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Identification of Genomic Clonal Types of *Actinobacillus actinomycetemcomitans* by Restriction Endonuclease Analysis

NAIMING HAN,[1,2]† * CHARLES I. HOOVER,[3] JAMES R. WINKLER,[2]
CLIFFORD Y. NG,[3] AND GARY C. ARMITAGE[2]

Department of Periodontology, School of Stomatology, Beijing Medical University, Beijing 100081, China,[1] and Division of Periodontology[2] and Division of Oral Biology,[3] Department of Stomatology, School of Dentistry, University of California, San Francisco, California 94143

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To evaluate its utility in discriminating different strains, restriction endonuclease analysis was applied to 12 strains of *Actinobacillus actinomycetemcomitans* (3 serotype a, 5 serotype b, and 4 serotype c strains). DNA isolated from each strain was digested by 12 different restriction endonucleases, and the electrophoretic banding patterns of the resulting DNA fragments were compared. The DNA fragment patterns produced by *SalI*, *XhoI*, and *XbaI* for the 12 *A. actinomycetemcomitans* strains were simple (<30 bands) and allowed us to recognize easily 10 distinct genomic clonal types. The three serotype a strains exhibited distinctly different clonal types from one another, the five serotype b strains exhibited an additional four distinct clonal types, and the four serotype c strains showed another three different clonal types. The other endonucleases tested were less useful in typing *A. actinomycetemcomitans*. We conclude that restriction endonuclease analysis is a powerful tool for typing and discerning genetic heterogeneity and homogeneity among *A. actinomycetemcomitans* strains. It should, therefore, be very useful for epidemiologic studies.

Actinobacillus actinomycetemcomitans is considered to be a primary pathogen of juvenile periodontitis in humans and can cause other serious infections throughout the body, including septicemia, meningitis, endocarditis, and abscesses in the brain and abdomen (20).

It is important in epidemiologic studies to be able to discriminate between different strains of *A. actinomycetemcomitans* because differences in pathogenicity among *A. actinomycetemcomitans* strains have been reported (1, 22), juvenile periodontitis appears to have a familial tendency (14, 19), and periodontally healthy individuals are also often colonized with *A. actinomycetemcomitans* (16, 21). Traditional techniques such as serotyping (23), polyacrylamide gel electrophoresis of cellular proteins (4, 9), cellular fatty acid analysis (2), and lipopolysaccharide subtyping (8) have limited epidemiological value since they compare only a few phenotypic characteristics and differentiate *A. actinomycetemcomitans* strains into relatively few groups (e.g., there are only three serotypes).

Restriction endonuclease analysis (REA) has proven useful in identifying and typing a variety of microorganisms (6, 11, 13, 15). Restriction endonucleases recognize and cleave double-stranded DNA at specific base pair sequences. The DNA fragments generated are separated by electrophoresis, stained with ethidium bromide, and visualized with UV light. The genetic heterogeneity and homogeneity of strains can then be evaluated by comparing the number and size (electrophoretic patterns) of the DNA fragments obtained. These DNA fragment patterns constitute a specific fingerprint to characterize each strain.

In a preliminary study (7), fragment patterns resulting from *SalI* and *XhoI* digestion of DNA from two *A. actinomycetemcomitans* strains and one *Haemophilus aphrophilus* strain were compared. The patterns of the two *A. actinomycetemcomitans* strains (one serotype a and one serotype b) were different from that of the *H. aphrophilus* strain and were different from one another. This suggested that *SalI* and *XhoI* could be of value for genomic clonal typing of *A. actinomycetemcomitans*. In the present study, we examined the use of 12 different restriction endonucleases, including *SalI* and *XhoI*, for clonal typing of 12 *A. actinomycetemcomitans* strains.

MATERIALS AND METHODS

Bacterial strains and growth. We studied 12 *A. actinomycetemcomitans* strains (3 serotype a, 5 serotype b, and 4 serotype c [Table 1]). The purity and identity of each strain were verified by standard microbiological techniques (5, 10).

