NIH Public Access
Author Manuscript
J Periodontol. Author manuscript; available in PMC 2012 December 17.

Published in final edited form as:
J Periodontol. 1998 September ; 69(9): 998–1007.

Relationship Between Conversion of Localized Juvenile Periodontitis-Susceptible Children From Health to Disease and *Actinobacillus actinomycetemcomitans* Leukotoxin Promoter Structure*

Lina C. Bueno, **Marcia P.A. Mayer**, and **Joseph M. DiRienzo**

Abstract

The periodontal pathogen *Actinobacillus actinomycetemcomitans* produces a leukotoxin that is considered a primary virulence factor in localized juvenile Periodontitis (LJP). Select strains of the bacterium contain a 530-bp deletion in the promoter region of the leukotoxin gene operon which results in enhanced transcription of the leukotoxin. DNA hybridization and polymerase chain reaction (PCR) were used to examine genetic variants of *A. actinomycetemcomitans* in 24 LJP-susceptible children from 21 families having a history of the disease and 34 control children from non-LJP families. A significant association was found between the detection of variants that had a deletion in the leukotoxin promoter region, indicative of a high level expression leukotoxin genotype, and conversion from a healthy periodontal status to disease. Subjects harboring *A. actinomycetemcomitans* of this genotype were more likely to convert to LJP than those subjects who had variants containing the full length leukotoxin promoter region (odds ratio = 22.50, 95% C.I.). These findings support the concept that highly virulent strains or clonal types of periodontal pathogens play a major role in the initiation of periodontal disease in susceptible hosts.

Keywords

Periodontitis; juvenile/epidemiology; *Actinobacillus actinomycetemcomitans*; leukotoxin; epidemiology; polymerase chain reaction; polymorphism; restriction fragment length

Localized juvenile Periodontitis (LJP) is a particularly aggressive form of periodontal disease that causes rapid periodontal destruction in juveniles and young adults. The facultative Gram-negative bacterium *Actinobacillus actinomycetemcomitans* is considered to be the primary pathogen in this disease.[1–3] Clonal analysis of this species has resulted in the identification of six major lineages or electrophoretic type (ET) divisions.[4] One of the major virulence determinants produced by this bacterium is a leukotoxin which is specifically cytotoxic for neutrophils and macrophages.[5–7] The leukotoxin is part of an operon that is highly conserved among the 6 ET divisions.[8,9] A deletion in the promoter region of the leukotoxin gene operon, found in a relatively small percentage of strains, results in a significant increase in leukotoxin expression.[10] These “high leukotoxin-producing” strains appear to cluster in a single ET division.[4]

The world-wide distribution of the high leukotoxin-producing strains has been a subject of great interest. Haubek et al.[11] screened 148 isolates of *A. actinomycetemcomitans* from

*Department of Microbiology, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA.

Send reprint requests to: Dr. Joseph DiRienzo, Department of Microbiology, University of Pennsylvania School of Dental Medicine, 4001 Spruce St., Philadelphia, PA 19104-6002. Fax: 215/898-8385. dirienzo@pobox.upenn.edu..

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Scandinavian and northern European patients and failed to identify high leukotoxin-producing strains. Brogan and coworkers[10] found that only 3 of 17 strains examined produced high levels of leukotoxin. Zambon and coworkers[12] found that the high leukotoxin-producing strain was detected in just over half of 21 LJP patients from a North American population. Haubek et al.[13] found that 11 of 17 subjects of African ancestry had the high leukotoxin-producing strains and proposed that strains of this genotype may cluster in people of African origin.

It is likely that the reason for the differences in these studies is related to differences in the distribution of the high leukotoxin-producing strains in various regions of the world. Most studies to date have been cross-sectional associations of the virulent genotype with disease. To investigate the hypothesis that the virulent genotypes of *A. actinomycetemcomitans* are causal, a longitudinal study of a well characterized geographically homogeneous population of LJP-susceptible children was undertaken. Using restriction fragment length polymorphism (RFLP) analysis of *A. actinomycetemcomitans* isolated from members of these families, we showed that a single genetic variant, designated RFLP group II, correlated with the conversion of LJP-susceptible children from a healthy to diseased periodontal status.[14] In addition, we found that isolates containing the deletion in the leukotoxin promoter region were present in some of these subjects.[15] Since the subjects in our longitudinal study were young children from predominantly African-American families, this population was ideally structured to address the question of a possible correlation between the presence of high leukotoxin-producing strains of *A. actinomycetemcomitans* and LJP. In the present study we have used DNA hybridization and PCR to characterize the leukotoxin promoter region in *A. actinomycetemcomitans* isolated from the longitudinal study subjects. We provide clinical evidence that strains of *A. actinomycetemcomitans* that exhibit the high leukotoxin-producing genotype prevail in young members of LJP families who convert from a healthy periodontal status to disease.

MATERIALS AND METHODS

Subject Population

The patient population examined in this study consisted of 21 families that contained at least one member, child or adult, with clinical signs of 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. Twenty-seven siblings (mean age 10.2 years at the time of enrollment) who had no evidence of LJP when accessioned into the study were defined as the LJP-susceptible subjects during the time that they were between the ages of 10 and 16 years. These subjects were deemed at risk for LJP because the disease appears to be subject to familial inheritance.[16,17] Thirty-five subjects (mean age 10.3 years at the time of enrollment), matched by age, gender, and race to the LJP-susceptible children, were recruited from families with no prior history of LJP and served as the control group in the longitudinal study.

