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Strain identification of *Actinobacillus actinomycetemcomitans* using the polymerase chain reaction

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A molecular assay that distinguishes among strains of the periodontal pathogen *Actinobacillus actinomycetemcomitans* was developed by identifying DNA restriction site polymorphisms in the highly variable transcribed spacer region between the 16S and 23S ribosomal genes. The polymerase chain reaction (PCR) was used to amplify this region from genomic DNA using primers within conserved regions of the 16S and 23S genes. This amplified region was digested using a series of restriction enzymes and electrophoresed. Examination of restriction fragment length polymorphisms obtained by separate digestion with RsaI and NciI allowed the 7 strains examined to be divided into 4 genetic groups. This assay provides a more precise and reproducible method of strain identification than whole genomic methods and should be useful as a method for studying the epidemiology of *A. actinomycetemcomitans* strains in human subjects. The genetic variability detected supplies strong evidence that direct sequence analysis of the region could provide extremely precise and potentially definitive identification of strains.

Key words: *Actinobacillus actinomycetemcomitans;* polymerase chain reaction; ribosomal genes; molecular genetics; epidemiology

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A definitive assay that can distinguish among strains of *Actinobacillus actinomycetemcomitans* is needed to track the transmission of bacterial strains within human populations to determine the mode of acquisition of this pathogen and to study the pathogenicity of specific strains. Traditional methods such as immunologic typing and biotyping tests (1, 12) have limited utility for identifying strains, and can be cumbersome and time-consuming to use. Molecular techniques can provide more precise methods of strain identification, and whole genomic DNA has been examined for restriction fragment length polymorphisms (RFLPs) that have proved to have some utility in differentiating genetically distinct groups of oral microbes (10, 13); however, this approach has limitations. Analysis of a specifically defined genetic region provides a more precise method for distinguishing similar strains (5). In our study the polymerase chain reaction (PCR) method (7) was used to amplify a defined region of the genome for analysis. This region was restriction-mapped to provide the basis for a fingerprint to be used for strain identification. The specific region chosen for amplification has been shown to be significantly variable among strains in other species (2) and is flanked by highly conserved regions for which species-specific sequences are known (6). By using these as primers, it should be possible in future studies to use this assay to identify strains of *A. actinomycetemcomitans* directly from a mixed clinical sample, eliminating the need for isolating and culturing bacteria.

Material and methods

Seven reference strains of *A. actinomycetemcomitans* (ATCC 29522, ATCC 29523, ATCC 29524, ATCC 33384, ATCC 43717, ATCC 43718, ATCC 43719) obtained from the American Type Culture Collection, Rockville, MD, were grown on brain-heart infusion broth (Becton Dickinson, Cockeysville, MD) at 34–37°C under anaerobic conditions with 5% CO_2. Genomic DNA was isolated by a modification of the procedure of Ausubel et al. (4). A 200-ml culture was grown to saturation and centrifuged for 20 min at 4000 *g*. The supernatant was discarded and the pellet was resuspended in 9.5 ml TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). The mixture was incubated at 37°C for 2 h with the addition of 0.5 ml of 10% sodium dodecyl sulfate (SDS) and 50 μl of 20 mg/ml proteinase K (American Research Products, Solon, OH). After incubation, 1.8 ml of 5 M NaCl was added followed by 1.5 ml CTAB/NaCl solution (10% CTAB in 0.7 M NaCl), and the mixture was incubated for 20 min at 65°C. The DNA was extracted with an equal volume of chloroform/isoamyl alcohol (24 : 1) and centrifuged 10 min at 6000 *g*. The aqueous phase was transferred to a fresh tube and extracted with phenol/chloroform/isoamyl alcohol (25/24/1) and centrifuged as described above. The aqueous phase was again transferred to a fresh tube and the DNA precipitated with 0.6 volumes of isopropanol. The precipitated DNA was centrifuged,

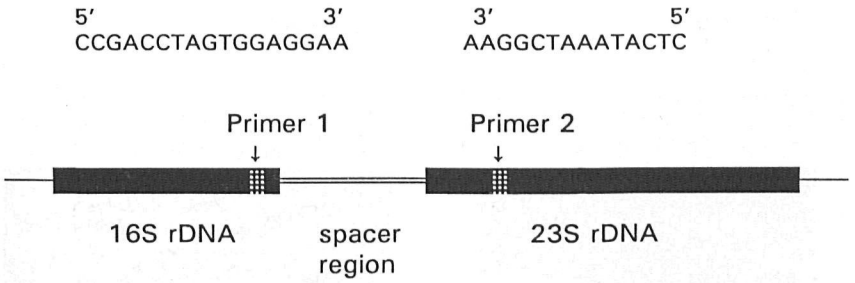

Fig. 1. Map of the procaryotic ribosomal gene. The variable spacer region is located between the 16S and 23S genes. The location, orientation and sequence of primers used to amplify the spacer region are indicated.

washed twice with 70% ethanol, and dissolved in 400 μl TE buffer.

