Identification and Characterization of Genetic Cluster Groups of *Actinobacillus actinomycetemcomitans* Isolated from the Human Oral Cavity

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Actinobacillus actinomycetemcomitans is recognized as a primary pathogen in localized juvenile periodontitis (LJP). Restriction fragment length polymorphisms (RFLP) within a collection of subgingival plaque isolates of this bacterium were identified and characterized as the first step in understanding the pathogenesis of LJP. Over 800 isolates, from members of 18 families (LJP families) with at least one member with active LJP or a documented history of the disease and one or more siblings, less than 13 years of age, having no clinical evidence of LJP and 32 healthy control subjects, were assigned to one of 13 distinct RFLP groups (II to XIV) by using a previously characterized 4.7-kb DNA probe cloned from the reference strain FDC Y4. Isolates belonging to RFLP groups II, IV, V, and XIII predominated subgingival sites in the subjects. Members of RFLP groups II, IV, VII, VIII, X, and XI were recovered only from LJP family subjects, while group XIII and XIV variants were found exclusively in healthy controls. A synthetic oligonucleotide, homologous to the 5' end of the leukotoxin gene (*lktA*), and the *A. actinomycetemcomitans* plasmid, pVT745, were tested for their abilities to subdivide the 13 RFLP groups. The leukotoxin probe specifically identified all RFLP group II variants because of the absence of a *Hind*III site in the upstream noncoding region of the *lkt* gene complex. The plasmid probe was not as selective but may be useful for identifying clinical isolates belonging to RFLP group I. The use of these probes for the identification of genetic variants of *A. actinomycetemcomitans* that may preferentially colonize diseased and healthy subjects will facilitate the study of the role of this important pathogen in periodontal diseases.

The bacterium *Actinobacillus actinomycetemcomitans* is a small capnophilic, gram-negative coccobacillus. This species was first recognized as an important periodontal pathogen in studies that examined the microflora of a specific type of periodontal disease known as localized juvenile periodontitis (LJP) (20, 24, 28). The first clinical signs of LJP are usually detected in children about 11 to 13 years of age and are characterized by the rapid breakdown of the supporting tissues of the first permanent molars and incisors (3, 17). There is increasing evidence to support early impressions that LJP is readily transmitted within families, with some families appearing to be more susceptible than others to the disease (4, 23).

On the basis of available evidence suggesting the familial transmission of LJP and the suspected role of *A. actinomycetemcomitans* in the disease, our objective was to identify genetic variants of the bacterium that may preferentially colonize periodontally diseased versus healthy sites in LJP subjects. In previous studies, restriction fragment length polymorphisms (RFLP) were identified in laboratory strains of *A. actinomycetemcomitans*, and this provided a reliable method for identifying genetic variants (6, 7).

In this article, we describe and compare the various RFLP groups of *A. actinomycetemcomitans* isolated, over a 5-year period, from 73 members of LJP families (see below) and 32 healthy control subjects. The LJP families were part of a clinical longitudinal model of the disease (5). This identi-

cation and characterization of RFLP types of clinical isolates of *A. actinomycetemcomitans* is the first step in determining whether specific genetic variants of the bacterium correlate with the onset, progression, and transmission of LJP.

MATERIALS AND METHODS

Bacterial strains, isolation methods, and growth conditions. Reference strains used in this study were FDC Y4, JP2, ATCC 29523, NCTC 9710, and SX48. These strains were members of RFLP groups I, II, I, V, and XV, respectively, as determined with the 4.7-kb probe (6). The clinical bacterial isolates examined in this study were obtained from subgingival plaque samples from periodontally diseased and healthy subjects ($n = 73$) who were members of 18 LJP families. The families chosen for the original study contained at least one member (child or adult) with active LJP or a documented history of the disease and one or more siblings, less than 13 years of age, having no clinical evidence of LJP. The study population was recruited between 1987 and 1989 from the West Philadelphia area adjacent to the University of Pennsylvania campus. A more detailed description of this population and the LJP model will appear elsewhere (8). Bacteria were also isolated from subgingival plaque samples from periodontally healthy children ($n = 32$) who were members of families in which no member had LJP or a history of the disease.