The change in the periodontal status, from health to disease, that occurred during the course of the study was defined as a conversion. The subject in each family who had active LJP at accession or a documented history of the disease was defined as the proband. The clinical evaluation of the subjects, description of the criteria used to classify the conversion of a subject from a healthy to diseased periodontal status, microbiological sampling methods, and results of RFLP analyses were described previously.[14] Briefly, the plaque and gingival indexes; eruption status; decayed, missing and filled teeth; probing depth, attachment level from cemento-enamel junction (CEJ); and percentage of sites which bled on probing were collected as each subject entered the study and every 3 months thereafter from the LJP-susceptible and control children. Adults were monitored once a year. Radiographic films were taken at the time of accession and at yearly intervals. Bone loss was assessed by

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measuring the greatest distance between the most coronal level of the alveolar bone crest and the deepest part of the bony defect in over-laid tracings taken from the baseline and subsequent radiographs.[18] Interim radiographs were taken during active periodontal inflammation. Conversions were defined as an increase of ≥ 2 mm above the baseline value, in the distance from the CEJ to the bottom of the periodontal pocket or an increase of at least 2 mm in probing depth. This change, occurring over a 3-month interval, had to coincide with a loss of attachment ≥ 1 mm above the baseline value. Bone loss was also checked at the time of surgery as supporting evidence of periodontal destruction. Over the course of the 5-year longitudinal study 9 LJP-susceptible children (mean age 12.8 years) and 3 control children (mean age 14.7 years) converted from a healthy to a diseased periodontal state.[14]

Subgingival microbiological samples were obtained every 3 months from 24 and 34 of the LJP-susceptible and control children, respectively. These samples were obtained from the mesial surfaces of the 4 permanent first molars, the maxillary first premolars, and any additional sites that converted from a healthy to diseased state during the study. Supragingival plaque was removed with sterile cotton pellets and subgingingival plaque was collected by inserting 3 sterile paper points to the depth of the periodontal pocket. The subgingival samples were placed in prereduced VMGA III anaerobic transport medium.[19] *Actinobacillus actinomycetemcomitans* was isolated on trypticase soy-serum-bacitracin-vancomycin (TSBV) agar.[20] The isolates were identified according to conventional colony morphology and biochemical criteria and by DNA hybridization.

Human use protocols were approved by the University of Pennsylvania Committee on Studies Involving Human Beings (assurance M1025).

Leukotoxin Promoter Deletion Assays

Total DNA was prepared from *A. actinomycetemcomitans* isolates according to standard methods.[21] A representative isolate of each RFLP type, that was found in each family member that had *A. actinomycetemcomitans*, was used. Microbial analysis was limited in this manner because we have previously found that multiple isolates exhibiting the same RFLP type were often replicates of the same genetic variant or genotype.[14,15] The RFLP types were previously identified based on the hybridization of a 4.7 kb *Eco*RI chromosomal DNA fragment, cloned from strain FDC Y4, to total DNA.[15]

To identify the leukotoxin promoter deletion by DNA hybridization, approximately 1 μg of total DNA from each isolate was digested to completion with *Hin*dIII, according to the manufacturer's instructions, and the restriction fragments were separated and analyzed on Southern blots. Hybridizations were performed with an oligonucleotide 5′-GGCAGTGTAGTAGTTGCCAT-3′ (LKTA), homologous to the 5′ end of the *lktA* gene (Fig. 1A), to detect restriction site polymorphism in the upstream non-coding region of the leukotoxin promoter.[15] The probe was end labeled with [γ-^{32}P]ATP (>5,000 Ci/mmol) as previously described.[22] To obtain molecular size markers on the autoradiograms, bacteriophage λ DNA was digested to completion with *Hind*III and run on the Southern blots. A portion of the λ DNA sample was labeled by nick translation and used as a hybridization probe (5×10^6 cpm/blot). Hybridizations were performed as described previously.[15] A single hybridization-positive fragment of approximately 8.4 or 2.3 kb was observed if the *A. actinomycetemcomitans* isolate contained or lacked, respectively, the leukotoxin promoter deletion (Fig. 1A). This heterogeneity was due to a *Hind*III recognition site that resided within the sequence marked by the deletion.

The PCR was also used to identify the leukotoxin promoter deletion. Approximately 100 pg of total DNA and 100 pmol of the primers 5′-GGAGTCGACTTGAGAAATATGACAGT-3′ (LKTP-1) and 5′-

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GGCGAATTCTCTATGCAAAGGAGAAT-3′ (LKTP-2) (Nucleic Acid Facility, University of Pennsylvania) were used. The primers flank the promoter region and are homologous to positions −1002 to −1018 and +56 to +72, respectively, in the leukotoxin operon (position +1 is the first base of the *lktC* start codon) (Fig. 1B). Recognition sites (underlined bases) for the restriction endonucleases *SalI* and *Eco*RI were incorporated into LKTP-1 and LKTP-2, respectively, to facilitate future cloning experiments. PCR reactions were performed in 50 or 100 μl volumes using a PCR reagent kit[†] and DNA Polymerase.[‡] Reagent concentrations used were the same as recommended in the manufacturer's instructions. Reactions were run in a DNA thermal cycler.[§] The samples were heated at 97°C for 4 minutes and amplifications were performed at 95°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute for 25 cycles. The amplified products were extended at 72°C for 10 minutes. Twenty μl of the amplified products were analyzed on 1% agarose gels in 0.5× TBE buffer [1×x TBE buffer contains 89 mM Tris base, 89 mM boric acid and 2.5 mM EDTA (pH 8.3)] and were detected by staining with 0.5 μg/ml of ethidium bromide. Lambda DNA digested with *Hind*III and *Hind*III/*Eco*RI was run on the gels as molecular size standards. A single PCR product of approximately 560 or 1050 bp was observed if the isolate contained or lacked, respectively, the leukotoxin promoter deletion (Fig. 1B).

