JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1997, p. 3037–3042 Vol. 35, No. 12
0095-1137/97/$04.00+0

Racial Tropism of a Highly Toxic Clone of *Actinobacillus actinomycetemcomitans* Associated with Juvenile Periodontitis

DORTE HAUBEK,[1,2] JOSEPH M. DIRIENZO,[3] EDUARDO M. B. TINOCO,[4] JYTTE WESTERGAARD,[5] NÉSTOR J. LÓPEZ,[6] CHONG-PYOUNG CHUNG,[7] KNUD POULSEN,[2] AND MOGENS KILIAN[2]*

Department of Oral Biology[1] and Department of Medical Microbiology and Immunology,[2] University of Aarhus, Aarhus, and Department of Periodontology, University of Copenhagen, Copenhagen,[5] Denmark; Department of Microbiology, School of Dental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania[3]; Department of Periodontology, University of Oslo, Oslo, Norway[4]; Department of Periodontics, School of Dentistry, University of Chile, Santiago, Chile[6]; and Department of Periodontology, College of Dentistry, Seoul National University, Seoul, South Korea[7]

Received 3 July 1997/Returned for modification 27 August 1997/Accepted 9 September 1997

Actinobacillus actinomycetemcomitans **strains with enhanced levels of production of leukotoxin are characterized by a 530-bp deletion from the promoter region of the leukotoxin gene operon. Previous isolates with this deletion constituted a single clone belonging to serotype b, although they displayed minor differences among each other. We have analyzed the geographic dissemination of this clone by examining 326** ***A. actinomycetemcomitans*** **isolates from healthy and periodontally diseased individuals as well as from patients with different types of extraoral infections originating from countries worldwide. A total of 38 isolates, all belonging to the same clone, showed the 530-bp deletion. Comparison of a 440-bp sequence from the promoter region of the leukotoxin gene operon from 10 of these strains revealed complete identity, which indicates that the deletion originates from a single mutational event. This particular clone was exclusively associated with localized juvenile periodontitis (LJP). In at least 12 of 28 families from which the clone was isolated, more than one family member had LJP. Notably, all the subjects carrying this clone had a genetic affiliation with the African population. These observations suggest that juvenile periodontitis in some adolescents with an African origin is associated with a disseminating clone of** ***A. actinomycetemcomitans*****.**

During the last two decades the leukotoxin expressed by the gram-negative organism *Actinobacillus actinomycetemcomitans* has been considered an important virulence factor implicated in the pathogenesis of juvenile periodontitis and possibly other forms of periodontal disease. The leukotoxin, which is a member of the RTX family of toxins, specifically lyses human polymorphonuclear leukocytes (1, 34) and thus is assumed to enable the bacterium to evade an important part of the innate host immune system. The toxin is encoded by a gene operon (*ltx*) consisting of four genes, *ltxC*, *ltxA*, *ltxB*, and *ltxD*, of which *ltxA* is the structural gene (17, 18).

Differences in virulence may be one explanation for the fact that *A. actinomycetemcomitans* may be isolated from periodontally diseased subjects as well as from apparently healthy carriers (30, 38). Although all strains carry the *ltx* operon (9, 25), significantly different levels of leukotoxin expression have been demonstrated among *A. actinomycetemcomitans* strains (1, 3, 32). Brogan et al. (3) demonstrated a 530-bp deletion from the promoter region of the leukotoxin gene operon of strains that showed 10- to 20-fold higher levels of leukotoxin activity and ascribed the enhanced activity to a second strong promoter upstream from the deletion. Recently, it has been described that leukotoxin expression in the minimally leukotoxic strain *A. actinomycetemcomitans* 652 is directed by a single promoter which does not correspond to either of the two promoters driving *ltx* expression in the highly toxic strain JP2, suggesting that different sequence elements regulate *ltx* expression in highly toxic and minimally toxic *A. actinomycetemcomitans* strains (13). Furthermore, it has been observed that leukotoxin expression can be induced by anaerobic conditions (13, 33). In addition, there is evidence that *cis* elements as well as *trans*-acting factors are involved in strain-specific regulation of the leukotoxin gene (17). These findings suggest that a variety of regulatory mechanisms may be involved in leukotoxin expression in the genetically diverse population of *A. actinomycetemcomitans.*

We had previously analyzed the population structures of two collections of *A. actinomycetemcomitans* from Caucasians and found that no particular clone is associated with periodontal disease (10, 25). More recently, a unique clone with the mutational 530-bp deletion was isolated from 16 African patients with localized juvenile periodontitis (LJP) living in the United States and two European countries (11). Likewise, Zambon et al. (39) observed that 12 of 21 North American LJP patients examined harbored highly leukotoxic strains with the same deletion. Furthermore, *A. actinomycetemcomitans* strains characterized by a distinct restriction fragment length polymorphism (RFLP) pattern (RFLP group II) have been shown to predominate in LJP patients in the United States and were not found in healthy individuals (7).

