JOURNAL OF CLINICAL MICROBIOLOGY, May 1994, p. 1288–1294
0095-1137/94/$04.00+0

Vol. 32, No. 5

Detection and Strain Identification of *Actinobacillus actinomycetemcomitans* by Nested PCR

EUGENE J. LEYS,[1] ANN L. GRIFFEN,[2]* SHERYL J. STRONG,[1] AND PAUL A. FUERST[1]

Department of Molecular Genetics, College of Biological Sciences,[1] and Department of Pediatric Dentistry, College of Dentistry,[2] The Ohio State University, Columbus, Ohio 43210-1241

Received 23 November 1993/Returned for modification 27 January 1994/Accepted 4 February 1994

By using PCR, *Actinobacillus actinomycetemcomitans* strains were identified directly from plaque samples without the need to isolate or culture bacteria. DNA fragments were generated by a nested, two-step PCR amplification of the ribosomal spacer region between the 16S and 23S rRNA genes. For the first amplification, primers homologous to sequences common to all bacterial species were used. This was followed by a second amplification with primers specific to *A. actinomycetemcomitans*. The ribosomal DNA spacer region was amplified from as few as 10 bacterial cells within a total population of 10^8 cells (0.00001%), and cross-reactivity between species was not observed. DNA fragments specific for *Porphyromonas gingivalis* were generated from the same samples by using a *P. gingivalis*-specific primer, and equivalent sensitivity and specificity were observed. *A. actinomycetemcomitans* was detected in 60% and *P. gingivalis* was detected in 79% of 52 subjects tested. Sequence analysis of the spacer region DNA fragment for *A. actinomycetemcomitans* gave precise strain identification, producing unique sequences for seven reference strains and identification of nine plaque-derived isolates. A phylogenetic tree based on quantitative sequence relationships was constructed. Two-step PCR amplification directly from plaque samples combined with sequence analysis of the ribosomal DNA spacer region provides a sensitive assay for detection and strain identification of multiple species directly from a single plaque sample. This simplified approach provides a practical method for large-scale studies on the transmission and pathogenicity of periodontitis-associated bacteria.

Actinobacillus actinomycetemcomitans and *Porphyromonas gingivalis* have been implicated as pathogens in periodontal disease (24). However, both organisms can be detected with some frequency in periodontally healthy individuals of all ages, even children (13, 19). This raises the question of whether the bacteria are opportunistic in nature, with all or most strains having the potential to produce disease under hospitable conditions, or whether only some strains have pathogenic potential. In addition, little is known about the stability of colonization within individuals or the transmission of strains between individuals. To develop meaningful prognostic tests and to determine to what extent periodontitis is a contagious disease, studies of the pathogenicity and means of transmission of these organisms are needed. Because there are many clones of each pathogen (4, 16, 17), these studies must include large numbers of subjects. The ability to carry out large-scale studies requires simplified and highly sensitive methods for the detection and identification of strains. The ideal assay would reproducibly discriminate between closely related strains, allow the comparison of strains over time and between laboratories, detect low levels of bacteria, and be rapidly and easily performed. Various approaches have been used, and each of these approaches satisfies some of these criteria. Serotyping has been used to identify groups within species for *A. actinomycetemcomitans* and *P. gingivalis* (20, 30). Although some variation has been seen depending on the culture conditions used (2), serotyping is reproducible, and results can be cataloged and compared. The primary shortcoming is that only a limited number of serotypes have been detected, and strains that can be differentiated by other methods cannot be distinguished by serotyping. Restriction fragment polymorphisms found in whole genomic DNA have been used to distinguish strains of both *A. actinomycetemcomitans* and *P. gingivalis* (17, 27, 31), and multiple genetic groups have been identified. Southern blot analysis of whole genomic DNA (7, 25) and random priming of the PCR (21) have also been used to distinguish strains of *A. actinomycetemcomitans*, and multilocus enzyme electrophoresis has been used to distinguish strains of both organisms (4, 16). These techniques have identified many more clonal types than could be determined by serotyping, but the disadvantage is that samples must be compared directly and cannot be precisely cataloged for comparison over time or between laboratories. All of these techniques require that the bacteria of interest first be isolated and cultured. This is a cumbersome and time-consuming step and selects only those organisms that will grow under the culture conditions. If the organism of interest occurs at a level low enough to elude detection by culturing techniques, it cannot be identified by those methods. Information about the presence of strains that occur in low numbers may be crucial to answering questions about the association of particular strains with the disease process and the transmission of strains between individuals. Methods that circumvent the need to isolate and culture bacteria are needed both to simplify and to increase the sensitivities of assays for strain identification.

Earlier (11), we reported an approach to strain identification that relies upon restriction fragment length polymorphism analysis of the ribosomal DNA (rDNA) spacer region. We have further developed this technique to generate a DNA fragment directly from a plaque sample without the need to isolate or culture bacteria, and sequencing of the fragment provided a more precise means of strain identification than restriction fragment length polymorphism analysis. This assay was accomplished by using a nested, two-step PCR amplification of the rDNA spacer region located between the 16S and 23S ribosomal genes. The use of species-specific primers allowed us to amplify the spacer region from a single species in

* Corresponding author.

the presence of DNAs from the multiple species found in a plaque sample with high sensitivity and specificity. The two-step amplification provided adequate material for the analysis of multiple species from a single sample. The sequence information obtained provided a unique identifier for all reference strains examined and was used to quantitatively estimate the genetic relatedness of the reference and patient-derived strains.