Oligonucleotide primers for amplification were obtained from the Ohio State University Biochemical Instrumentation Center. Genomic DNA (0.5 μg) was amplified using Taq polymerase and the GeneAmp reagent kit (Perkin Elmer Cetus, Norwalk, CT) according to the manufacturer's directions in a total volume of 100 μl for 30 cycles of amplification (denaturation at 94° for 1 min, annealing at 42° for 2 min, and primer extension at 72° for 3 min) in an automated thermal cycler (Perkin Elmer Cetus). PCR product was purified using Geneclean (BIO 101, LaJolla, CA) according to the manufacturer's directions. The resulting DNA was digested with a series of restriction endonucleases (approximately 10% of the product of 1 PCR reaction was used for each digest) including AccI, AluI, BstI, DdeI, DraI, EcoRV, HaeIII, HhaI, HinCII, HinFI, MboI, MboII, MspI, NciI, RsaI, SspI, TaqI, ThaI and XbaI. The DNA fragments were separated by electrophoresis in a 3% agarose gel or an 8% acrylamide gel in TBE (0.1 M Tris-Borate, pH 8.3, 2 mM EDTA). Gels were stained with ethidium bromide and photographed using ultraviolet (UV) light.

Results

To distinguish among strains of *A. actinomycetemcomitans,* the spacer region between the 16S and 23S ribosomal RNA genes from 7 reference strains was amplified by PCR and examined for restriction length polymorphisms (RFLPs). Highly conserved oligonucleotide sequences in the 16S and 23S rRNA genes were selected as primer sites for amplification. Amplified products were obtained that included the entire spacer region plus approximately 420 bases of the 23S gene. Primer 1 was located at the 3′ end of the 16S rRNA gene and primer 2 was located within the 23S rRNA gene approximately 420 bases from the 5′ end (Fig. 1). Amplification resulted in 2 major bands for all strains of *A. actinomycetemcomitans* examined. One band was approximately 880 base pairs and the second approximately 1000 base pairs (Fig. 2).

Amplified DNA from the 7 strains was digested with 19 different restriction endonucleases. Ten enzymes (AccI, BstI, EcoRV, HhaI, HinCII, MboI, MboII, TaqI, ThaI and XbaI) produced patterns that appeared identical for all strains. Digestion with the 9 remaining enzymes revealed fragment size differences and/or restriction site polymorphisms (Table 1). Based on differences in the restriction patterns, we were able to identify 4 genetic groups from the 7 strains examined.

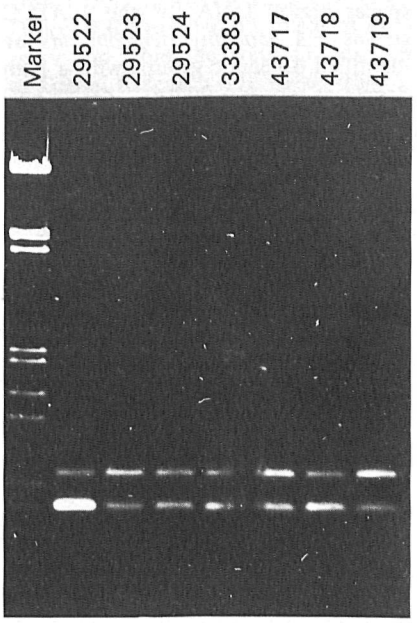

Fig. 2. PCR amplification product of the rDNA spacer region of 7 ATCC strains of *A. actinomycetemcomitans.* The 2 amplified products appear at approximately 880 and 1000 base pairs on 1% agarose gel. Markers are EcoRI, HindIII digestion products of lambda DNA.

A minimum of 2 enzymes (RsaI, NciI) provided sufficient information for distinguishing the 4 groups. Digestion with RsaI distinguished strain ATCC 29524 from all others tested (group II, Table 1) on the basis of a small size difference in the 2 bands running at about 600 and 410 base pairs (Fig. 3A). This difference was probably due to the presence of a second RsaI site in ATCC 29524. The extra fragment generated would be too small to detect by ethidium bromide staining. Four enzymes, NciI (Fig. 3B), MspI, SspI and AluI, gave a different restriction pattern for strains ATCC 29523 and ATCC 43717 compared with the other 5 strains, establishing a distinct genetic group (group IV, Table 1). A small difference in fragment size estimated to be approximately 20–25 base pairs, seen in digestions with NciI and MspI, and also with HaeIII, DdeI, DraI and HinFI, separated the strains into 2 additional groups. This difference appears in the bands running at about 150 bases (lowest-molecular-weight bands) in the HaeIII digest (Fig. 3C). The size variation observed with these 6 enzymes appears to be caused by a single genetic event. This size difference separated the remaining 4 strains into 2 additional genetic groups (groups I and III, Table 1).