To further determine the dissemination of the highly toxic clone of *A. actinomycetemcomitans* and its genetic relationships to U.S. strains, we collected 326 isolates from subjects originating in countries worldwide and analyzed them for the presence of the 530-bp deletion in the promoter region of the

* Corresponding author. Mailing address: Department of Medical Microbiology and Immunology, The Bartholin Building, University of Aarhus, DK-8000 Aarhus C, Denmark. Phone: (45) 8942 1735. Fax: (45) 8619 6128. E-mail: mogens.kilian@svfcd.aau.dk.

leukotoxin gene. We report that 38 of these 326 isolates belonged to a single clone of serotype b and were characterized by a 530-bp deletion from the promoter region of the leukotoxin gene. This disseminating clone was associated exclusively with LJP in adolescents with an African origin or a historically close relationship to the African continent.

MATERIALS AND METHODS

Bacterial strains. A total of 326 clinical isolates of *A. actinomycetemcomitans* were included in this study. Of these, 56, 81, and 16 isolates were included in previously published studies by Poulsen et al. (25) and Haubek et al. (10, 11), respectively. The individuals from whom the strains were isolated originated from at least 29 countries worldwide: 47 individuals from 11 countries in Asia, 171 individuals from 8 countries in Europe, 32 individuals from 7 countries in Africa, 44 individuals from 2 countries in South America, and 32 individuals from at least five states in the United States (Table 1). In 23 instances *A. actinomycetemcomitans* isolates from two members of a family were included, in 6 instances isolates from 3 family members were included, and in one instance isolates from 4 family members were included. The majority of isolates ($n = 225$) were from dental plaque from patients with different forms of periodontal disease, e.g., LJP, adult periodontitis (AP), rapidly progressing periodontitis, and prepubertal periodontitis. Fifty-two isolates were from individuals with apparently healthy periodontia or moderate gingivitis. Six isolates were from patients with endocarditis, 2 were from mucosal lesions, 1 was from blood, 12 were from actinomycotic or other abscesses, 13 were from pus, 3 were from expectorates, 1 was from a periapical granuloma, 1 was from saliva, and 10 were of unknown clinical origin. The diagnosis of LJP was defined according to the disease characteristics reported by Baer (2). Subjects with AP included patients with periodontal destructions not resembling those in LJP. The AP patients did not have a history of a previous diagnosis of or treatment for LJP. The diagnosis of rapidly progressing periodontitis was used when the patients were younger than age 35 years and showed relatively severe periodontal destruction relative to their age, e.g., very high bleeding tendency and angular bony defects. The diagnosis of prepubertal periodontitis was used when the periodontal disorder involved teeth of a prepubertal child without systemic diseases.

A. actinomycetemcomitans was cultivated on TSBV agar (31) in air supplemented with 5% CO_2 at 37°C for 4 to 5 days. The basis for species identification was typical colony morphology; catalase activity; porphyrin synthesis; lack of indole production; urease, ornithine, lysine decarboxylase, and β-galactosidase (*o*-nitrophenyl-β-D-galactopyranoside) activities; and lack of V-factor dependency (15). In addition, fermentation of glucose but not sucrose or lactose was used to verify the diagnosis. Examination of hemolytic activity was done by cultivation on 5% horse blood agar (Statens Serum Institut, Copenhagen, Denmark) and in some cases by growing the bacteria in Lewinthal broth added to holes cut in a blood agar plate. Isolates were preserved at −70°C in skim milk.