MATERIALS AND METHODS

Bacterial strains. *A. actinomycetemcomitans* ATCC 29522, ATCC 29523, ATCC 29524, ATCC 33384, ATCC 43717, ATCC 43718, and ATCC 43719 and *Haemophilus aphrophilus* ATCC 33389 were obtained from the American Type Culture Collection (ATCC; Rockville, Md.). *P. gingivalis* W50 was provided by Joseph Zambon (Buffalo, N.Y.). Growth of *A. actinomycetemcomitans* reference strains was as described previously (11). *Escherichia coli* RR1 cell numbers were determined by measurement of the A_{600}. *A. actinomycetemcomitans* and *P. gingivalis* cells were enumerated by visualization in a hemocytometer, or cell equivalents were estimated by determining the A_{260} of purified genomic DNA (18).

Plaque sampling procedure. A sample of 52 subjects ranging in age from 2 to 72 years was selected from the pediatric, periodontal, and emergency clinics of the Ohio State University College of Dentistry. All subjects were informed concerning the nature and procedures involved in the study, and consent was obtained. The number of sites sampled from each subject ranged from 1 to 12, and samples from all sites from a single subject were pooled for analysis. Saliva and supragingival plaque were removed from the sampling site with a cotton roll or gauze pad. Specimens were collected by inserting one to four sterile, medium endodontic paper points (Johnson and Johnson, Windsor, N.J.) into the sulcus for 10 s. The points were removed and placed in a sterile 1.5-ml microcentrifuge tube, and the tubes were transported to the laboratory, where they were refrigerated and processed within 1 week. Bacteria were removed from the paper point by adding 750 μl of sampling buffer [150 mM NaCl, 20 mM Tris (pH 7.5), 10 mM EDTA, 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EGTA] and vortexing for 1 min, after which the paper points were removed.

Isolation and purification of DNA. Suspended plaque samples or 1 ml of cultured bacterial cells were centrifuged at 10,000 × *g* for 30 s, after which the supernatant was removed and discarded. The pellet was suspended in 300 μl of 50 mM Tris-HCl (pH 8.0)–1 mM EDTA–1% sodium dodecyl sulfate. Proteinase K was added to a concentration of 1 mg/ml, and the sample was incubated at 37°C for 1 to 2 h. The DNA was purified by the standard Geneclean (Bio 101, Inc., LaJolla, Calif.) protocol, with the addition of one additional washing step. This simplified purification method produced yields comparable to those of previously used methods (11) and provided DNA of suitable integrity for PCR amplification.

Amplification and sequencing of the rDNA spacer region. A schematic representation of the bacterial rRNA operon, the relative locations and orientations of the various primers, and the resulting DNA fragments is presented in Fig. 1. Primer sequences are listed in Table 1. Primers 011 (3), 317-R, and 422 (29) are universal procaryotic primers. Primer AA is a composite of the *A. actinomycetemcomitans*-specific oligonucleotides Aa2 developed by Dix et al. (8) and AaC-25 developed by Chuba et al. (5). This primer hybridizes to a region in the *A. actinomycetemcomitans* 16S gene that shows a high degree of variability between *A. actinomycetemcomitans* and other closely related species (6). Primer PG is homologous to the *P. gingivalis*-specific oligonucleotide developed by Dix et al. (8). Primer sp-1t, which is used to sequence the rDNA spacer, is homologous to a region located in the Glu-tRNA gene found in the spacer region of the small *A. actinomycetemcomitans* ribosomal operon. Oligonucleotide primers were synthesized by the Ohio State University Biochemical Instrumentation Center.

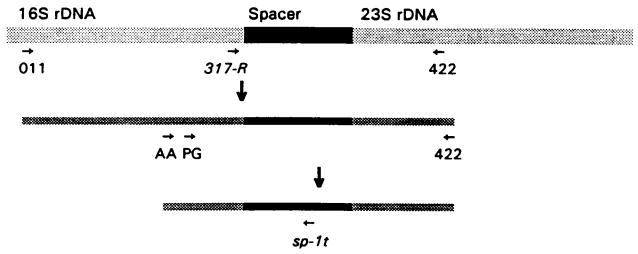

FIG. 1. Schematic representation of the procaryotic ribosomal operon and the locations and orientations of the primers and the resulting DNA fragments. The variable spacer region is located between the 16S and 23S genes. Universal primers 011 and 422 were used to generate a DNA fragment in the first nonspecific amplification step, and species-specific primer AA or PG and the universal primer 422 were used in the second amplification step. Primers 317-R and sp-1t were used for sequencing.