Plaque samples were taken from all subjects at accession. Subjects older than 18 years were sampled at yearly intervals. Plaque samples were collected from all other subjects every 3 months throughout the study or until they reached 18 years of age. Supragingival plaque was removed with sterile

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cotton pellets, and subgingival plaque was collected by inserting three sterile paper points (Johnson and Johnson, East Windsor, N.J.) to the depth of the periodontal pocket, keeping them there for 10 s. The samples were taken from the mesial surfaces of the four permanent first molars (target sites 1 and 4 to 6), the maxillary first premolars (control sites 2 and 3), and from any site that converted from a healthy to a diseased state during the course of the study. In addition, a pooled sample was collected from all six specific sites. Only the pooled sample was collected from the adults during their yearly visits. The subgingival samples were transferred to 2 ml of prereduced VMGA III anaerobic transport medium (19). The bacteria were dispersed with a vortex mixer, at the maximum setting, for 60 s. Appropriate dilutions were spread on Trypticase soy-serum-bacitracin-vancomycin agar for the isolation of *A. actinomycetemcomitans* (25). After incubation of the plates in an atmosphere containing 5% CO₂, *A. actinomycetemcomitans* colonies were distinguished from *Haemophilus aphrophilus* by their distinctive colony morphology, positive catalase reaction, and negative lactose fermentation (25) and were counted. One colony of *A. actinomycetemcomitans* was selected from each subgingival sample and was inoculated on peptone-yeast extract-glucose medium (7) containing 1.5% agarose (Sigma Chemical Co., St. Louis, Mo.) in place of Bacto agar (Difco Laboratories, Detroit, Mich.). After growth, the bacteria were removed from one half of the plate, mixed with peptone-yeast extract-glucose medium containing 20% glycerol, and frozen at -70°C. Bacteria removed from the other half of the agar plate were used to isolate chromosomal DNA.

DNA methods and hybridization conditions. Chromosomal DNA was isolated as described elsewhere (7). The DNA samples (1 to 3 µg) were digested to completion with *Hind*III, according to the manufacturer's instructions, and the restriction fragments were separated on 0.7% agarose gels as described elsewhere (7).

The DNA fragments were transferred to a nylon membrane (Magnagraph; Micron Separations, Westboro, Mass.) by using a vacuum blotting apparatus (Bio-Rad Laboratories, Rockville, N.Y.). Hybridizations were performed with one of three DNA probes. The probe used to identify the basic RFLP groups was a 4.7-kb *Eco*RI fragment isolated, as a randomly cloned fragment, from *A. actinomycetemcomitans* FDC Y4 (7). This probe was restriction mapped, validated, and shown to detect significant genetic polymorphism in laboratory strains of *A. actinomycetemcomitans* (6, 7). In some hybridization experiments, *Hind*III fragments of the 4.7-kb *Eco*RI fragment were extracted from a 0.7% agarose gel as described elsewhere (9) and used as probes. Two additional probes, pVT745 and a synthetic oligonucleotide, were used to subgroup the basic RFLP groups. Plasmid pVT745 was extracted according to the method of Anderson and McKay (1). Oligonucleotide 3'-TACCGTT GATGATGTGACGG-5', homologous to the 5' end of the *lktA* gene, was made by M. Mitchell (Nucleic Acid Facility, University of Pennsylvania). The 4.7-kb probe and pVT745 were labeled with [α -³²P]dATP and [α -³²P]dCTP (6,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) by nick translation (22). The synthetic oligonucleotide was end labeled with [γ -³²P]ATP (>5,000 Ci/mmol) according to a standard procedure (22). Lambda DNA, digested to completion with *Hind*III, was labeled by nick translation, and 5 × 10⁶ cpm was hybridized to λ DNA digested with *Hind*III to provide molecular size markers on the autoradiograms.