Statistical Methods

A total of 58 subjects, 24 LJP-susceptible and 34 control, were analyzed. The Fisher's exact test was used to evaluate differences in distributions between LJP-susceptible subjects and control subjects who converted to LJP and those who remained healthy. This test was also used to assess differences in distribution between the families that had LJP-susceptible children convert to LJP during the course of the study and those families that did not. Odds ratios were calculated to evaluate the risk that subjects containing the high leukotoxin-producing variant would convert from a healthy to diseased periodontal status.[23]

RESULTS

The first objective was to establish if an association was evident between the detection of *A. actinomycetemcomitans* and conversion from a healthy to diseased periodontal status in a geographically homogeneous LJP family population. To more clearly present the data from these analyses, the 21 LJP families used in the study were divided into two categories. The first category, listed in Table 1, contained members of the 7 LJP families that had at least one child convert from a healthy to diseased periodontal state. The second category, listed in Table 2, contained all members of the 14 LJP families in which no child converted from health to disease. *A. actinomycetemcomitans* was detected in at least one member of each of the conversion subject families included in Table 1. The bacterium was not detected in members of 3 of the families shown in Table 2. Differences between families that had at least one new case of LJP (Table 1) and those that did not (Table 2), with respect to the detection of *A. actinomycetemcomitans*, were not statistically significant.

The 35 children who served as control subjects from non-LJP families are listed in Table 3. The presence or absence of *A. actinomycetemcomitans* could not be confirmed in one of the control subjects. Likewise, 3 of the LJP-susceptible subjects were not available for microbiological sampling. Therefore, among the 24 LJP-susceptible subjects (Tables 1 and 2) and 34 control subjects (Table 3) available for study, 12 subjects developed disease (9 LJP-susceptible and 3 control children). *Actinobacillus actinomycetemcomitans* was detected in 30 of the 58 children (Table 4). There were no statistically significant differences

[†]GeneAmp PCR Reagent Kit, Perkin Elmer Cetus, Norwalk, CT.
[‡]Amplitaq, Perkin Elmer Cetus.
[§]Perkin Elmer Cetus.

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between the proportion of subjects who developed LJP and those that did not, with respect to the detection of the bacterium either in LJP-susceptible subjects, the control group or the total sample (Table 5).

Since there was no association between the presence of *A. actinomycetemcomitans* and the conversion of a subject from a healthy to LJP status, at either the family or subject level, we proceeded to determine if an association existed between specific genotypes of the bacterium and the development of disease. DNA hybridization and PCR were used to distinguish between *A. actinomycetemcomitans* isolates containing and lacking a deletion in the promoter region of the leukotoxin operon representative of high and low leukotoxin-producing genotypes, respectively. The results are shown in Tables 1 through 3. Our early experiments employed the DNA hybridization method since a strain variable *Hind*III site in the leukotoxin locus had been reported.[3] Later investigations showed that this *Hind*III site was variable because it resided within the region encompassed by the leukotoxin promoter deletion. This meant that PCR could be used as the primary method for the analysis of high leukotoxin-producing strains.

The genetic variant containing the leukotoxin promoter region deletion was found in members of 10 of 21 LJP families as evidenced by a 8.4 kb hybridization-positive DNA fragment and a 560 bp PCR product (Tables 1 and 2). Five of these families had at least one LJP-susceptible child convert to disease. Two of 11 LJP families that did not have members infected with *A. actinomycetemcomitans* containing the leukotoxin promoter deletion had a member who converted to LJP.

Eight of 24 LJP-susceptible subjects and none of the 34 control subjects had *A. actinomycetemcomitans* with the leukotoxin promoter deletion genotype. Six of these 8 subjects converted to disease during the study period. Three LJP-susceptible and 3 control children who did not have genetic variants containing the leukotoxin promoter region deletion converted from a healthy to periodontally diseased status. There was a statistically significant association between the detection of genetic variants with the promoter deletion and conversion to LJP among the LJP-susceptible subjects as well as in the total sample (Table 6). Those subjects that had *A. actinomycetemcomitans* with the leukotoxin promoter deletion were at higher risk to develop LJP (odds ratio = 22.5).

Examination of *A. actinomycetemcomitans* isolates from the probands of the LJP families revealed that among 22 probands (2 probands in family 2), 16 had the bacterium and 7 of these contained the genotype with the leukotoxin promoter deletion (Table 4). Two of these 7 probands resided in LJP families that had an LJP-susceptible child convert from a healthy to diseased periodontal status. No significant association between the presence of the high leukotoxin-producing variant in the proband and conversion to disease of a LJP-susceptible sibling was observed ($P > 0.05$).

Seventeen of the 21 LJP families studied were African-American. This high proportion of African-American subjects in our study population precluded a statistical determination of an association between the presence of the high leukotoxin-producing genotype and disease on the basis of race. All 7 LJP families that had a conversion subject were African-American (Table 1). Eight of 46 African-American subjects harbored the promoter deletion variant and 6 of these 8 subjects developed LJP. In addition, 4 of 37 subjects who did not contain the promoter deletion variant, also converted to a diseased status. Two of the control subjects who converted to LJP did not have cultivable *A. actinomycetemcomitans* and were of undetermined ancestry (Table 3). The third control subject who converted to disease contained a genetic variant with the full length promoter region and was African-American.

Without exception, the *A. actinomycetemcomitans* isolates that exhibited the leukotoxin promoter deletion were members of RFLP group II (Tables 1 and 2). This genetic variant was not found in any of the control subjects (Table 3). Fifteen of 18 of the RFLP group II isolates contained the promoter deletion. The remaining 3 isolates that had the full length promoter region were obtained from the proband and each of two LJP-susceptible children in the same non-conversion LJP family (family 20).