All PCRs were performed with 2.5 U of *Taq* polymerase (Promega, Madison, Wis.) in a total volume of 100 μl in buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 3 mM $MgCl_2$, 0.1% Triton X-100, and 0.2 mM (each) deoxynucleoside triphosphates. Samples were subjected to 25 cycles (denaturation at 94°C for 1 min, annealing at 42°C for 2 min, and primer extension at 72°C for 3 min) in an automated thermal cycler (Perkin-Elmer Cetus). For the first amplification, genomic DNA isolated from plaque samples (20 to 25% of total isolated DNA) or reference strains was used as a template. The universal procaryotic primers 011 (0.036 μg) and 422 (0.36 μg) were used (Fig. 1). A minimum amount of primer 011 was used to avoid carryover into the second amplification.

To generate species-specific DNA fragments from mixed samples, a second amplification was performed. Aliquots consisting of 2% of the product from the first amplification were used as templates for a second amplification with a species-specific primer to obtain rDNA spacer region fragments specific for either *A. actinomycetemcomitans* or *P. gingivalis*. For amplification of *A. actinomycetemcomitans* DNA, primer AA (0.36 μg) was used, and for amplification of *P. gingivalis* DNA, primer PG (0.36 μg) was used. These primers were each used in combination with the universal primer 422 (0.36 μg) (Fig. 1).

DNA fragments were separated by electrophoresis in a 1% agarose gel in TBE (0.1 M Tris-borate [pH 8.3], 2 mM EDTA).

TABLE 1. Sequences of oligonucleotide primers

Primer	Sequence
011	GTTTGATCCTGGCTCAG
422	AAGGCTAAATACTCC
AA	GGATTGGGGTTTAGCCCTGG
PG	TGTAGATGACTGATGGTGAAAACC
317-R	GGCTGGATCACCTCCTT
sp-1t	CGAACCCCGGTTACGCCGTG

Gels were stained with ethidium bromide and were photographed by using UV light.

The spacer region of the small rDNA operon was sequenced from the product of the second amplification by using the ds DNA Cycle Sequencing System (BRL) according to the manufacturer's instructions. Primer sp-1t, which is homologous to the Glu-tRNA found in the small *A. actinomycetemcomitans* ribosomal operon and which is not present in the large operon (14), was used to avoid having to separate the DNA fragments from the small and large spacer regions. The sequence of the spacer region for the *A. actinomycetemcomitans* reference strains was confirmed by sequencing the other strand by using primer 317-R and the Sequenase system (U.S. Biochemicals) according to the manufacturer's instructions. Because primer 317-R is not specific for the smaller operon, the smaller band was isolated from a 1% agarose gel by using the standard Geneclean (Bio 101, Inc.) protocol.

Phylogenetic analysis. Two different phylogenetic analyses were performed to assess strain relationships. First, an unrooted network was developed from the matrix of nucleotide similarities by using the neighbor-joining analysis NEIGHBOR of the package PHYLIP (9). The data were also examined by a cladistic approach (parsimony), DNAPARS of PHYLIP (9), coding nucleotide changes and insertion or deletion changes as individual characters.

Nucleotide sequence accession number. The nucleotide sequences of the spacer region of the small ribosomal operon for *A. actinomycetemcomitans* ATCC 29522, ATCC 33384, and ATCC 29523 have been submitted to GenBank under accession numbers UO7778, UO7777, and UO7776.

RESULTS

Sensitivity of amplification of the rDNA spacer region. The sensitivity of detection of *A. actinomycetemcomitans* by PCR was tested by amplifying the rDNA spacer region by using decreasing amounts of template DNA. The pair of universal primers, 011 and 422, was used. This amplification generated a fragment that included the entire rDNA spacer region flanked by most of the 16S gene and a portion of the 23S gene.

Figure 2 is an ethidium bromide-stained agarose gel containing the rDNA spacer regions amplified from decreasing numbers of *A. actinomycetemcomitans* starting with 10^6 bacteria and decreasing in 10-fold dilutions down to 10 cell equivalents. Even when only 10 cell equivalents of the *A. actinomycetemcomitans* genome were available as starting material for rDNA spacer region amplification, amplified spacer region DNA was obtained. Similar results (data not shown) were obtained for *P. gingivalis*.

Specificity. A two-step amplification was used to examine the specificity of amplification of the rDNA spacer region from mixtures of bacteria. Increasing amounts of *E. coli* DNA were amplified in the presence of 10 cell equivalents of *A. actinomycetemcomitans* (Fig. 3) or *P. gingivalis* DNA (data not shown). The rDNA spacer region was first amplified by using the universal primers 011 and 422. This amplification generated a mixture of rDNA spacer region DNA fragments amplified from both species (data not shown). A second amplification was performed by using the product of the first amplification as a template. One of the species-specific 16S primers (AA or PG) paired with the universal 23S primer (universal primer 422) was used. This amplification produced spacer region DNA fragments only from the species determined by the primer. The fragments generated in this amplification did not include the first 800 bp of the 16S gene-coding region present in the fragment generated in the first amplification. *A. actinomycetemcomitans* or *P. gingivalis* DNA was amplified even in the presence of a 10^8-fold excess of *E. coli* DNA. No amplified DNA was observed by using primer AA with *E. coli*, *P. gingivalis*, or *H. aphrophilus* template DNA. No amplified DNA was observed by using primer PG and *E. coli* or *A. actinomycetemcomitans* template DNA.