Southern blots were prehybridized for a minimum of 3 h

FIG. 1. Hybridization patterns of isolates representative of the 13 RFLP cluster groups (II to XIV) identified for members of LJP families and healthy control subjects. Lanes I and XV, DNAs from reference strains FDC Y4 and SX48, respectively. Fragments of λ bacteriophage DNA (23.1 to 0.6 kb), from a *Hind*III digest, were used as size markers (lane L). The Southern blot was hybridized with the 4.7-kb DNA fragment from *A. actinomycetemcomitans* FDC Y4 and λ bacteriophage DNA labeled with ³²P. The hybridization probe was cloned from the chromosomal DNA used in lane I. Hybridization-positive bands in lane I correspond to fragments A to E from the physical map of the 4.7-kb probe (Fig. 3). Samples XIII and XV were from a separate Southern blot.

and then hybridized, with either the 4.7-kb DNA fragment or pVT745, overnight at 42°C in 50% formamide as described elsewhere (7). Usually 1 × 10⁷ to 2 × 10⁷ cpm of probe DNA (specific activity, 10⁸ cpm/µg of DNA) was used. Following hybridization, the blots were washed twice with 2× SSC (0.3 M sodium chloride-0.03 M sodium citrate, pH 7.0) for 15 min at room temperature, twice with 1× SSC-1% sodium dodecyl sulfate (SDS) for 30 min at 60°C, and twice with 0.1× SSC-0.1% SDS for 15 min at room temperature. The synthetic oligonucleotide was hybridized overnight, on dried agarose gels, at 55°C (5°C below the denaturation temperature) as described elsewhere (30). The posthybridization washes consisted of two washes with 6× SSC for 20 min at room temperature, one wash with 6× SSC for 4.5 h at room temperature, and one wash with 6× SSC for 2 min at 55°C. In some experiments, the stringency of the hybridization and posthybridization washes was changed by lowering the temperature to 38°C. The nylon membranes or dried gels were exposed to Kodak XAR-5 X-ray film between two Cronex Lightning Plus intensifying screens at -70°C.

RESULTS

RFLP cluster group analysis. The *A. actinomycetemcomitans* isolates obtained from periodontally diseased and healthy subjects in the LJP families and from the healthy control subjects were examined for genetic polymorphisms by using a previously characterized 4.7-kb DNA fragment cloned from reference strain FDC Y4 (7). Thirteen distinct hybridization patterns were identified with this probe and were designated RFLP groups II to XIV (Fig. 1). Three additional randomly cloned DNA fragments of 5.8, 3.0, and 4.8 kb from FDC Y4 detected only two, three, and two distinct RFLP patterns, respectively, among the 800 isolates examined (data not shown). The RFLP patterns obtained with the 4.7-kb probe were highly reproducible, such that no ambiguities were noted among any of the isolates identified within a particular RFLP group. Patterns I and XV were

TABLE 1. Grouping of *A. actinomycetemcomitans* isolates by RFLP

RFLP group	No. of isolates from:	
	LJP subjects ^a	Control subjects ^b
I ^c	0	0
II	186	0
III	10	12
IV	133	0
V	220	15
VI	2	6
VII	4	0
VIII	18	0
IX	22	1
X	5	0
XI	7	0
XII	14	58
XIII	0	98
XIV	0	6
XV ^d	0	0

^a Number of subjects, 73; number of isolates, 624; number of subjects lacking cultivable *A. actinomycetemcomitans*, 26.

^b Number of subjects, 32; number of isolates, 196; number of subjects lacking cultivable *A. actinomycetemcomitans*, 19.

^c RFLP pattern of reference strain FDC Y4.

^d RFLP pattern of reference strain SX48.

obtained with DNAs from reference strains Y4 and SX48. None of the isolates from the LJP family members or from the control subjects had the same hybridization pattern as these two strains.