DISCUSSION

We have shown that isolates of *A. actinomycetemcomitans* that have a deletion in the promoter region of the leukotoxin operon are prevalent in young LJP-susceptible children who are members of LJP families. In total, 15 subjects in 10 African-American families harbored this specific genotype and 6 of these were children who converted from a healthy to diseased periodontal status. Eight of the 15 subjects were probands infected with the specific promoter deletion genotype. This demonstrates a relationship between the leukotoxin promoter deletion and disease even in the non-conversion LJP families.

Actinobacillus actinomycetemcomitans was detected in 8 of 9 LJP-susceptible subjects who converted to LJP during the study (89%) and in 1 of 3 control subjects who exhibited attachment loss. However, these conversions in the control subjects appeared to be atypical since they occurred at a later age than those in the LJP-susceptible subjects.[14] Despite the association between *A. actinomycetemcomitans* and LJP reported by others, and as shown here, there was no statistical association between conversion to disease and the presence of the bacterium.[3,24,25] This outcome was most likely due to the frequent isolation of *A. actinomycetemcomitans* from the healthy subjects. Nine of 15 healthy subjects belonging to LJP families (60%) and 12 of 31 healthy control subjects (38.7%) harbored the bacterium. The percentage of healthy subjects positive for *A. actinomycetemcomitans* in this study was higher than that reported previously.[3,24,26] This may have been the result of higher sampling frequencies which increased the chances of detecting the bacterium. Several hypotheses, such as increased host resistance, presence of a neutralizing microflora,[26] or presence of a less virulent strain, could explain the presence of *A. actinomycetemcomitans* in healthy subjects.

There was a statistically significant association between the detection of the high leukotoxin-producing genotype of *A. actinomycetemcomitans* and the conversion of an LJP-susceptible subject from health to disease. These data indicate that the high leukotoxin-producing genotype of the species has the potential to contribute to the virulence of this genetic variant. It was also observed that the genetic variant with the leukotoxin promoter deletion was detected in only 2 of the probands in the 7 LJP families that had a child convert to disease (families 10 and 21). However, 6 of the 9 conversion subjects from the LJP families harbored this genotype. This difference may have been due to the sampling frequency. Probands were sampled only once a year while the LJP-susceptible subjects were sampled quarterly.[14] Also, some of the probands were adults and had been treated for LJP prior to their entry into the study. Treatment may have reduced numbers of *A. actinomycetemcomitans* in these subjects. The risk of acquiring this bacterium may be even higher in subjects belonging to LJP families in which no member has been treated for the disease.

The virulence potential of the high leukotoxin-producing variant of *A. actinomycetemcomitans* may not be due solely to the enhanced expression of the leukotoxin genes. We have shown previously that variants of a single specific RFLP group (II), out of 14 different groups, have a statistically significant correlation with the development of LJP in the same subject population.[14,15] While it is clear that the high leukotoxin-producing

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variants are unique members of RFLP group II, it is likely that additional genotypic differences account for the correlation between the members of this RFLP group and LJP. Increased expression of other potential vimlence genes, in addition to the leukotoxin, by the RFLP group II variants may contribute to the association between this variant and conversion from health to disease.

Our results are consistent with those of Zambon et al.[12] who found that 57% of LJP subjects contained the high leukotoxin-producing variant while adult Periodontitis and periodontally healthy subjects lacked this variant. Also, there was a significantly higher prevalence of the high leukotoxin-producing strains in our study population than in those examined by Haubek et al.[11] These investigators reported that strains that have the leukotoxin promoter deletion are rare. Their conclusion was based on the analysis of a collection of 88 isolates from Finnish dental patients and over 60 strains obtained from northern European patients. However, the strains they examined originated from subjects of varied clinical status including a significant number of healthy individuals and adult Periodontitis patients. Brogan and coworkers[10] showed that two strains obtained from the Haubek et al.[11] group were highly leukotoxic. Presumably, these strains were of northern European origin. More recently, Haubek et al.[13] reported a high prevalence of leukotoxin-producing strains in subjects originating from the Cape Verde Islands, Morocco, and Algeria. Our data are consistent with these findings and support epidemiological studies promoting a higher prevalence of LJP in people of African ancestry than in peoples of other origins.[27–29] Since our study population was composed of predominantly African-American families, we cannot show a statistical correlation between the high leukotoxin-producing genotype and LJP based on race. All other races were drastically underrepresented in the study population.

Studies reporting the relatively high prevalence of LJP in peoples of African origins, the data presented by Haubek et al.[13] and the results of our investigation suggest that the detection of the leukotoxin promoter deletion by PCR may be a useful diagnostic test for the identification of disease risk subjects in specific highly homogeneous patient populations.

Acknowledgments

We thank Terry McKay for providing able technical assistance during the early stages of the study and Fred Correia for critically reading the manuscript. Dr. Marcia Mayer was supported by grant 1996-3328-0 from the Fundação de Auxilio a Pesquisa de Estado de São Paulo, Brazil. This study was supported by a University of Pennsylvania Research Foundation award and National Institutes of Health grant DE/OD10891.

REFERENCES

1. Moore WEC. Microbiology of periodontal disease. J Periodont Res. 1987; 22:335–341. [PubMed: 2961863]
2. Slots J, Zambon JJ, Rosling BG, Reynolds HS, Christersson LA, Genco RJ. *Actinobacillus actinomycetemcomitans* in human periodontal disease: Association, serology, leukotoxicity, and treatment. J Periodont Res. 1982; 17:447–455. [PubMed: 6218267]
3. Zambon JJ, Christersson LA, Slots J. *Actinobacillus actinomycetemcomitans* in human periodontal disease: Prevalence in patient groups and distribution of biotypes and serotypes within families. J Periodontol. 1983; 54:707–111. [PubMed: 6358452]
4. Poulsen K, Theilade E, Lally ET, Demuth DR, Kilian M. Population structure of *Actinobacillus actinomycetemcomitans*: a framework for studies of disease-associated properties. Microbiol. 1994; 140:2049–2060.
5. Taichman, NS.; McArthur, WR.; Tsai, C-C., et al. Leukocidal mechanisms of *Actinobacillus actinomycetemcomitans*.. In: Genco, RJ.; Mergenhagen, SE., editors. Host-Parasite Interactions in Periodontal Diseases. American Society for Microbiology; Washington, DC: 1982. p. 261-269.