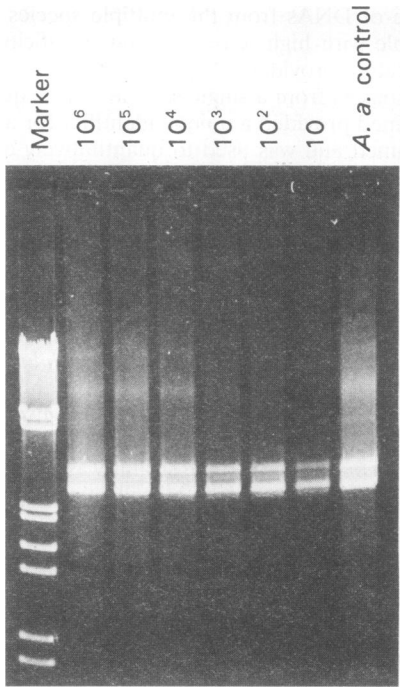

FIG. 2. Sensitivity of *A. actinomycetemcomitans* amplification. rDNA was amplified from a serial dilution. Lanes 2 to 7, 10^6 to 10 copies of the *A. actinomycetemcomitans* genome in 10× decrements. Markers are *Eco*RI and *Hin*dIII digestion products of bacteriophage lambda DNA; the last lane is a control amplification (*A. a.* is *A. actinomycetemcomitans*). The amplified products appear at approximately 2.45 and 2.30 kb.

Amplification with species-specific primers resulted in two products for all *A. actinomycetemcomitans* strains examined and one product for all *P. gingivalis* strains examined (Fig. 4). The two *A. actinomycetemcomitans* products are the result of two rRNA operons that differ in the lengths of their transcribed spacer regions. The small spacer region contains the coding region for the single tRNA Glu, and the large spacer region contains the coding region for at least the two tRNAs Ile and Ala (14). These tRNAs are the same as those that occur in two classes of ribosomal operons found in *E. coli* (12).

Direct amplification from plaque samples. Amplification of species-specific DNA fragments directly from plaque samples was achieved by a nested, two-step PCR. The first amplification, with the universal primers 011 and 422, generated a mixture of spacer region DNA fragments amplified from all procaryotic species present in the sample (data not shown). A second amplification was performed by using the product of the first amplification as a template and a species-specific 16S primer (AA or PG) and the universal 23S primer 422. This produced DNA fragments corresponding to the rDNA spacer region only from the species determined by the primer. Figure 4 shows an example of the amplification of the *A. actinomycetemcomitans* and *P. gingivalis* rDNA spacer regions from three plaque samples, demonstrating the results when both species

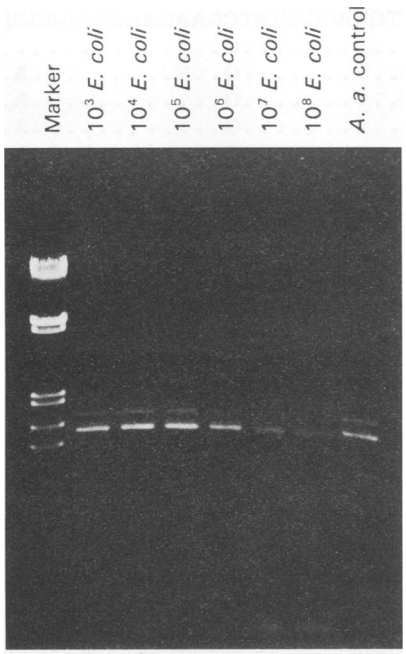

FIG. 3. Amplification of the *A. actinomycetemcomitans* spacer region from a mixed population. DNA was amplified from 10 copies of the *A. actinomycetemcomitans* genome in the presence of increasing concentrations of *E. coli* DNA. Lanes 2 to 7, the addition of 10^3 to 10^8 copies of *E. coli* in 10× increments. Markers are *Eco*RI and *Hin*dIII digestion products of bacteriophage lambda DNA; the last lane is a control amplification of *A. actinomycetemcomitans* (*A. a.*) DNA alone. The amplified products appear at approximately 1.50 and 1.65 kb.

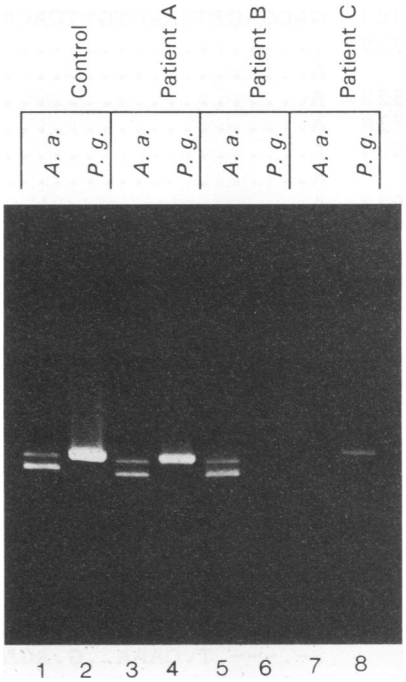

FIG. 4. rDNA amplified directly from plaque samples. Lanes 1 and 2, DNA amplified from reference strains of *A. actinomycetemcomitans* (*A. a.*) and *P. gingivalis* (*P. g.*), respectively; lanes 3 and 4, DNA amplified from a single plaque sample by using an *A. actinomycetemcomitans*-specific primer (lane 3) and a *P. gingivalis*-specific primer (lane 4). The plaque sample obtained from patient B contained detectable *A. actinomycetemcomitans* (lane 5) but not *P. gingivalis* (lane 6), while the sample obtained from patient C contained *P. gingivalis* (lane 8) but not *A. actinomycetemcomitans* (lane 7). The amplified *A. actinomycetemcomitans* products appear at approximately 1.50 and 1.65 kb. The amplified *P. gingivalis* product appears at approximately 1.70 kb.