The distribution of isolates within the 13 RFLP groups is shown in Table 1. The majority of the isolates had RFLP patterns II, IV, V, and XIII. These values were based on the analysis of only one isolate from each sampling site from each patient visit. Isolates having RFLP patterns II, IV, V, VII, VIII, X, and XI were recovered only from LJP family members. Isolates having patterns XIII and XIV were cultured exclusively from control subjects.

Molecular basis of restriction site heterogeneity among the RFLP cluster groups. The degree of similarity between the DNAs of isolates belonging to pairs of RFLP groups was determined by comparing the number of conserved *Hind*III fragments within the region of DNA specified by the 4.7-kb probe. These relationships are shown in a paired matrix in Fig. 2. The closer the value to 1, the greater the similarity between two RFLP groups. RFLP groups I, III, VII, and

XIII to XV appear to form a closely related cluster within the species.

Polymorphism within the region of the *A. actinomycetemcomitans* chromosome specified by the 4.7-kb probe was defined by restriction analysis and Southern blotting (Fig. 3). The probe recognized a maximum of five *Hind*III fragments in the *A. actinomycetemcomitans* chromosome. These restriction fragments were designated A to E in the RFLP group I pattern (Fig. 1, lane I, and Fig. 3). Each of these fragments was isolated from the cloned probe sequence. Each fragment was then used as a hybridization probe to identify the corresponding *Hind*III fragment in the chromosomal DNA from isolates representing each of the RFLP groups. The conserved regions and regions of polymorphism were determined by aligning the hybridization patterns obtained with the strains or variants that belonged to each group. Some regions of DNA, such as those specified by fragments C (0.8 kb) and D (0.7 kb), were highly conserved. Fragments C and D were present in representative members of 12 and 13 of 15 RFLP groups, respectively. Fragment A (3.5 kb) was the same size in isolates from 8 of 15 RFLP groups. The absence of *Hind*III site ii (Fig. 3) in the genomes of members of groups V, VI, VIII, XI, and XII created a single large restriction fragment, approximately 17 or 23 kb in length, which was composed of the combined fragments A and B. The most extensive polymorphism was evident in the regions of the chromosome corresponding to fragments B and E, such that small insertions or deletions appear to have formed new RFLP groups. Fragment E was missing, in entirety, in strains or variants representing cluster groups V, XII, and XIV. Strains or variants belonging to RFLP groups I, III, VII, and XIII differed only in the size of fragment E.

Subgrouping of the primary RFLP cluster groups. Restriction site polymorphism exists among *A. actinomycetemcomitans* strains because of the presence or absence of a *Hind*III site located approximately 300 bp upstream of the *lktC* gene (27) (Fig. 4A). To determine whether this restriction site heterogeneity was useful for subgrouping the 14 RFLP groups, a synthetic oligonucleotide homologous to the 5' end of the *lktA* gene from strain JP2 was used to assess polymorphism defined by that *Hind*III site. Chromosomal DNA from members of each of the RFLP groups was digested to completion, with *Hind*III, for Southern blot analysis. A conserved *Hind*III site is located approximately at the midpoint in the *lktA* gene from strain JP2 (14) (Fig. 4A). The results of the hybridization experiments are shown in Fig. 4B. The probe recognized a single 8.4-kb DNA fragment in

RFLP GROUP	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV
I	1.00	0.60	0.80	0.60	0.40	0.25	0.80	0.44	0.22	0.22	0	0	0.80	0.89	0.80
II		1.00	0.60	0.60	0.40	0.25	0.60	0.67	0.22	0.22	0	0	0.60	0.67	0.60
III			1.00	0.80	0.40	0.25	0.80	0.44	0.22	0.22	0	0	0.80	0.89	0.60
IV				1.00	0.40	0.25	0.60	0.44	0.22	0.22	0	0	0.60	0.67	0.60
V					1.00	0.25	0.40	0.44	0.22	0.22	0.29	0	0.40	0.44	0.40
VI						1.00	0.25	0.57	0.57	0.29	0	0.33	0.25	0.29	0.25
VII							1.00	0.44	0.22	0.22	0	0	0.80	0.89	0.60
VIII								1.00	0.25	0.25	0	0.29	0.44	0.50	0.44
IX									1.00	0.25	0	0	0.22	0.25	0.22
X										1.00	0	0	0.22	0.25	0.22
XI											1.00	0	0	0	0
XII												1.00	0	0	0
XIII													1.00	0.89	0.60
XIV														1.00	0.67
XV															1.00