Table 1. Banding patterns observed after digestion of *A. actinomycetemcomitans* strains with various restriction endonucleases. Genetic groups are shown with the corresponding serotype.*

Genetic group	I	II	III	IV
ATCC strain	29522 43718	29524	33384 43719	29523 43717
Serotype	b	–	c	a
RsaI	1	2	1	1
NciI MspI	1	1	2	3
SspI AluI	1	1	1	2
HaeIII DdeI DraI HinFI	1	1	2	2

* from Zambon et al. (1983).

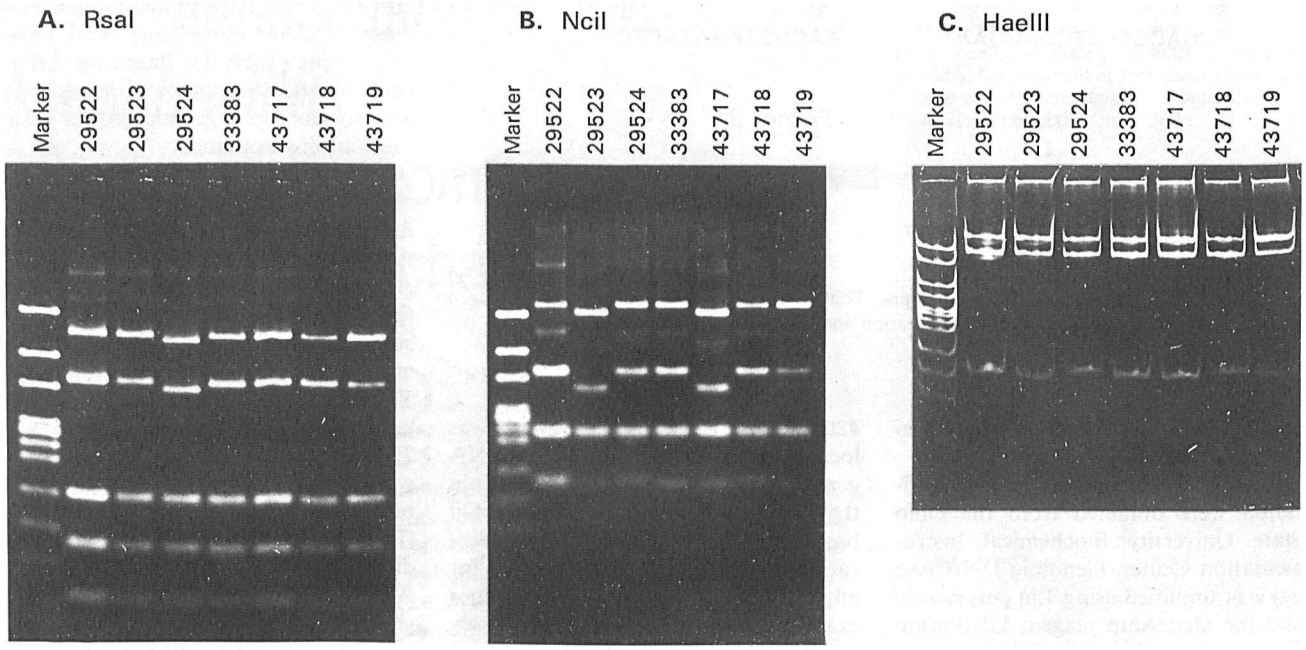

Fig. 3. Restriction endonuclease digestions of PCR-amplified rDNA spacer regions from 7 ATCC strains of *A. actinomycetemcomitans.* **A.** 3% agarose gel of the RsaI-digested rDNA spacer region. **B.** 3% agarose gel of NciI-digested rDNA spacer region. **C.** 8% acrylamide gel of HaeIII-digested rDNA spacer region. Marker sizes are 718, 501, 404, 301, 295–288 doublet, 267, 242, 190, 147, and 100 base pairs.