were detected together in the same sample and when each species was detected alone. The first two lanes of Fig. 4 contain DNAs amplified from reference strains of *A. actinomycetemcomitans* and *P. gingivalis*; lanes 3 and 4 show that both species were detected in the plaque sample obtained from patient A; lanes 5 and 6 show that only *A. actinomycetemcomitans* was detected in the sample obtained from patient B, while lanes 7 and 8 show that only *P. gingivalis* was detected in the sample obtained from patient C. Table 2 demonstrates the frequency of detection of *A. actinomycetemcomitans* and *P. gingivalis* DNA fragments from the 52 subjects in our study sample. *A. actinomycetemcomitans* was detected in 60% of subjects and *P. gingivalis* was detected in 79% of subjects.

Strain identification from spacer DNA sequence. The rDNA spacer regions for seven ATCC strains and nine subject-derived isolates of *A. actinomycetemcomitans* were sequenced. The aligned sequences for the 5′ region of the spacer are shown in Fig. 5. Sequences of two of the ATCC strains were found among the nine patient-derived isolates examined: a sequence identical to that of ATCC 29523 appeared in three of the subjects' samples, and a sequence identical to that of ATCC 43719 was identified from one subject's sample. The remaining five clinically derived isolates were found to have unique sequences. Thirty-nine of the 130 nucleotide positions aligned were variable in the 16 sequences examined.

Phylogenetic analysis. Quantitative comparisons of differences in nucleotide sequence were used to ascertain the phylogenetic relationship between strains by using two different analyses. In the unrooted network developed from the matrix of nucleotide similarities by using the neighbor-joining analysis, several clusters appeared. The samples formed a closely related grouping which included the following clusters: (i) ATCC 43717 and ATCC 29523 (identical to CMH8, CMH12, and CMH13), (ii) ATCC 33384 and ATCC 43719 (identical to CMH11), and (iii) ATCC 29522, ATCC 29524, and ATCC 43718. Isolate CMH15 appeared equidistant from clusters i and iii, while CMH9 and CMH5 seemed to fall just outside of the combined cluster. A divergent fourth cluster also existed; it was made up of CMH1 and CMH4. The consensus tree generated by using a cladistic approach, coding nucleotide changes and insertion or deletion changes as individual characters, is presented in Fig. 6. The consensus tree was directed by placing CMH1 and CMH4 as outliers, but was unrooted.

TABLE 2. Frequency of detection of *A. actinomycetemcomitans* and *P. gingivalis* DNA fragments from plaque by PCR amplification[a]

A. actinomycetemcomitans	No. (%) of subjects in whom *P. gingivalis* was detected (n = 52)[b]	
	+	−
+	28 (54)	3 (6)
−	13 (25)	8 (15)

[a] Thirty-one subjects (60%) were positive for *A. actinomycetemcomitans*; 41 subjects (79%) were positive for *P. gingivalis*.

[b] Number (percentage) of subjects in whom the indicated species was detected (+) or not detected (−) in the plaque.

```
ATCC33384  GACGACTTTAAGTGTTCACACAGTTTGTTTGAT-G-ATTGTAGGCAATCGAAGAACAAAAGCAAA 65
ATCC43719  .....................................................G.............
CMH9       A.....................................G...........G..N.......G.A...
ATCC29522  A.....................................................G.........G.A...
ATCC43718  A.....................................................G.........G.A...
ATCC29524  A.....................................................G.........G.A...
CMH15      A.....................................................G.........G.A...
ATCC29523  A.....................................G...........G.........G.A...
ATCC43717  C.....................................G...........G.........G.A...
CMH5       AG....................................G......A....G.........G.A...
CMH1       AT..................................G.TG......A...-G...-----.G.AN...
CMH4       AT....................................G......A...-G...-----.G..N...

ATCC33384  AGTGCAAG-----GATAGT-----ATCTTTACATGTTgtccccatcgtctagaggcctaggacat 130
ATCC43719  ...................................................................
CMH9       ..................................C..N............................
ATCC29522  ........GCAAA..........................................................
ATCC43718  ....-...G.AAA..........................................................
ATCC29524  ...-....G.AAA..........................................................
CMH15      ....----..AAA..........................................................
ATCC29523  ....----..AAA..........................................................
ATCC43717  ....----..AAA..........................................................
CMH5       ....----..AAA.....C.....................................................
CMH1       .-.---.C.TAAA..G.AGAGAGC.......T...C....................................
CMH4       .-.---.T.CAAA..G.AGAGAGC.......T...C....................................
```

FIG. 5. Comparison of *A. actinomycetemcomitans* sequences at the 5′ end of the spacer region of the small ribosomal operon for seven ATCC strains and five plaque samples. The first 28 bp of the Glu-tRNA sequence are indicated in lowercase letters.