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6. Taichman NS, Simpson DL, Sakurada S, Cranfield M, DiRienzo J, Slots J. Comparative studies on the biology of *Actinobacillus actinomycetemcomitans* leukotoxin in primates. Oral Microbiol Immunol. 1987; 2:97–104. [PubMed: 3507626]

7. Tsai C-C, Taichman NS. Dynamics of infection by leucotoxic strains of *A. actinomycetemcomitans* in juvenile Periodontitis. J Clin Periodontol. 1986; 11:330–331. [PubMed: 3458728]

8. Kraig E, Dailey Kolodrubetz D. Nucleotide sequence of the leukotoxin gene from *Actinobacillus actinomycetemcomitans*: homology to the alpha-hemolysin/leukotoxin gene family. Infect Immun. 1990; 58:920–929. [PubMed: 2318535]

9. Lally ET, Golub EE, Kieba IR, et al. Analysis of the *Actinobacillus actinomycetemcomitans* leukotoxin gene. J Biol Chem. 1989; 264:15451–15456. [PubMed: 2670940]

10. Brogan JM, Lally ET, Poulsen K, Kilian M, Demuth DR. Regulation of *Actinobacillus actinomycetemcomitans* leukotoxin expression: analysis of the promoter regions of leukotoxic and minimally leukotoxic strains. Infect Immun. 1994; 62:501–508. [PubMed: 8300209]

11. Haubek D, Poulsen K, Asikainen S, Kilian M. Evidence for absence in northern Europe of especially virulent clonai types of *Actinobacillus actinomycetemcomitans*. J Clin Microbiol. 1995; 33:395–401. [PubMed: 7714199]

12. Zambon JJ, Haraszthy VI, Harihara G, Lally ET, Demuth DR. The microbiology of early-onset Periodontitis: Association of highly toxic *Actinobacillus actinomycetemcomitans* strains with localized juvenile Periodontitis. J Periodontol. 1996; 67(Suppl.):282–290.

13. Haubek D, Poulsen K, Westergaard J, Dahlèn G, Kilian M. Highly toxic clone of *A. actinomycetemcomitans* in geographically widespread cases of juvenile Periodontitis in adolescents of African origin. J Clin Microbiol. 1996; 34:1576–1578. [PubMed: 8735124]

14. DiRienzo JM, Slots J, Sixou M, Sol M-A, Harmon R, McKay TL. Specific genetic variants of *A. actinomycetemcomitans* correlate with disease and health in a regional population of localized juvenile Periodontitis families. Infect Immun. 1994; 62:3058–3065. [PubMed: 7913695]

15. DiRienzo JM, McKay TL. Identification and characterization of genetic cluster groups of *A. actinomycetemcomitans* isolated from the human oral cavity. J Clin Microbiol. 1994; 32:75–81. [PubMed: 7907346]

16. Beaty TH, Boughman JA, Yang R, Astemborski JA, Suzuki JB. Genetic analysis of juvenile Periodontitis in families ascertained through an affected proband. Am J Hum Genet. 1987; 40:443–452. [PubMed: 3578282]

17. Saxén L. Juvenile Periodontitis. J Clin Periodontol. 1980; 7:1–19. [PubMed: 6988466]

18. Rosling B, Nyman S, Lindhe J. The effect of systemic plaque control on bone regeneration in infrabony pockets. J Clin Periodontol. 1976; 3:38–53. [PubMed: 767366]

19. Möller ARJ. Microbial examination of root canals and periapical tissues of human teeth. Odontol Tidskr. 1966; 74:1–38. [PubMed: 5335186]

20. Slots J. Selective medium for isolation of *Actinobacillus actinomycetemcomitans*. J Clin Microbiol. 1982; 15:606–609. [PubMed: 7068837]

21. Stull TL, LiPuma JJ, Edlind TD. A broad-spectrum probe for the molecular epidemiology of bacteria: ribosomal RNA. J Infect Dis. 1988; 167:280–286. [PubMed: 2447202]

22. Sambrook, J.; Fritsch, EF.; Maniatis, T. Molecular Cloning: a Laboratory Manual. 2nd ed. Vol. 2. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 1989. p. 11.31-11.34.

23. Greenberg, RS.; Daniels, SR.; Flanders, WD.; Eley, JW.; Boring, JR. Medical Epidemiology. 2nd ed. Appleton & Lange; Norwalk CT: 1996.

24. Genco RJ, Zambon JJ, Christersson LA. Use and interpretation of microbiological assays in periodontal diseases. Oral Microbiol Immunol. 1986; 1:73–79. [PubMed: 3295682]

25. Asikainen S, Jousimes-Somer H, Kanervo A, Summanen P. Certain bacterial species and morphotypes in localized juvenile Periodontitis and in matched controls. J Periodontol. 1987; 58:224–230. [PubMed: 3473219]

26. Asikainen S, Alaluusua S, Kleemola-Kujala E. A 2-year follow-up on the clinical and microbiological condition of periodontium in teenagers. J Clin Periodontol. 1991; 18:16–19. [PubMed: 2045514]

27. Harley AF, Floyd PD. Prevalence of juvenile Periodontitis in school-children in Lagos, Nigeria. Community Dent Oral Epidemiol. 1988; 16:299–301. [PubMed: 3263253]