The tested strains were grown in 75 ml of thioglycolate broth (trypticase soy broth, 15 g/liter; yeast extract, 5 g/liter; dextrose, 7.5 g/liter; sodium chloride, 2.5 g/liter; L-cysteine, 0.75 g/liter; sodium thioglycolate, 0.5 g/liter). Sodium bicarbonate was added to a final concentration of 0.4% immediately before inoculation. Bacterial cells were harvested by centrifugation and washed three times in 0.15 M phosphate-buffered saline, pH 7.3.

Preparation of DNA. DNA of *A. actinomycetemcomitans* was extracted by the method of Marshall et al. (11) with modifications. Briefly, the final pellet of bacterial cells (wet weight, 0.2 g) was resuspended in 1 ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), and then 0.5 mg of lysozyme was added. After incubation at 37°C for 15 min with gentle shaking, 100 μl of a 10% aqueous solution of sodium dodecyl sulfate and 10 μl of an aqueous solution of proteinase K (10 mg/ml) were added. Incubation was continued until the solution became clear. Sodium perchlorate (5 M) was added to a final concentration of 1 M; the lysate was incubated for 1 h at 50°C and was then increased to a volume of 3 ml with STE buffer (150 mM NaCl, 10 mM Tris, 1 mM EDTA). Each sample was then extracted repeatedly with equal volumes of

* Corresponding author.
† Present address: Department of Periodontics, Dental School, The University of Texas Health Science Center at San Antonio, San Antonio, TX 78284-7894.

TABLE 1. Sources of *A. actinomycetemcomitans* strains tested

Serotype	Strain	Isolation site	Location
a	ATCC 29523[a]	Blood	Denver, Colo.
	SUNYaB 75[b]	Mouth	Buffalo, N.Y.
	GA 3[c]	Mouth	San Francisco, Calif.
b	FDC Y4[d]	Mouth	Boston, Mass.
	ATCC 29522	Mandible (abscess)	Chicago, Ill.
	ATCC 29524	Chest (aspirate)	Seattle, Wash.
	SAC 11A[b]	Mouth	Arizona
	JP2[e]	Mouth	Philadelphia, Pa.
c	SUNYaB 67	Mouth	Buffalo, N.Y.
	SAC 5A	Mouth	Arizona
	SAC 6A	Mouth	Arizona
	SAC 12A	Mouth	Arizona

[a] American Type Culture Collection, Rockville, Md.
[b] J. J. Zambon and H. S. Reynolds, State University of New York at Buffalo.
[c] C. I. Hoover, University of California, San Francisco.
[d] S. S. Socransky, Forsyth Dental Center, Boston, Mass.
[e] N. A. Taichman, University of Pennsylvania, Philadelphia.

a mixture of phenol (previously saturated with TE buffer), chloroform, and isoamylalcohol (25:24:1). DNA was precipitated with 2 volumes of alcohol and dissolved in TE buffer. RNase A was added to a final concentration of 100 μl/ml, and the mixture was incubated at 37°C for 1 h and then extracted with the phenol-chloroform-isoamylalcohol (25:24:1) mixture as mentioned above.

The DNA concentration of each extract was determined by measuring the A_{260}. The purity of each extract was assessed by measuring the ratio of UV radiation at A_{260} to that at A_{280} ($A_{260/280}$).

Restriction endonuclease digestion of DNA. Individual portions containing 0.8 μg of genomic DNA from each strain were digested with *Eco*RI, *Hin*dIII, *Kpn*I, *Not*I, *Sac*I, *Sal*I, *Sfi*I, *Xho*I (all from Stratagene, La Jolla, Calif.), *Cla*I, *Pst*I, *Sau*3AI, and *Xba*I (all from New England BioLabs, Inc., Beverly, Mass.) for 2 h in the digestion buffer provided by the supplier and at the digestion temperature indicated by the supplier. Ten units of each enzyme was used (except *Xba*I, [20 U]).

Gel electrophoresis and photography. Digests of DNA were electrophoresed in horizontal slabs of 0.5% agarose immersed in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3). The gels were run for 10 h at 3 V/cm. After electrophoresis, the gels were stained for 30 min with 0.5 μg of ethidium bromide per ml. Stained gels were illuminated with UV light and photographed with a Polaroid camera.