FIG. 2. Conserved fraction of probe-homologous *Hind*III restriction fragments between pairs of isolates representative of the RFLP groups. The fraction of fragments conserved between two RFLP groups was calculated as $2a/(x + y)$, where a is the number of pairs of conserved fragments in the restriction digests and $(x + y)$ is the total number of hybridization-positive fragments (13). Relationships were determined by using the 4.7-kb probe from strain FDC Y4.

FIG. 3. Physical maps of the chromosomal region recognized by the 4.7-kb probe in DNAs from members of the various RFLP groups. Fragments A to E were identified by isolating each of these fragments from the cloned 4.7-kb *EcoRI* fragment and independently hybridizing to chromosomal DNAs digested with *HindIII*. Only the *HindIII* sites are designated on the maps. For individual fragments differing in size from the corresponding fragments in the group I map, the length (in kilobases) of the fragment is shown in parentheses.

the DNAs from strains JP2 and 42BD under stringent and nonstringent hybridization conditions. Both of these strains belong to RFLP group II. A single 2.3-kb hybridization-positive fragment was detected in strains or variants representative of each of the remaining RFLP groups. To determine whether the presence of the 8.4-kb hybridization-positive fragment was a general characteristic of RFLP group II strains, the DNAs from 12 members of this group were hybridized to the oligonucleotide probe. Only three of the group II strains contained the smaller hybridization-positive fragment found in the non-group II strains (Fig. 4C).

It has been reported that sequences homologous to an *A. actinomycetemcomitans* plasmid, pVT745, are distributed in the chromosomes of various strains of this bacterium (18). To determine whether the distribution of these sequences was extensive enough to be useful for subgrouping the RFLP groups, pVT745 DNA was hybridized to DNA from representative members of the 14 basic RFLP groups (Fig. 5). The plasmid DNA hybridized to chromosomal DNA from two group I strains (FDC Y4 and ATCC 29523), one group V strain (NCTC 9710) and one group XIII strain (1020012).

Strain 1020012 originated from a healthy control subject. The hybridization patterns of the two group I strains were identical. No hybridization was obtained with DNAs from six group II strains.

DISCUSSION

RFLP was used to assess the extent of polymorphism, in a heterogeneous region of the *A. actinomycetemcomitans* chromosome, within a comprehensive collection of *A. actinomycetemcomitans* from a well characterized LJP subject population. The objective was to use RFLP analysis to identify and group genetic variants of *A. actinomycetemcomitans* from clinical sources to facilitate studies of the role of this bacterium in the pathogenesis of LJP. Thirteen distinct RFLP groups were identified within a collection of *A. actinomycetemcomitans* isolates obtained, over a 5-year period, from a regional population of 73 subjects from 18 LJP families and 30 control subjects. These LJP family members and control subjects formed the basis of a longitudinal study designed to monitor the earliest events associated with the

FIG. 4. Hybridization of isolates representative of the RFLP groups with an oligonucleotide probe homologous to the 5' end of the *lktA* gene. (A) Physical map of the *lkt* gene complex and the noncoding upstream region. Only the *Hind*III sites are shown. Information for the construction of the map was obtained from the sequences deposited in GenBank (accession numbers X16829, X53955, and X53956). The location of the strain-variable *Hind*III site (boxed) was inferred from hybridization data in reference 27 and this study. The sequence of the oligonucleotide probe is shown below the map. (B) Autoradiogram of the Southern blot depicting patterns of hybridization of representative members of RFLP groups I to XV with the oligonucleotide probe. Strain JP2 is a member of RFLP group II. For each of RFLP groups V and XII, two representative strains or variants were run on the blot. There is no sample for RFLP group XIV. Lane XV contains DNA from reference strain SX48. The DNA samples were digested with *Hind*III. The hybridization and posthybridization washes were done at 38°C. The sizes of the major hybridization-positive fragments are given in kilobases. (C) Autoradiogram of the Southern blot showing the hybridization patterns of 12 members of RFLP group II. Lane 1 contains DNA from strain JP2. The electrophoresis and hybridization conditions were the same as for panel B.