Serotyping of strains. Serotyping was performed by double immunodiffusion in a 1% (wt/vol) agarose gel with autoclaved extracts of bacteria harvested from a 10-ml broth culture and resuspended in 0.5 ml of saline (0.9%). Five reference strains, strains OMZ 300 and OMZ 295 (B. Guggenheim, Zürich, Switzerland), NCTC 9710 (National Collection of Type Cultures, Colindale, London, United Kingdom), and HK 928 and HK 929 (M. Kilian, own isolates), were used to raise antisera against serotype antigens a through e by intravenous immunization of rabbits.

Detection of the 530-bp deletion by PCR. The characteristic 530-bp deletion from the promoter region of the leukotoxin gene operon was detected by PCR (3, 11). Briefly, a single colony was boiled for 5 min in 85 μl of water. PCR was performed in a total volume of 100 μl with *Taq* DNA polymerase as recommended by the manufacturer (Life Technologies, Roskilde, Denmark), with 30 cycles of a program including denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and polymerization for 2 min at 72°C. The primers were designed from the published *ltx* sequences (3, 18). The primer upstream from the deletion had the sequence 5′-CAGATCAAAACCTGATAACAGTATT-3′, and the primer downstream from the deletion had the sequence 5′-TTTCTCCATATTAAATC TCCTTGT-3′ (3, 11). The PCR fragment was 504 bp when genomic DNAs from strains with the deletion were used as templates, whereas the size of the PCR fragment from strains without the deletion was 1,034 bp. The sizes of the amplification products were compared by agarose gel electrophoresis (11).

MLEE. *A. actinomycetemcomitans* isolates characterized by the 530-bp deletion were analyzed by multilocus enzyme electrophoresis (MLEE) as described previously (10) by using the principles described by Selander et al. (28). After electrophoresis of bacterial extracts the starch gels were selectively stained for the following six intracellular metabolic enzymes, which in our previous studies revealed polymorphism among *A. actinomycetemcomitans* strains: phosphoglucomutase, glucose-6-phosphate dehydrogenase, malate dehydrogenase, adenylate kinase, phosphoglucose isomerase, and mannose phosphate isomerase. Mobility variants of each enzyme were determined. Strain JP2 was used as a reference strain for the highly toxic clonal type of *A. actinomycetemcomitans.*

Restriction endonuclease fingerprinting of genomic DNA. Whole-cell DNA from *A. actinomycetemcomitans* isolates was extracted from 10-ml fluid cultures. The cells were harvested by centrifugation, and the pellet was resuspended in 0.7 ml of 0.1 M NaCl–0.05 M Tris-HCl–0.05 M EDTA (pH 8); the cells were lysed by adding 15 μl of 10% sodium dodecyl sulfate. Proteinase K (1 mg/ml) digestion was performed in the tubes with lysed cells while shaking the tubes at 37°C for about 24 h. After several extractions with phenol-chloroform and one extraction with chloroform alone, nucleic acids were precipitated with ethanol and were finally resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7.6]). Approximately 2 μg of whole-cell DNA was digested with 20 U of the restriction endonuclease *Msp*I for 2 h as recommended by the manufacturer (Boehringer Mannheim, Mannheim, Germany). The DNA samples were treated with 0.05 μl of DNase-free RNase (Boehringer Mannheim) for 15 min. Separation of the fragments of cleaved DNA was performed in 1% agarose gels by electrophoresis in 1× TAE (Tris-acetate, EDTA) buffer (27) for 20 h at 4 V/cm, and the fragments were visualized by staining with ethidium bromide.

Ribotyping. For Southern blot analysis 1 to 2 μg of whole-cell DNA was cleaved with 20 U of the restriction endonuclease *Eco*RI for 2 h as recommended by the manufacturer (Boehringer Mannheim). The digested DNA samples were treated with RNase, and separation of fragments by size was performed as described above for 22 h at 4.5 V/cm. The gels were denatured and neutralized, and the DNA fragments were transferred to nylon membranes as described previously (27). Cross-linking of the DNA fragments to the nylon filters was performed by UV irradiation (UV-Stratalinker 1800).

Hybridization with plasmid pKK 3535 as the probe was performed as described previously (27). The plasmid contains the rRNA operon from *Escherichia coli* (4, 26).

DNA sequencing. The 504-bp PCR product from individual strains with the deletion was purified by agarose gel electrophoresis followed by elution with the Gene Clean Kit (Bio 101, Inc., Vista, Calif.). The sequencing reactions were performed with the *Taq* DyeDeoxy-Terminator Cycle Sequencing Kit (Perkin-Elmer, Foster City, Calif.) and with approximately 30 ng of the DNA fragment, together with each of the oligonucleotides used for PCR as sequencing primers. The reactions were analyzed on an Applied Biosystems model 373A DNA sequencer.