Discussion

Methods that yield a precise strain fingerprint are needed to track strains among cohorts and geographic locations and to study potential strain pathogenicity differences. Phenotypic characteristics such as serotype and biotype are useful markers, but they provide limited information for strain identification and may even be altered by laboratory growth conditions (3). Molecular genetic approaches that rely on DNA sequence differences provide more powerfull methods for fingerprinting closely related organisms. Whole genomic *A. actinomycetemcomitans* DNA has been examined for restriction site polymorphisms by Zambon et al. (13), but only 3 genetic groups were identified from among the 70 strains examined. The analysis of RFLPs in a DNA sample representing the entire genome of a bacteria is subject to a number of confounding factors, including electrophoretic migration anomalies, difficulties in obtaining complete digestion of genomic DNA, the inability to resolve nonhomologous restriction fragments with similar mobilities, and insensitivity. DiRienzo et al. (5) performed a Southern blot analysis of a whole genomic digestion using a randomly cloned chromosomal probe. This refinement revealed more strain heterogeneity than had previously been demonstrated. It should be possible to discriminate still more precisely among strains by examining a genetically defined region that is known to be highly variable. Using RFLP analysis of amplified rDNA gene spacer region DNA for the 7 ATCC strains of *A. actinomycetemcomitans,* we identified 4 genetic groups with a high degree of reproducibility.

The transcribed spacer region between the 16S and 23S ribosomal RNA genes is suitable for strain differentiation analysis because it is a variable region that is flanked by 2 conserved regions. The ribosomal RNA gene region contains 2 major genes, one coding for the 16S rRNA found in the small ribosomal subunit and the other coding for the 23S rRNA found in the large ribosomal subunit. These 2 genes are separated by a transcribed spacer sequence that usually contains at least one tRNA gene (Fig. 1). Because most of the spacer region is not under tight evolutionary constraints, it tends to be highly variable. It was selected for this analysis because of the high probability of variation, even among different strains of the same species (2). In contrast, the 16S and 23S coding regions flanking the spacer are highly conserved, even among species, making it possible to use oligonucleotides that hybridize to conserved sequences in these regions as primers for PCR amplification of the variable spacer region. These universal primers were used to amplify the spacer region between the 16S and 23S rDNA for all strains tested.

Most bacterial genomes contain several copies of the rDNA genes. Genetic mechanisms keep the separate copies of the rDNA repeat similar in sequence within an individual (8). Based on Southern analysis, each *A. actinomycetemcomitans* strain possesses at least 3 ribosomal operons (E. Leys, unpublished). The uncut PCR products of the *A. actinomycetemcomitans* spacer region appear as 2 bands of approximately 880 and 1000 base pairs on a 1% agarose gel (Fig. 2). This size difference (120 base pairs) is consistent with the presence of an additional tRNA gene in some but not all copies of the rDNA operon, as has been observed in other bacterial species (9).

By examining RFLPs in the rDNA spacer region, the 7 strains examined in this study could be divided into 4 genetic groups (Table 1). The classification determined by spacer region RFLP analysis correlates with the previously identified serotype classification of *A. actinomycetemcomitans* (11) (Table 1) and is not inconsistent with the 3 genetic groups identified by Zambon et al. (13),

although the serotype b and c strains selected for this analysis are clearly genetically distinct.

RFLP analysis of the spacer region allows a preliminary estimate to be made of the genetic relatedness of the strains examined. Strains in group IV were differentiated from strains in the other 3 groups by either 4 or 6 polymorphisms, whereas the other 3 groups were differentiated from each other by only 1 polymorphism. This suggests that genetic groups I, II, and III are more closely related to each other than they are to group IV.

These studies directly examined differences in only a small portion of the spacer region. Based on the number of restriction sites analyzed and the length of the recognition sequences, the RFLP approach in effect provides a direct assay of less than 150 of the approximately 1000-base-pair amplified region. Examination of ribosomal RNA gene spacer region DNA sequences deposited in the GENBANK database shows that, in bacterial species for which the spacer sequences from different strains are known, the genetic variability is 1–5%. Based on our data, the variability found among *A. actinomycetemcomitans* strains is comparable or greater. This suggests that direct sequence analysis should make it possible to distinguish even more precisely among strains of *A. actinomycetemcomitans*. Future studies should examine the entire DNA sequence of this region from a series of *A. actinomycetemcomitans* strains to determine the level of genetic variability among strains in an attempt to provide a definitive method for strain identification even between closely related strains within the same group.

It should also be possible to amplify the spacer region directly from a clinical sample by PCR amplification using species-specific oligonucleotides as primers. Appropriate species-specific probes to the 16S rDNA gene have been developed by Dix et al. (6). This approach would make it possible to bypass the need to isolate and culture each strain by amplifying directly from clinical samples, thus providing a very powerful and convenient tool for strain identification.

The transcribed spacer region between the 16S and 23S ribosomal rDNA genes of *A. actinomycetemcomitans* is a good candidate sequence with which to examine variation among different strains. RFLP analysis of this region in 7 strains revealed 4 genotypes and provides a rapid method of strain grouping that is more precise and reproducible than whole genomic methods. The genetic variability detected supplies strong evidence that more detailed analysis using direct sequencing could distinguish closely related strains within a genetic group and potentially provide definitive identification of strains.

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