onset of the disease. The RFLP groups were identified by using a defined 4.7-kb *Eco*RI fragment cloned from the *A. actinomycetemcomitans* FDC Y4 genome. This probe was validated and shown to detect significant restriction site heterogeneity in laboratory strains of *A. actinomycetemcomitans* (6, 7). Previous methods used to determine the distribution of strains of *A. actinomycetemcomitans* among LJP-infected and healthy subjects have included immunofluorescence using serotype-specific antibodies (2, 21, 31),

FIG. 5. Hybridization of pVT745 to strains or variants belonging to selected RFLP groups. DNA samples were digested with *Hinc*II. Chromosomal DNA samples were from strains FDC Y4 (group I), ATCC 29523 (group I), NCTC 9710 (group V), and 1020012 (group XIII) and plasmid pVT745. Molecular size markers are the same as those described in the legend to Fig. 1.

biotype analyses (26), and DNA fingerprinting (32). The RFLP method has several advantages over these methods for examining the epidemiology of *A. actinomycetemcomitans* in LJP. Serotyping and biotyping are dependent on gene expression, which may be influenced by environmental pressures or in vitro growth conditions. For example, Asikainen et al. (2) found that strains reacted with more than one typing serum when the bacteria were prepared for analysis by several methods. The use of restriction endonuclease fingerprinting by Zamboni et al. (32) overcame the limitations imposed by the phenotypic analyses. However, the poor physical resolution of the large number of DNA fragments characteristic of chromosomal digests resulted in the identification of only three distinct restriction patterns among 70 strains of *A. actinomycetemcomitans* examined.

The RFLP groups identified in the current study were considered to represent genetic cluster groups. The organization of these groups was dependent on the use of the 4.7-kb probe. Since this probe recognized a relatively small portion of the bacterial chromosome, the RFLP group scheme could potentially change if other fragments of chromosomal DNA were used as probes. However, other randomly cloned DNA fragments, from strain FDC Y4, did not detect significant restriction site heterogeneity when hybridized to DNAs from various *A. actinomycetemcomitans* laboratory strains or clinical isolates. Guthmiller et al. (11) used three randomly cloned probes, a leukotoxin gene probe, and a CAMP gene probe as part of a typing scheme for *A. actinomycetemcomitans* isolated from human and nonhuman primates. These probes identified 12 RFLP patterns, respectively, among 35 human strains examined. The randomly cloned probes detected only four, five, and two RFLP patterns, respectively.

Selectivity was observed in the distribution of strains belonging to the various RFLP groups identified with the 4.7-kb DNA fragment. RFLP pattern I, representative of *A. actinomycetemcomitans* FDC Y4, was not detected in any of the LJP family or control subjects. This strain was one of the first isolates of *A. actinomycetemcomitans* recovered, in the late 1970s, from the human oral cavity (29). Other isolates representative of RFLP groups II, IV, V, VII, VIII, X, and XI were found exclusively in LJP family members. In contrast, isolates belonging to RFLP groups XIII and XIV were found only in control subjects. The significance of this selective distribution may be related to the initial colonization of disease-susceptible individuals by putative "virulent" genetic variants and the subsequent transmission of these variants to other disease-susceptible family members.