RESULTS

The initial examinations revealed that among 326 *A. actinomycetemcomitans* isolates examined, 38 had the characteristic 530-bp deletion from the *ltx* operon. All 38 isolates belonged to serotype b. Among the remaining 288 strains the distribution of serotypes a through e were 66, 75, 95, 10, and 19 strains, respectively, and 23 isolates showed no reaction with the typing sera (Table 1). No significant association was found between any particular serotype and periodontal disease (data not shown). The genetic similarities of the 38 isolates with the 530-bp deletion were further analyzed by four additional methods (see below). Notably, these analyses revealed that 9 of 38 cultures received from different laboratories were found to contain a mixture of two strains, one with and one without the deletion. This emphasizes that purification of *A. actinomycetemcomitans* is problematic, presumably due to the significant aggregations of fresh isolates.

MLEE. For 37 of the strains tested by MLEE, the patterns for the enzyme loci examined were identical to that for JP2. Due to poor growth, one of the strains from Brazil could not be examined by MLEE.

Restriction enzyme analysis. Digestion of whole-cell DNA from all 38 strains characterized by the 530-bp deletion with the restriction enzyme *Msp*I revealed DNA fingerprints identical to that of strain JP2. Among 14 *A. actinomycetemcomitans* strains belonging to RFLP group II described by DiRienzo et al. (7), 12 had the characteristic 530-bp deletion, whereas 2 did not. These two strains showed an *Msp*I fingerprint different from, although very similar to, those of strains with the deletion (Fig. 1, lanes 8 and 9).

Ribotyping. Limited variation within the highly toxic clonal type of *A. actinomycetemcomitans* was previously revealed by ribotyping with rRNA from *E. coli* as the hybridization probe on Southern blots of *Eco*RI-restricted whole-cell DNA (11). Seven different ribotypes were detected among the 38 *A. actinomycetemcomitans* strains characterized by the 530-bp deletion. One predominating pattern was observed in 19 strains. In three other patterns, representing six, five, and five strains, respectively, one of five bands in the predominating ribotype pattern varied, whereas the remaining three strains, all isolated

TABLE 1. Geographic distribution of a highly toxic clonal type of *A. actinomycetemcomitans* characterized by a 530-bp deletion in the promoter region of the leukotoxin gene, and serotype distributions of 326 *A. actinomycetemcomitans* analyzed

Country of origin	No. of strains	No. of strains belonging to JP2 clone	Ribotype of JP2 clone	No. of strains of the following serotype:					
				a	b	c	d	e	Nontypeable
Asia									
India[a]	1	0		1	0	0	0	0	0
Pakistan[a]	1	0		0	0	1	0	0	0
Turkey[a]	1	0		0	0	1	0	0	0
Iran[a]	1	0		0	0	1	0	0	0
Israel[b]	1	1		0	1	0	0	0	0
Vietnam[c]	11	0		3	3	5	0	0	0
China	11	0		2	0	4	0	3	2
South Korea[d]	7	0		1	0	0	0	0	6
Indonesia	7	0		0	6	1	0	0	0
Thailand	1	0		0	0	1	0	0	0
Japan	5	0		1	0	1	0	2	1
Europe									
Denmark	42	0		13	7	19	0	2	1
Sweden	12	0		1	7	1	1	0	2
Finland	82	0		22	20	21	7	8	4
Iceland	4	0		0	3	0	0	0	1
Germany	3	0		2	1	0	0	0	0
Switzerland	6	0		0	3	1	0	2	0
Holland	21	0		9	8	4	0	0	0
Macedonia[a]	1	0		0	0	1	0	0	0
Africa									
Morocco[a,e]	11	7	a (6),[f] c (1)	2	7	1	0	0	1
Algeria[a]	2	2	a	0	2	0	0	0	0
Ghana[a]	1	1	a	0	1	0	0	0	0
Cape Verde Isles[g]	6	6	a	0	6	0	0	0	0
Kenya	10	0		0	1	5	0	2	2
Tanzania[a]	1	0		0	0	1	0	0	0
Somalia	1	0		0	1	0	0	0	0
South America									
Chile	13	0		1	6	3	2	0	1
Brazil[h]	31	4	a (2), d (2)	3	6	20	0	0	2
North America									
United States[i]	32	17	a (2), b (5), c (4), d (3), e (1), f (1), g (1)	5	24	3	0	0	0
Total	326	38		66	113	95	10	19	23