The clusters identified by the examination of nucleotide similarity are present in the consensus tree shown in Fig. 6.

DISCUSSION

In order to study the epidemiology of periodontal disease-associated pathogens, we developed a method to generate an identifying DNA fragment directly from a plaque sample without the need to culture or isolate bacteria. This fragment is then sequenced, resulting in the sensitive, precise genetic identification of individual strains. The assay relies upon a two-step, nested PCR amplification of the highly variable transcribed spacer region between the 16S and 23S rRNA genes. Specific regions of the 16S and 23S rRNA genes flanking the spacer region are known to be highly conserved among procaryotic species (3, 29). In the first amplification, oligonucleotides homologous to these universally conserved sequences were used as primers for PCR to amplify the variable spacer region from all bacterial species present in a plaque sample.

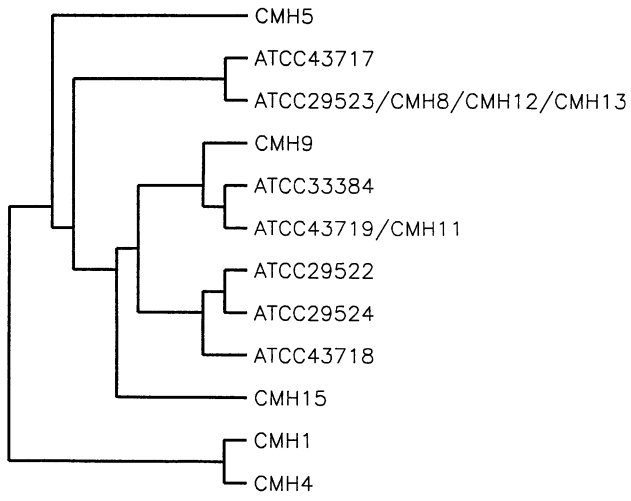

FIG. 6. Phylogenetic relationships between strains of *A. actinomycetemcomitans*. The tree represents the consensus network obtained from a parsimony analysis (DNA PARS program from PHYLIP [9]).

In addition to the universally conserved sequences, the 16S gene-coding region also contains sequences unique to a single species, including *A. actinomycetemcomitans*, *P. gingivalis*, and several other species associated with periodontal disease (5, 6, 8). These sequences were used as primers for a second amplification to selectively generate a fragment from the rDNA spacer region only from the species of interest. The specificities of the oligonucleotides used in the present study to detect *A. actinomycetemcomitans* and *P. gingivalis* have been established in previous investigations. Dewhirst et al. (6) have sequenced this region for 54 representative strains of species in the family *Pasteurellaceae*. Sequence diversity of at least 20% is found in the region of interest between *A. actinomycetemcomitans* and all species examined, including the closely related organism *H. aphrophilus*. Dix et al. (8) and Chuba et al. (5) found no cross-reactivity with other species from the oral cavity using these oligonucleotides in hybridization assays. Given the level of sequence diversity observed between species in previous investigations, no cross-reactivity was expected even between closely related species when these oligonucleotide sequences were used as primers for PCR. This was confirmed by the absence of any DNA product when the *A. actinomycetemcomitans*-specific primer was used with *H. aphrophilus* DNA as the template. The level of diversity observed in the sequence analysis of strains from patients was consistent with the level of diversity expected within a species, also confirming the species specificity of the assay.

In a survey of 52 patients of various ages and with various degrees of periodontal health, *A. actinomycetemcomitans* was detected in 60% of subjects and *P. gingivalis* was detected in 79% of subjects by PCR amplification of the rDNA spacer region. Direct comparison with previous studies that used sensitive detection methods are not possible because of differences between studies in the number of sites sampled and the ages and the disease states of the subjects. Savitt and Kent (22) detected *A. actinomycetemcomitans* in 54% of diseased sites and *P. gingivalis* in 78% of diseased sites using a DNA hybridization assay, and Loesche et al. (15) detected *A. actinomycetemcomitans* in 55.1% of diseased sites and *P. gingivalis* in 88.6% of diseased sites using immunologic assays. Since many of the subjects included in the current study were periodontally healthy, it appears, as expected, that the PCR assay may be more sensitive than previously used assays. Other investigators have reported the development of PCR assays for the detection of *A. actinomycetemcomitans* or *P. gingivalis* (10, 26, 28), but the prevalence of bacteria determined by these assays has not been reported. The sensitivity of the PCR assay described here, 10 cell equivalents, may be somewhat greater than that reported by Watanabe and Frommel (28), who did not report detecting fewer than 100 bacterial cells.