RESULTS

The amount of DNA obtained from each strain was in the range of 200 to 400 μg. The $A_{260/280}$s of the DNA extracts ranged from 1.84 to 1.94. The DNA extracted was intact (>25 kb), and no plasmids were present in any of the strains tested (data not shown).

Our standard digestion conditions (10 U for 2 h) completely digested the DNA extracts into discrete fragments. Increasing digestion time up to 16 h or doubling the amount of restriction enzymes did not change the patterns.

Of the 12 different endonucleases we evaluated for genomic clonal typing of 12 *A. actinomycetemcomitans* strains, *Sal*I, *Xho*I, and *Xba*I proved to be the most useful. The results of agarose gel electrophoresis of DNA fragments produced by *Sal*I digestion of DNAs from the 12 *A. actinomycetemcomitans* strains are shown in Fig. 1. The fragment patterns obtained with the *A. actinomycetemcomitans* strains were relatively simple, and 8 distinct restriction fragment patterns (eight genomic clonal types) were easily recognized among the 12 *A. actinomycetemcomitans* strains. Two serotype a strains, ATCC 29523 (Fig. 1a, lane 1) and GA 3 (lane 3), exhibited identical DNA fragment patterns and were designated *Sal*I type I. The third serotype a strain, SUNYaB 75

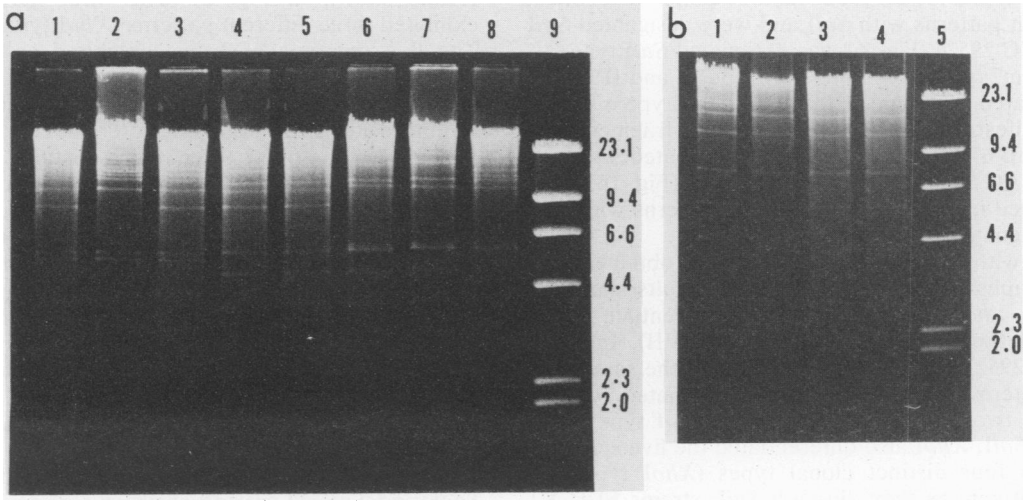

FIG. 1. (a) Fragment patterns after *Sal*I digestion of DNA. Lanes 1 to 3, *A. actinomycetemcomitans* serotype a strains ATCC 29523 (lane 1), SUNYaB 75 (lane 2), and GA 3 (lane 3); lanes 4 to 8, *A. actinomycetemcomitans* serotype b strains FDC Y4 (lane 4), ATCC 29522 (lane 5), ATCC 29524 (lane 6), SAC 11A (lane 7), and JP2 (lane 8); lane 9, *Hin*dIII digest of lambda phage DNA. The molecular sizes of fragments are indicated in kilobases at the right. (b) Fragment patterns after *Sal*I digestion of DNA. Lanes 1 to 4, *A. actinomycetemcomitans* serotype c strains SUNYaB 67 (lane 1), SAC 5A (lane 2), SAC 6A (lane 3), and SAC 12A (lane 4); lane 5, *Hin*dIII digest of lambda phage DNA. The molecular sizes of fragments are indicated in kilobases at the right.