If there is a relationship between certain virulence phenotypes and genetic identity of the RFLP groups of *A. actinomycetemcomitans*, then probes specifying putative virulence genes may prove useful for subgrouping clinical isolates of the bacterium. At the present time, the *lkt* genes are the only putative virulence genes for which sequence data are available (10, 12, 14, 15). Published evidence, based on the examination of two leukotoxin-positive, one leukotoxin-variable, and two leukotoxin-negative strains, suggested that the leukotoxin gene complex of *A. actinomycetemcomitans* JP2 is distributed throughout the species (27). However, one major difference between strains that produce the active toxin and those lacking it appears to be the absence and presence, respectively, of a *Hind*III site located approximately 300 bp upstream of the *lktC* gene (27). Taking advantage of this difference, we examined representative members of each of the RFLP groups for the presence of this variable *Hind*III site. The results clearly showed that the group II strains, in general, were similar to strain JP2 (leukotoxin positive), because they lacked the extra *Hind*III site. In contrast, representative members of the remaining RFLP groups were similar to strains FDC Y4 (leukotoxin positive), ATCC 29523 (leukotoxin variable), and ATCC 33384 and SUNYAB 67 (both leukotoxin negative). The results of the hybridization experiments suggest that RFLP group II variants have a unique feature specified by the DNA sequence in the noncoding region upstream of the *lkt* gene complex. When 12 RFLP group II variants were examined by hybridization, 9 of the variants lacked the extra *Hind*III site. Experiments are under way to determine whether there is a difference in the expression of the leukotoxin among RFLP group II variants that are differentiated by the oligonucleotide probe. This genetic heterogeneity among the RFLP group II isolates suggests that the oligonucleotide probe may be useful for subgrouping this particular RFLP group. It is possible that mutations that result in either the creation or loss of the *Hind*III site alter expression of the *lktA* gene or transport of the gene product to the cell surface. The *Hind*III site in question is located within the theoretical transcriptional control region of the *lkt* gene complex (14) and near the proposed start sites of the 9.3- and 4.3-kb mRNA transcripts (27). Therefore, this polymorphism among RFLP groups may contribute to differences in the virulence potentials of clinical isolates of the bacterium isolated from diseased and healthy subjects.

The rare occurrence of plasmids in *A. actinomycetemcomitans* (2 of 39 strains examined) as discussed by LeBlanc et al. (16) suggests that existing plasmid sequences would not be useful, as DNA probes, for subgrouping clinical isolates. However, Madison and LeBlanc (18) showed that homologous sequences of the recently identified *A. actinomycetem-*

comitans plasmid pVT745 resided in the chromosomes of 15 strains of the bacterium obtained from geographically diverse sources. These data suggested that the integrated plasmid sequences could be potentially useful for subgrouping clinical isolates of *A. actinomycetemcomitans*. We found a much lower incidence of integrated plasmid sequences in representative members of the RFLP groups found in the LJP family population. However, identical hybridization patterns were obtained with the only two members of RFLP group I identified to date. In addition, one of the few strains that showed positive hybridization with the pVT745 probe was a group XIII strain. RFLP groups I and XIII are very similar, on the basis of hybridizations with the 4.7-kb probe, since they differ only in the size of fragment E. Additional isolates representative of group I have to be identified to determine whether the integrated plasmid sequences are characteristic of this RFLP group. The existence of identical integrated plasmid fragments in FDC Y4 and ATCC 29523 strengthens the possibility that these strains are closely related and belong in the same genetic cluster group. Our analyses showed that RFLP groups I, III, VII, XIII, and XV constitute a closely related cluster in the species.

In conclusion, a defined set of 13 genetic cluster (RFLP) groups of *A. actinomycetemcomitans* were identified in a well characterized regional population of LJP family subjects and healthy control children. This study is the first to show the genetic relationships of clinical isolates of *A. actinomycetemcomitans* obtained from a large, well characterized LJP population. The ability to identify genetic variants of *A. actinomycetemcomitans* that may preferentially colonize diseased and healthy subjects will facilitate the study of the role of this important pathogen in periodontal diseases.

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