Most of the rDNA spacer region is noncoding, so it is not under tight evolutionary constraint and tends to be highly variable, even among different strains of the same species (1). This variability makes it possible to distinguish among strains of the same species by sequence analysis of this DNA fragment generated by PCR. Sequence analysis of the first 130 bp of the rDNA spacer region included a highly variable region and yielded sufficient information to distinguish among all reference strains tested. Examination of this region from patient-derived samples showed sequences that matched those of the reference strains as well as new sequences. This bacterial population distribution pattern is consistent with that observed for other human pathogens, in which both common and uncommon strains are observed (23). The level of diversity observed among the strains from the present study population suggests that examination of the first 130 bp of the rDNA spacer region provides good discrimination between isolates. However, additional sequence information from the entire rDNA spacer region for both operons might provide additional information for discriminating among isolates.

Quantitative comparisons of differences in nucleotide sequences can be used to ascertain the phylogenetic relationship between strains. We compared the groupings generated by both a neighbor-joining analysis and a cladistic approach. The only major difference in the consensus trees produced by these two methods is the clustering of CMH9 with ATCC 33384, ATCC 43719, and CMH11 in the parsimony analysis because of shared insertion or deletion events. Collection of additional sequences should provide us with a better picture of the variation present in populations of *A. actinomycetemcomitans* and indicate whether this spacer sequence will provide useful phylogenetic information of strain similarity.

The aim of the present study was to develop a method for the detection and identification of strains of periodontal pathogens that is suitable for use in the epidemiologic studies that are needed to address questions concerning the pathogenicity and transmission of these bacteria. Previous methods for strain differentiation have all relied on the culture and isolation of the bacteria of interest. Although the rDNA spacer region can be amplified from cultured bacteria for analysis (11), the fragment can be generated directly from a plaque sample by using species-specific primers for PCR amplification as described here. Direct amplification offers several advantages. It eliminates the need to maintain the viability of anaerobes for culture, simplifies the process of analysis, and increases the sensitivity of detection. We were able to store samples for at least 1 week before processing by this assay, whereas culture-based assays require immediate processing. Despite careful culturing technique, if an organism of interest occurs at a very low level or as a small percentage of the total bacterial flora or if a strain is particularly fastidious, it may not be detected by culturing. Information about the presence of strains that occur in low numbers may be crucial to answering questions about the association of particular strains with the disease process and the transmission of strains between individuals. By using PCR amplification, as few as 10 cells can be analyzed in the presence of 10^8 cells of another species. Furthermore, by using a two-step amplification procedure, several species from the same plaque sample can be analyzed.

Methods that have previously been used for strain identification, with the exception of serotyping, require direct comparison of individual samples, and therefore assays must be performed concurrently. Although serotype analysis does allow indirect comparisons to be made, it cannot distinguish strains that can be differentiated by other methods. Sequence analysis of the DNA fragment from the ribosomal spacer region provides good discrimination between strains and allows strain identification information to be cataloged so that comparisons can be made over time and between laboratories. The assay also provides quantitative sequence information that can be used to construct the phylogenetic relationships among strains. Information about these relationships may prove to be important in separating nonpathogenic and pathogenic groups of strains.

A nested, two-step PCR amplification and sequence analysis provides a simplified, sensitive, precisely catalogable strain detection and identification methodology suitable for the epidemiologic studies that are needed to determine whether specific strains of periodontal pathogens are associated with disease and to track the transmission of strains among human contacts.

ACKNOWLEDGMENT

This study was supported by NIH grant DEO9807-01.

REFERENCES

1. **Appels, R., and R. L. Honeycutt.** 1986. rDNA evolution over a billion years, p. 81–135. *In* S. K. Dutta (ed.), DNA systematics. CRC Press, Inc., Boca Raton, Fla.
2. **Asikainen, S., C.-H. Lai, S. Alaluusua, and J. Slots.** 1991. Distribution of *Actinobacillus actinomycetemcomitans* serotypes in periodontal health and disease. Oral Microbiol. Immunol. **6**:115–118.
3. **Brosius, J., M. L. Palmer, P. J. Kennedy, and H. F. Noller.** 1978. Complete nucleotide sequence of a 16S rRNA gene from *Escherichia coli*. Proc. Natl. Acad. Sci. USA **75**:4801–4805.
4. **Caugant, D. A., R. K. Selander, and I. Olsen.** 1990. Differentiation between *Actinobacillus* (*Haemophilus*) *actinomycetemcomitans*, *Haemophilus aphrophilus* and *Haemophilus paraphrophilus* by multilocus enzyme electrophoresis. J. Gen. Microbiol. **136**:2135–2141.
5. **Chuba, P. J., K. Pelz, G. Krekeler, T. S. de Isele, and U. Gobel.** 1988. Synthetic oligodeoxynucleotide probes for the rapid detection of bacteria associated with human periodontitis. J. Gen. Microbiol. **134**:1931–1938.
6. **Dewhirst, F. E., B. J. Paster, I. Olsen, and G. J. Fraser.** 1992. Phylogeny of 54 representative strains of species in the family *Pasteurellaceae* as determined by comparison of 16S rRNA sequences. J. Bacteriol. **174**:2002–2013.
7. **DiRienzo, J. M., and J. Slots.** 1990. Genetic approach to the study of epidemiology and pathogenesis of *Actinobacillus actinomycetemcomitans* in localized juvenile periodontitis. Arch. Oral Biol. **35**(Suppl.):79S–84S.