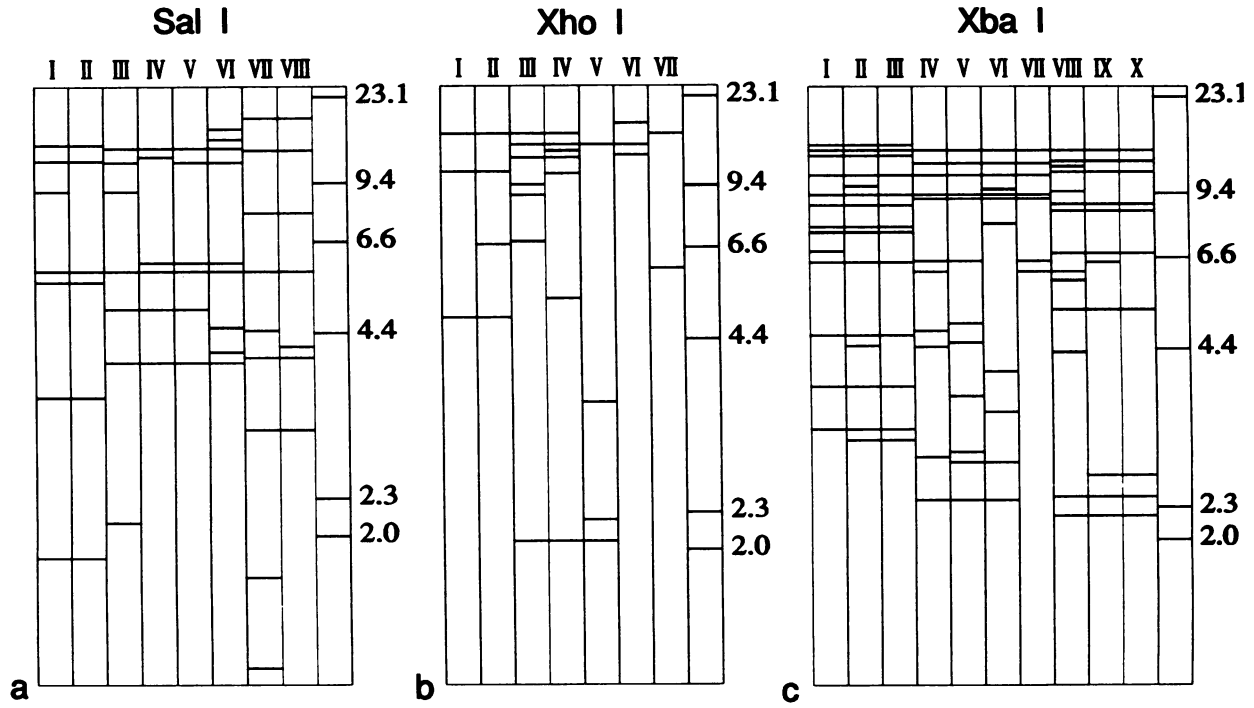

FIG. 2. DNA fragment patterns generated by restriction endonuclease treatment of 12 strains of *A. actinomycetemcomitans*. (a) *Sal*I DNA fragment types. Type I, ATCC 29523 and GA 3; type II, SUNYaB 75; type III, FDC Y4 and ATCC 29522; type IV, ATCC 29524; type V, SAC 11A; type VI, JP2; type VII, SUNYaB 67; type VIII, SAC 5A, SAC 6A, and SAC 12A. (b) *Xho*I DNA fragment types. Type I, ATCC 29523; type II, SUNYaB 75 and GA 3; type III, FDC Y4 and ATCC 29522; type IV, ATCC 29524; type V, SAC 11A; type VI, JP2; type VII, SUNYaB 67, SAC 5A, SAC 6A, and SAC 12A. (c) *Xba*I DNA fragment types. Type I, ATCC 29523; type II, SUNYaB 75; type III, GA 3; type IV, FDC Y4 and ATCC 29522; type V, ATCC 29524; type VI, SAC 11A; type VII, JP2; type VIII, SUNYaB 67; type IX, SAC 5A; type X, SAC 6A and SAC 12A. The molecular sizes of fragments are indicated in kilobases at the right.

