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, V, 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 HindIII 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 cariophilic, 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 were 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 identifi-
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-agar. Bacteria were grown for 24 h at 37°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 I 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

![Fig. 1. Hybridization patterns of isolates representative of the 13 RFLP cluster groups (I to XIV) identified for members of LJP families and healthy control subjects. Lanes I and XV, DNA from reference strains FDC Y4 and SX48, respectively.](http://jcm.asm.org/)

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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 I, II, IV, 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 HindIII 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 HindIII 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 HindIII 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 HindIII 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 chromosomal 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 HindIII 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 HindIII site. Chromosomal DNA from members of each of the RFLP groups was digested to completion, with HindIII, for Southern blot analysis. A conserved HindIII 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

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

<table>
<thead>
<tr>
<th>RFLP group</th>
<th>No. of isolates from:</th>
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<tr>
<td></td>
<td>LJP subjectsa</td>
</tr>
<tr>
<td>I</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>186</td>
</tr>
<tr>
<td>III</td>
<td>10</td>
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<tr>
<td>IV</td>
<td>133</td>
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<td>V</td>
<td>220</td>
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<td>XIII</td>
<td>5</td>
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<tr>
<td>XIV</td>
<td>0</td>
</tr>
<tr>
<td>XVd</td>
<td>0</td>
</tr>
</tbody>
</table>

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\[ a \] 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.

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**FIG. 2. Conserved fraction of probe-homologous HindIII restriction fragments between pairs of isolates representative of the RFLP groups.** The fraction of fragments conserved between two RFLP groups was calculated as \( \frac{a}{a + y} \), 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 (15). Relationships were determined by using the 4.7-kb probe from strain FDC Y4.
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
onset of the disease. The RFLP groups were identified by using a defined 4.7-kb EcoRI 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), 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 Zambon 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-negative, 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. actinomycetemcomitans* 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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