28. Löe H, Brown LJ. Early-onset Periodontitis in the United States of America. J Periodontol. 1991; 62:608–616. [PubMed: 1770420]

29. Saxby M. Prevalence of juvenile Periodontitis in a British school population. Community Dent Oral Epidemiol. 1984; 12:185–187. [PubMed: 6589110]

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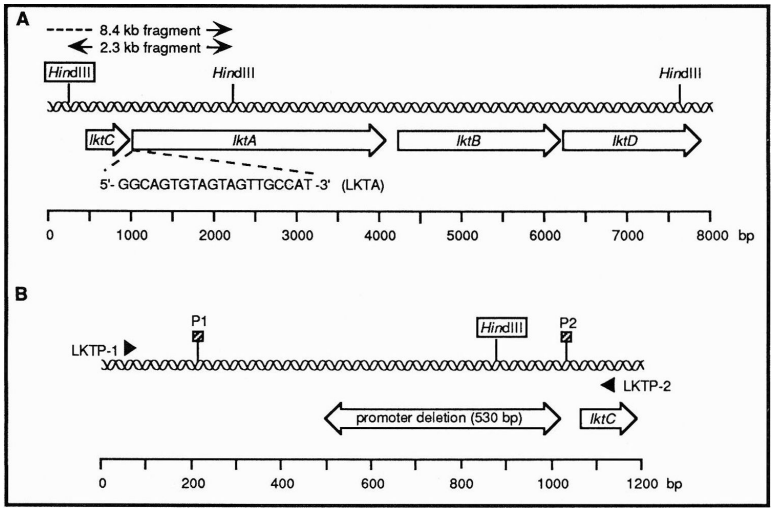

Figure 1.

DNA hybridization probe and PCR primers used in this study. (**A**) Physical map of the leukotoxin operon showing the location of the hybridization probe sequence (LKTA). The boxed HindIII site is present (2.3 kb hybridization-positive fragment) or absent (8.4 kb hybridization-positive fragment) in low or high leukotoxin-producing strains, respectively. Information for the construction of the map was obtained from sequences deposited in GenBank (accession numbers X16829, X53955, and X53956). (**B**) Physical map of the leukotoxin promoter region. The portion of the sequence missing in high leukotoxin-producing strains is indicated by the double-headed arrow. The binding sites of the PCR primers, LKTP-1 and LKTP-2, used to detect the deleted region, are shown. The boxed HindIII site is the same one designated in panel A. The proposed transcriptional start sites are labeled P1 and P2. Information for the construction of the map was obtained from reference 10.

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J Periodontol. Author manuscript; available in PMC 2012 December 17.

Table 1

Leukotoxin Promoter Deletion Analysis of Isolates of *A. actinomycetemcomitans* From LJP Families Having 1 or More Conversion Subjects

LJP Family (N = 7)	Subject (N = 27)	Familial Relation	Age at Baseline*	Race†	Clinical Status	RFLP of Isolate‡	Hybridization-positive lkt DNA Fragment (kb)§	lkt PCR Product (bp)‖
6	060025	Father	34	AA	NA	XI	2.3	1050
6	060026	Mother	34	AA	Proband	IV	ND	1050
6	060027¶	Son	13	AA	Conversion	IV	2.3	1050
6	060028¶	Son	9	AA	Healthy	IV	ND	1050
6	060029	Son	1	AA	Healthy	IV	ND	1050
7	070030	Mother	29	AA	Proband	None	NA	NA
7	070031¶	Daughter	12	AA	Conversion	II	8.4	560
7	070032¶	Daughter	6	AA	Healthy	None	NA	NA
7	070084¶	Son	<1	AA	Healthy	None	NA	NA
10	100039	Mother	25	AA	Proband	II	8.4	560
10	100040¶	Son	11	AA	Conversion	II	8.4	560
						V	2.3	1050
10	100054	Father	40	AA	NA	VI	2.3	1050
17	170070	Mother	34	AA	NA	ND	NA	NA
17	170072	Daughter	14	AA	Proband	None	NA	NA
17	170073¶	Son	11	AA	Conversion	None	NA	NA
17	170074¶	Son	9	AA	Conversion	II	8.4	560
18	180075	Mother	27	AA	Proband	V	ND	1050
18	180076¶	Son	10	AA	Conversion	V	ND	1050
21	210090	Mother	38	AA	NA	None	NA	NA
21	210091	Son	16	AA	Proband	II	8.4	560
21	210092¶	Son	12	AA	Conversion	II	8.4	560
						ND	NA	NA
23	230125	Mother	33	AA	NA	IX	ND	1050
23	230124	Daughter	9	AA	Proband	II	8.4	560

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LJP Family (N = 7)	Subject (N = 27)	Familial Relation	Age at Baseline*	Race†	Clinical Status	RFLP of Isolate‡	Hybridization-positive lkt DNA Fragment (kb)§	lkt PCR Product (bp)‖
23	230126¶	Son	9	AA	Conversion	VIII	2.3	1050
						II	8.4	560
23	230127¶	Daughter	11	AA	Conversion	IX	ND	1050
						VI	ND	1050
23	230128	Father	35	AA	NA	ND	NA	NA
23	230129	Daughter	18	AA	Healthy	None	NA	NA

*The time at which the subject entered the study is defined as the baseline.

†A, Asian; AA, African-American; C, Caucasian; O, other.

‡Determined on Southern blots using a 4.7 kb *EcoRI* DNA fragment cloned from strain Y4.[15] ND, not determined; these subjects were not included in the statistical analysis due to the lack of microbiological data.

§Determined by RFLP analysis using *Hin*dIII-digested DNA and an oligonucleotide (LKTA) homologous to the 5′-end of *lktA* gene. ND, not determined. NA, not applicable.

‖Amplified using primers LKTP-1 and LKTP-2.