(lane 2), differed from the other two by the absence of one DNA fragment band at about 9.4 kb and was designated *Sal*I type II. The five serotype b strains (Fig. 1a, lanes 4 to 8) manifested an additional four different DNA fragment patterns and were designated *Sal*I types III through VI. Strains FDC Y4 (lane 4) and ATCC 29522 (lane 5) exhibited identical DNA fragment patterns with *Sal*I and were designated *Sal*I type III. ATCC 29524 (lane 6) was designated *Sal*I type IV, SAC 11A (lane 7) was designated *Sal*I type V, and JP2 (lane 8) was designated *Sal*I type VI. The four serotype c strains (Fig. 1b) manifested two more distinct DNA fragment patterns. SUNYaB 67 (Fig. 1b, lane 1) was designated *Sal*I type VII. Strains SAC 5A, SAC 6A, and SAC 12A (Fig. 1b, lanes 2 to 4) produced identical DNA fragment patterns with *Sal*I and were designated *Sal*I type VIII.

The results with *Xho*I were similar to those obtained with *Sal*I (photographs of the gels not shown; results shown in Fig. 2). With *Xho*I, we were able to differentiate seven distinct clonal types (*Xho*I types I through VII). Interestingly, ATCC 29523 and GA 3, which shared the same *Sal*I restriction pattern (*Sal*I type I), were differentiated by *Xho*I digestion into two different clonal types, *Xho*I type I and type II. Like *Sal*I, *Xho*I also differentiated the five serotype b strains into four distinct clonal types (*Xho*I types III through VI). Again, as they did with *Sal*I, strains FDC Y4 and ATCC 29522 exhibited identical DNA fragment patterns with *Xho*I. These two strains were designated *Xho*I type III. ATCC 29524 was designated *Xho*I type IV, SAC 11A was designated *Xho*I type V, and JP2 was designated *Xho*I type VI. All four serotype c strains exhibited the same DNA fragment pattern with *Xho*I digestion and were designated *Xho*I type VII.

*Xba*I proved to be the most discriminating endonuclease we studied in that we were able to recognize 10 clonal types among the 12 strains (photographs of the gels not shown; results shown in Fig. 2). The three serotype a strains exhibited three different patterns (*Xba*I types I through III). The five serotype b strains exhibited an additional four distinct patterns (*Xba*I types IV through VII). With *Xba*I, as with *Sal*I and *Xho*I, strains FDC Y4 and ATCC 29522 produced identical fragment patterns. The four serotype c strains demonstrated three more distinct patterns (*Xba*I types VIII through X). A single restriction fragment polymorphism with *Xba*I differentiated serotype c strain SAC 5A from the other two serotype c strains (SAC 6A and SAC 12A) that had formed identical patterns with *Sal*I and *Xho*I. Since some faint DNA bands present in the gels do not show up well in photographs, and to aid in the interpretation of the results, we have provided line drawings of the fragment patterns produced by *Sal*I, *Xho*I, and *Xba*I digestion of the 12 *A. actinomycetemcomitans* strains (Fig. 2). In addition, the *Sal*I, *Xho*I, and *Xba*I types of each strain are shown in Table 2.

The results of REA with *Cla*I, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Sac*I, and *Sau*3AI are too complex for easy visual analysis because there were too many densely distributed fragments (photographs of the gels not shown). Either the DNAs from the *A. actinomycetemcomitans* strains were not digested by *Sfi*I and *Not*I or the DNA fragments produced were too large to be separated by our experimental conditions.