8. **Dix, K., S. M. Watanabe, S. McArdle, D. I. Lee, C. Randolph, G. Moncla, and D. E. Schwartz.** 1990. Species-specific oligodeoxynucleotide probes for the identification of periodontal bacteria. J. Clin. Microbiol. **28:**319–323.
9. **Felsenstein, J.** 1989. PHYLIP (Phylogeny Inference Package), version 3.2 manual. University of Washington, Seattle.
10. **Goncharoff, P., D. H. Figurski, R. H. Stevens, and D. H. Fine.** 1993. Identification of *A. actinomycetemcomitans*: polymerase chain reaction amplification of *lktA*-specific sequences. Oral Microbiol. Immunol. **8:**105–110.
11. **Griffen, A. L., E. J. Leys, and P. A. Fuerst.** 1992. Strain identification of *Actinobacillus actinomycetemcomitans* using the polymerase chain reaction. Oral Microbiol. Immunol. **7:**240–243.
12. **Jinks-Robertson, S., and M. Nomura.** 1987. Ribosomes and tRNA, p. 1358–1385. *In* F. C. Neidhardt, J. L. Ingraham, K. Brooks Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
13. **Kisby, L. E., E. D. Savitt, C. K. French, and W. J. Peros.** 1989. DNA probe detection of key periodontal pathogens in juveniles. J. Pedodontics **13:**222–229.
14. **Leys, E. J., A. L. Griffen, and P. A. Fuerst.** Unpublished data.
15. **Loesche, W. J., D. E. Lopatin, J. Stoll, N. Van Poperin, and P. P. Hujoel.** 1992. Comparison of various detection methods for periodontopathic bacteria: can culture be considered the primary reference standard? J. Clin. Microbiol. **30:**418–426.
16. **Loos, B. G., D. W. Dyer, T. S. Whittam, and R. K. Selander.** 1993. Genetic structure of populations of *Porphyromonas gingivalis* associated with periodontitis and other oral infections. Infect. Immun. **61:**204–212.
17. **Loos, B. G., D. Mayrand, R. J. Genco, and D. P. Dickinson.** 1990. Genetic heterogeneity of *Porphyromonas* (*Bacteroides*) *gingivalis* by genomic DNA fingerprinting. J. Dent. Res. **69:**1488–1493.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 468. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Mombelli, A., D. Buser, N. P. Lang, and H. Berthold.** 1990. Suspected periodontpathogens in erupting third molar sites of periodontally healthy individuals. J. Clin. Periodontol. **17:**48–54.
20. **Nagata, A., T. Man-yoshi, M. Sato, and R. Nakamura.** 1991. Serological studies of *Porphyromonas* (*Bacteroides*) *gingivalis* and correlation with enzyme activity. J. Periodont. Res. **26:**184–190.
21. **Preus, H. E., V. Haraszthy, and J. J. Zambon.** 1992. Amplification of DNA polymorphisms using PCR and random primers. J. Dent. Res. **71**(AADR Abstracts)**:**122. (Abstract 130.)
22. **Savitt, E. D., and R. L. Kent.** 1991. Distribution of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* by subject age. J. Periodontol. **62:**490–494.
23. **Selander, R. K., and J. M. Musser.** 1990. The population genetics of bacterial pathogenesis, p. 11–36. *In* B. H. Iglewski and V. L. Clark (eds.), Molecular basis of bacterial pathogenesis. Academic Press, Inc., Orlando, Fla.
24. **Slots, J., and M. A. Listgarten.** 1988. *Bacteroides gingivalis*, *Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans* in human periodontal diseases. J. Clin. Periodontol. **15:**85–93.
25. **Socransky, S. S., and L. Martin.** 1992. Ribotyping of *P. gingivalis* and *C. rectus*. J. Dent. Res. **71**(AADR Abstracts)**:**246. (Abstract 1127.)
26. **Tønjum, T., and R. Haas.** 1993. Identification of *A. actinomycetemcomitans* by leukotoxin gene-specific hybridization and polymerase chain reaction assays. J. Clin. Microbiol. **31:**1856–1859.
27. **van Steenbergen, T. J. M., M. D. A. Petit, U. van der Velden, and J. de Graaff.** 1992. Transmission of *Porphyromonas gingivalis* between spouses. J. Dent. Res. **71**(AADR Abstracts)**:**217. (Abstract 890.)
28. **Watanabe, K., and T. O. Frommel.** 1993. Detection of *P. gingivalis* in oral plaque samples by use of the polymerase chain reaction. J. Dent. Res. **72:**1040–1044.
29. **Weisburg, W., S. Barns, D. Pelletier, and D. Lane.** 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173:**697–703.
30. **Zambon, J. J., J. Slots, and R. J. Genco.** 1983. Serology of oral *Actinobacillus actinomycetemcomitans* and serotype distribution in human periodontal disease. Infect. Immun. **41:**19–27.
31. **Zambon, J. J., G. J. Sunday, and J. S. Smutko.** 1990. Molecular genetic analysis of *Actinobacillus actinomycetemcomitans* epidemiology. J. Periodontol. **61:**75–80.