¶LJP-susceptible subjects used in the statistical analysis (see Table 4).

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Table 2

Leukotoxin Promoter Deletion Analysis of Isolates of *A. actinomycetemcomitans* from LJP Families Having No Conversion Subjects

LJP Family (N = 14)	Subject (N = 56)	Familial Relation	Age at Baseline*	Race†	Clinical Status	RFLP of Isolate‡	Hybridization-positive lkt DNA Fragment (kb)§	lkt PCR Product (bp)‖
1	010001	Father	41	A	NA	V	ND	1050
1	010002	Mother	41	A	NA	III	ND	1050
						V	ND	1050
1	010003	Daughter	16	A	Proband	III	2.3	1050
						V	2.3	1050
1	010004¶	Son	13	A	Healthy	VII	2.3	1050
2	020005	Grandmother	31	AA	NA	None	NA	NA
2	020006	Daughter	16	AA	Proband	X	2.3	1050
2	020007	Son	15	AA	Proband	II	8.4	560
2	020008	Daughter	11	AA	Healthy	ND	NA	NA
2	020050	Son	<1	AA	Healthy	None	NA	NA
3	030009	Father	39	C	Proband	III	ND	1050
3	030010	Mother	36	C	NA	III	ND	1050
3	030011	Son	16	C	Healthy	None	NA	NA
3	030012	Son	12	C	Healthy	ND	NA	NA
4	040013	Grandmother	42	AA	NA	XI	ND	1050
4	040014	Daughter	25	AA	NA	None	NA	NA
4	040015	Granddaughter	3	AA	Healthy	IV	ND	1050
4	040016¶	Grandson	6	AA	Healthy	IV	ND	1050
						IX	ND	1050
4	040017	Daughter	25	AA	Proband	III	ND	1050
						XI	ND	1050
4	040018	Granddaughter	2	AA	Healthy	IV	ND	1050
4	040019	Grandson	<1	AA	Healthy	None	NA	NA
4	040020	Daughter	16	AA	Healthy	None	NA	NA
4	040021¶	Daughter	13	AA	Healthy	XI	ND	1050
5	050022	Mother	30	C	Proband	None	NA	NA

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LJP Family (N = 14)	Subject (N = 56)	Familial Relation	Age at Baseline*	Race†	Clinical Status	RFLP of Isolate‡	Hybridization-positive lkt DNA Fragment (kb)§	lkt PCR Product (bp)#
5	050023	Father	29	C	NA	None	NA	NA
5	050024¶	Daughter	8	C	Healthy	None	NA	NA
8	080033	Father	28	AA	Proband	XII	ND	1050
8	080034¶	Daughter	7	AA	Healthy	IV	ND	1050
						V	ND	1050
8	080056	Mother	30	AA	NA	IV	ND	1050
9	090035	Mother	47	AA	NA	None	NA	NA
9	090036¶	Daughter	15	AA	Healthy	None	NA	NA
9	090037¶	Son	12	AA	Healthy	None	NA	NA
9	090038	Son	18	AA	Proband	II	8.4	560
11	110041	Mother	36	AA	NA	None	NA	NA
11	110042	Daughter	16	AA	Proband	None	NA	NA
11	110043¶	Son	8	AA	Healthy	IV	ND	1050
12	120044	Mother	36	AA	NA	None	NA	NA
12	120045	Son	15	AA	Proband	II	8.4	560
12	120046	Son	8	AA	Healthy	ND	NA	NA
12	120047	Son	3	AA	Healthy	IV	ND	ND
13	130048	Mother	29	AA	Proband	None	NA	NA
13	130049¶	Son	8	AA	Healthy	None	NA	NA
13	130055	Aunt	27	AA	NA	None	NA	NA
14	140052	Son	14	AA	Proband	II	8.4	560
14	140053¶	Son	12	AA	Healthy	II	8.4	560
16	160066	Mother	39	C	Proband	None	NA	NA
16	160067	Father	37	C	NA	None	NA	NA
16	160068¶	Son	8	C	Healthy	None	NA	NA
16	160069	Son	3	C	Healthy	None	NA	NA
19	190077	Mother	31	AA	NA	XII	ND	1050
19	190078¶	Son	10	AA	Healthy	II	8.4	560

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LJP Family (N = 14)	Subject (N = 56)	Familial Relation	Age at Baseline*	Race†	Clinical Status	RFLP of Isolate‡	Hybridization-positive lkt DNA Fragment (kb)§	lkt PCR Product (bp)‖
						XII	2.3	1050
						XII	2.3	1050
19	190079	Daughter	14	AA	Proband	II	8.4	560
						XII	ND	1050
20	200085¶	Daughter	12	AA	Healthy	II	2.3	1050
20	200086	Grandmother	33	AA	NA	ND	NA	NA
20	200087	Daughter	3	AA	Healthy	II	2.3	1050
20	200088	Daughter	16	AA	Proband	II	2.3	1050
20	200089	Grandson	<1	AA	Healthy	IX	2.3	1050

*The time at which the subject entered the study is defined as the baseline.

†A, Asian; AA, African-American; C, Caucasian; O, other.

‡Determined on Southern blots using a 4.7 kb *EcoRI* DNA fragment cloned from strain Y4.[15] ND, not determined; these subjects were not included in the statistical analysis due to the lack of microbiological data.

§Determined by RFLP analysis using *Hin*dIII-digested DNA and an oligonucleotide (LKTA) homologous to the 5′-end of *lktA* gene. ND, not determined. NA, not applicable.

‖Amplified using primers LKTP-1 and LKTP-2.

¶LJP-susceptible subjects used in the statistical analysis (see Table 4).