TABLE 2. Differentiation of genomic clonal types of *A. actinomycetemcomitans*

Serotype	Strain	DNA fragment band types			Genomic clonal types
		*Sal*I	*Xho*I	*Xba*I	
a	ATCC 29523	I	I	I	1
	SUNYaB 75	II	II	II	2
	GA 3	I	II	III	3
b	FDC Y4	III	III	IV	4
	ATCC 29522	III	III	IV	4
	ATCC 29524	IV	IV	V	5
	SAC 11A	V	V	VI	6
	JP 2	VI	VI	VII	7
c	SUNYaB 67	VII	VII	VIII	8
	SAC 5A	VIII	VII	IX	9
	SAC 6A	VIII	VII	X	10
	SAC 12A	VIII	VII	X	10

DISCUSSION

REA is a powerful technique for demonstrating genetic heterogeneity and homogeneity among bacterial strains. If two strains exhibit a single restriction fragment polymorphism with a single endonuclease, they may be considered to be genetically distinct (different clonal types). If the DNA fragment patterns of two strains are identical with several different endonucleases, the two strains can be considered to be genetically indistinguishable (the same clonal type). For simple visual interpretation, we believe that restriction enzymes that produce less than 30 fragment bands are ideal. Restriction endonucleases that produce large numbers of DNA fragments create difficulties in interpretation, especially when the bands are densely distributed. Some studies (17, 18) have used laser densitometry and multivariate analyses to interpret very complex DNA fragment patterns. However, we found that, as demonstrated by Collins and Ross (3), if appropriate enzymes are chosen the similarities and differences among strains are clear and can be easily determined by visual examination.

In this study, we evaluated the utility of 12 different endonucleases for the clonal typing of *A. actinomycetemcomitans* strains. *Sal*I, *Xho*I, and *Xba*I proved to be the most useful and allowed us to separate easily the 12 *A. actinomycetemcomitans* strains into 10 distinct clonal types (Table 2). It is interesting that two strains, FDC Y4 and ATCC 29522, exhibited identical DNA fragment patterns with all the endonucleases tested. Since these two strains were isolated from diseased sites in different individuals in different geographic locations, one could speculate that they represent a particularly pathogenic clonal type of *A. actinomycetemcomitans*. However, analysis of more strains isolated from diseased and healthy sites is required to establish whether any particular clonal type(s) is associated with virulence. Another three strains also exhibited extremely similar DNA fragment patterns. *Sal*I and *Xho*I digestion produced identical patterns with strains SAC 5A, SAC 6A, and SAC 12A. Digestion with *Xba*I indicated a single restriction fragment polymorphism among SAC 5A and the other two strains. These three strains were isolated from subgingival plaque from Pima Indians in Arizona. The close genetic similarity of these strains may indicate the transmission of a prevalent genomic clonal type within this sequestered population of individuals. These observations indicate the potential value of REA for epidemiologic studies of transmission. Clearly it would be of interest to determine the clonal types of *A. actinomycetemcomitans* present in affected and unaffected members of families with a history of juvenile periodontitis.

Our results and conclusions are in contrast to those of a recent report by Zambon et al. (24). These investigators evaluated the use of 16 different endonucleases (including *Xho*I and *Xba*I) for REA of 70 *A. actinomycetemcomitans* strains (124 isolates). They concluded that *Eco*RI and *Hin*dIII were the most appropriate enzymes for REA of *A. actinomycetemcomitans* strains. However, they were only able to recognize three different restriction fragment patterns among the 70 strains that they examined. All serotype a strains produced a common restriction fragment pattern, 58% of the serotype b strains produced another restriction pattern, and the remaining serotype b strains and all the serotype c strains shared a third restriction pattern. Direct comparison of our results with those of Zambon et al. is difficult since their figures do not include DNA molecular size standards nor indicate which endonuclease was used. The choice of appropriate endonucleases is critical for REA. If we had based our clonal typing on *Eco*RI and *Hin*dIII, we might have reached conclusions similar to those reported by Zambon et al.

The results of our study indicate that REA is the most discriminating technique available for typing *A. actinomycetemcomitans*. REA is a simple, rapid, sensitive, and highly reproducible technique which utilizes readily available commercial restriction endonucleases. In contrast, serotyping separates *A. actinomycetemcomitans* strains into only three types and requires the production of serotype-specific antisera by individual laboratories; in addition, in some instances nearly 50% of isolates cannot be assigned to a serotype (12). In conclusion, we believe that epidemiologic studies utilizing REA to investigate the horizontal and vertical transmission of *A. actinomycetemcomitans* are warranted.

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