[a] First- and second-generation immigrants living in Denmark.
[b] The subject is a first-generation immigrant raised in Switzerland.
[c] Ten Vietnamese children who had just arrived in Finland were sampled by Höltta et al. (12).
[d] One isolate was obtained from a first-generation immigrant in Denmark; the remaining were obtained from patients in South Korea.
[e] One isolate from a first-generation immigrant living in The Netherlands.
[f] Values in parentheses are numbers of isolates.
[g] Isolates from first- and second-generation immigrants living in Sweden.
[h] Isolates were obtained from Brazilian patients who were of either African or mixed (African-Caucasian or African-South American Indian) races. The isolates were selected strains from the study of Tinoco et al. (37).
[i] Twenty-one isolates are known to have been obtained from African Americans; 17 of these had the 530-bp deletion. The remaining isolates were obtained from patients of unknown race.

in the United States, each had a unique pattern that differed by two bands. In all instances in which isolates from more than one family member were studied, the isolates showed identical ribotypes. Whereas 15 of 16 isolates from Africans living in European countries belonged to the predominant ribotype (ribotype a), all seven ribotypes were represented among isolates from African Americans (Table 1). Two strains without the deletion and belonging to RFLP group II had a ribotype identical to that of the majority of the *A. actinomycetemcomitans* strains with the deletion (Fig. 2, lane 2).

DNA sequencing. Ten of the 38 strains characterized by the 530-bp deletion were selected as representative strains with the observed variations in ribotype and source of isolation. The DNA sequence obtained stretched from 421 bp upstream to 19 bp downstream of the site of the deletion. The 440-bp sequence was identical for all 10 strains.

Hemolytic activity. All 38 isolates characterized by the 530-bp deletion showed clear β-hemolytic zones on blood agar after incubation for 3 to 4 days. Likewise, growth for 3 days in broth added to holes in the blood agar resulted in clear zones with diameters of 1 to 2 mm (Fig. 3). The remaining strains induced a weak greening of the blood agar or gave no color change. The same results were obtained after incubation in air plus carbon dioxide and under anaerobic conditions.

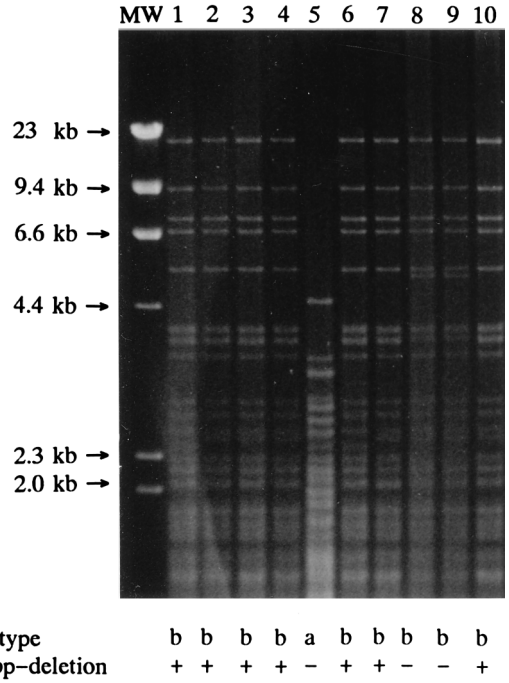

FIG. 1. Restriction endonuclease analysis of whole-cell DNA from 10 *A. actinomycetemcomitans* strains with the restriction enzyme, *MspI*. Lane MW, molecular size markers; lane 1, RAP-1; lane 2, HG1709; lane 3, P5926-1; lane 4, OMZ 678; lane 5, UP23; lane 6, UP18; lane 7, UP15; lane 8, UP14; lane 9, UP12; lane 10, UP11. All but one of the *A. actinomycetemcomitans* strains are of serotype b; lane 5 contains one serotype a strain. The *A. actinomycetemcomitans* strains were examined for the occurrence of a 530-bp deletion in the promoter region of the leukotoxin gene, marked by a plus or a minus sign.