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Table 3

Leukotoxin Promoter Deletion Analysis of Isolates of *A. actinomycetemcomitans* From Control Subjects

Subject (N = 35)	Gender	Age at Baseline*	Race†	Clinical Status	RFLP of Isolate‡	Hybridization-positive lkt DNA Fragment (kb)§	lkt PCR Product (bp)//
1020064	Female	11	AA	Healthy	XIII	2.3	1050
1020102	Female	11	AA	Healthy	XIII	2.3	1050
1030093	Male	11	C	Healthy	None	NA	NA
1030094	Male	11	C	Healthy	XIII	2.3	1050
1040133	Male	7	AA	Healthy	XIII	2.3	1050
1040123	Male	7	AA	Healthy	None	NA	NA
1060097	Male	13	O	Conversion	None	NA	NA
1060096	Male	8	O	Healthy	None	NA	NA
1070117	Female	12	AA	Healthy	None	NA	NA
1070099	Female	7	AA	Healthy	V	ND	1050
					XIII	2.3	1050
1070101	Female	6	AA	Healthy	None	NA	NA
1080081	Female	7	AA	Healthy	XIII	2.3	1050
1080113	Female	9	AA	Healthy	VI	ND	1050
					IX	ND	1050
1090063	Female	13	AA	Healthy	XII	ND	1050
					XIII	2.3	1050
					XIV	2.3	1050
1090100	Female	14	AA	Conversion	III	ND	1050
1090109	Female	14	AA	Healthy	None	NA	NA
1090111	Male	12	AA	Healthy	None	NA	NA
1100062	Male	11	AA	Healthy	None	NA	NA
1100119	Male	11	AA	Healthy	XII	ND	1050
1110112	Male	8	AA	Healthy	XII	ND	1050
					XIII	2.3	1050
1120114	Male	8	AA	Healthy	ND	NA	NA
1130107	Male	9	AA	Healthy	XIII	2.3	1050
1140098	Male	14	O	Conversion	None	NA	NA

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Subject (N = 35)	Gender	Age at Baseline*	Race†	Clinical Status	RFLP of Isolate‡	Hybridization-positive lkt DNA Fragment (kb)§	lkt PCR Product (bp)‖
1140108	Male	12	AA	Healthy	XIV	2.3	1050
1160095	Male	7	C	Healthy	None	NA	NA
1160122	Male	9	C	Healthy	None	NA	NA
1170110	Male	11	AA	Healthy	None	NA	NA
1170120	Male	9	O	Healthy	None	NA	NA
1170134	Male	10	AA	Healthy	None	NA	NA
1180115	Male	ND	AA	Healthy	None	NA	NA
1190121	Male	11	AA	Healthy	None	NA	NA
1200131	Female	12	AA	Healthy	None	NA	NA
1210118	Male	13	AA	Healthy	None	NA	NA
1230132	Male	10	AA	Healthy	None	NA	NA
1230130	Female	11	O	Healthy	None	NA	NA

*The time at which the subject entered the study is defined as the baseline.

†A, Asian; AA, African-American; C, Caucasian; O, other.

‡Determined on Southern blots using a 4.7 kb *EcoRI* DNA fragment cloned from strain Y4.[15] ND, not determined; these subjects were not included in the statistical analysis due to the lack of microbiological data.

§Determined by RFLP analysis using *Hin*dIII-digested DNA and an oligonucleotide (LKTA) homologous to the 5′ -end of *lktA* gene. ND, not determined. NA, not applicable.

‖Amplified using primers LKTP-1 and LKTP-2.

¶LJP-susceptible subjects used in the statistical analysis (see Table 4).

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Table 4

Percentage of Subjects Containng *A. actinomycetemcomitans* and Variants With the Leukotoxin (lkt) Promoter Deletion

Category	*A. actinomycetemcomitans*	lkt Promoter Region Deletion
LJP-susceptible subjects from families with conversion subjects*	81.8(9/11)	54.5(6/11)
LJP-susceptible subjects from families without conversion subjects†	61.5 (8/13)	15.4 (2/13)
Probands‡	72.7 (16/22)	31.8 (7/22)
Control subjects§	38.2(13/34)	0 (0/34)

*Subjects from Table 1.

†Subjects from Table 2.

‡Subjects from Tables 1 and 2.

§Subjects from Table 3.

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Table 5

Distribution of LJP-Susceptible and Control Subjects According to Detection of *A. actinomycetemcomitans* and Conversion From a Healthy to Diseased Periodontal Status

	LJP-Susceptible Family Members*		Control Subject Population†		Total‡	
M	**Healthy**	**Conversion**	**Healthy**	**Conversion**	**Healthy**	**Conversion**
A. actinomycetem-comitans detected	9	8	12	1	21	9
A. actinomycetem-comitans not detected	6	1	19	2	25	3
Total	15	9	31	3	46	12

*Subjects from Tables 1 and 2. Not significant (Fisher's exact test, $P < 0.05$).

†Subjects from Table 3. Not significant (Fisher's exact test, P 0.05).

‡Not significant (Fisher's exact test, $P > 0.05$).

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Table 6

Distribution of LJP-Susceptible and Control Subjects According to Detection of the Leu-kotoxin (lkt) Promoter Deletion Variant and Conversion From a Healthy to Diseased Periodontal Status

	LJP-Susceptible Family Members*		Control Subject Population†		Total‡	
	Healthy	Conversion	Healthy	Conversion	Healthy	Conversion
lkt promoter deletion variant detected§	2	6	0	0	2	6
lkt promoter deletion variant not detected	13	3	31	3	44	6
Total	15	9	31	3	46	12

*Subjects from Tables 1 and 2.Statistically significant differences (Fisher's exact test, $P < 0.05$).

†Subjects from Table 3.

‡Statistically significant differences (Fisher's exact test, $P < 0.05$).

§Subjects contain *A. actinomycetemcomitans* that yield the 560 bp PCR product.