 13, respectively). Because of the low yield of conventional ribotype 15, the PCR-ribotype cannot be seen on Fig. 3.

For the 49 clones studies, the information content as determined by PCR-ribotyping was 131.67, compared with 132.21 by conventional ribotyping; both methods together were even more discriminatory, with an information content of 137.83. The additional information gained is statistically significant [$\chi_2^2 = 2(137.83 - 132.21) = 11.25$; $P < 0.01$].

As conventional ribotyping is based on RFLPs that lie both within and adjacent to ribosomal genes, while PCR-ribotyping is based on RFLPs entirely within the ribosomal operon, it is surprising that the concordance was so high. There were 44 instances in which clones were concordant by the two typing methods. This apparent rate of concordance (44/49 = 90%) is inflated because of the examples that were represented only once. After adjustment for this possible selection bias by removing the first example of each concordance, there were 28 clones concordant by the two typing methods, compared with 2.24 clones expected to be concordant by chance alone. A difference of this magnitude could not possibly be due to chance ($\chi_1^2 = 296$; $P < 10^{-6}$), so that concordance is real, even though the true rate is likely to be less than the crude estimate of 90%.

The high apparent concordance in this study is also inflated because most concordant clones were derived from serial swabs from the same individual, giving less time for genetic variation. The concordance rates for clones from different children were lower and not statistically significant in this small sample ($P \approx 0.11$). Of two children who carried clones of conventional ribotype 3, one had PCR-ribotypes 3 and 18 and one had PCR-ribotype 3; of two children with clones of conventional ribotype 7, one had PCR-ribotype 5 and one had PCR-ribotype 13. This lesser concordance presumably reflects a greater evolutionary separation between clones which are derived from different individuals.

DISCUSSION

Epidemiological investigation of microbial pathogens has been hampered by the lack of a quick, discriminatory method of strain characterization. The introduction of RAPDs has been useful for this purpose. This study developed a method based on PCR amplification of the ribosomal operon followed by RFLP analysis done conveniently on ethidium bromide-stained gels. When applied to NTHi strains, this novel method produces clear, distinguishable patterns which are easy to interpret. We have found the technique to be highly reproducible, and this may be an advantage over RAPD analysis, which can be subject to artifactual variation (7) and nonreproducibility between thermal cyclers and between laboratories (9). There was sufficient heterogeneity in the ribosomal operons targeted for typing to enable distinction between separate infections and demonstrate a temporal pattern of variation of NTHi strains in the three infants studied. A comparison of PCR-ribotyping and conventional ribotyping allowed the initiation of PCR-ribotyping as a powerful new typing tool, as this new technique gave almost as much discrimination as the established ribotyping method. Although the combination of the two typing methods is significantly more informative than either alone, the small information gain would not be worth the effort of adding conventional ribotyping to PCR-ribotyping; the latter is clearly preferable because of its convenience, speed, and reproducibility. PCR-ribotyping can therefore be used for strain characterization in epidemiological investigations of NTHi and should be applicable to a wide range of other bacterial pathogens.

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