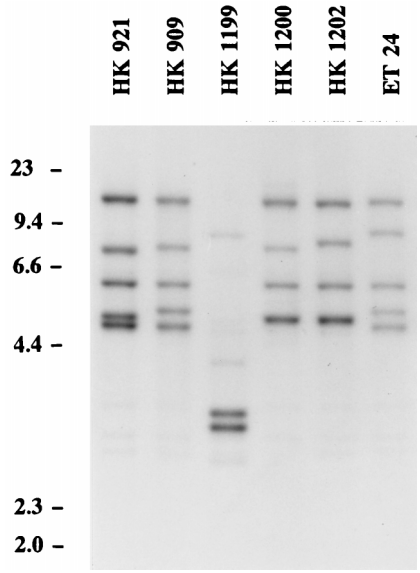

FIG. 2. *A. actinomycetemcomitans* strains with the 530-bp deletion are not identical. The Southern blot of *Eco*RI-restricted whole-cell DNA hybridized with rRNA sequences shows that six of seven different ribotypes can be found among *A. actinomycetemcomitans* strains characterized by the 530-bp deletion (lanes marked strains HK 921 [JP2], HK 909, HK 1199, HK 1200, HK 1202, and ET 24, respectively). Nineteen of the 38 strains with the deletion had a ribotype like the one demonstrated in the lane labeled HK 909. Numbers to the left refer to the sizes (in kilobases) of fragments of the DNA molecular weight marker II (Boehringer Mannheim).

Origin and disease category of the subjects from whom the highly toxic clonal type of *A. actinomycetemcomitans* was obtained. Among the 38 isolates with the deletion, 36 were from LJP patients, 1 was isolated from a 10-year-old healthy younger brother of a sister with LJP, and 1 was isolated from a 37-year-old mother of a son with LJP. The mother had mild AP. Six of the 38 isolates were obtained in Sweden from first- and second-generation immigrants from the Cape Verde Islands, and 10 were isolated from 1995 to 1997 from first- and second-generation immigrants to Denmark or The Netherlands from Morocco, Algeria, and Ghana. One isolate was from an LJP patient raised in Switzerland but originating in Israel. Further information about the race of this individual was not available. Four isolates were from Brazilian patients that were of either African or mixed (African-Caucasian or African-South American Indian) races. Of 17 isolates from the United States, 12 were from African-American subjects living in the west Philadelphia, Pa., area, and patients were examined between 1987 and 1989. Among the remaining five isolates from subjects in the United States, four were from African-American individuals, whereas the race of one subject was unknown. Collectively, this means that at least 34 of the 38 isolates were from LJP patients with a genetic background in an African population. In comparison, only 7 of the remaining 78 LJP patients who carried *A. actinomycetemcomitans* strains without the deletion were of African race, not including patients of mixed race.

Among the 28 families from which the highly toxic clone was isolated, at least 12 had more family members (two to four family members) with LJP or a history of treatment for diagnosed LJP. The ratio of female-to-male LJP patients carrying the clone with the deletion was 1.6:1.

DISCUSSION

Recent studies of several bacterial pathogens have revealed that, in contrast to strains carried by healthy individuals, a very limited number of clones are usually responsible for infections worldwide (23, 29), provided that the bacterial population structure is not blurred by frequent recombination (6, 20). The putative etiologic agent of LJP, *A. actinomycetemcomitans*, does show a strictly clonal population structure (10, 25). Therefore, our previous observation that virtually all isolates of this species from patients with LJP, various extraoral infections, and healthy individuals, all from Caucasians, were distinct led us to conclude that *A. actinomycetemcomitans* is an opportu-

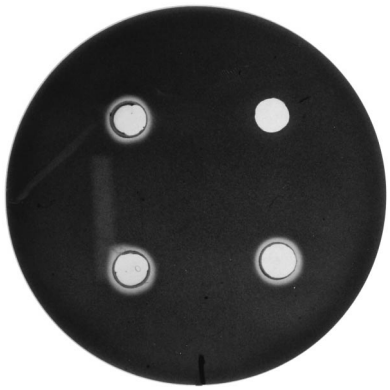

FIG. 3. All *A. actinomycetemcomitans* strains representing the JP2 clone (upper left and lower right and left) showed clear β-hemolytic activity on horse blood agar plates, in contrast to the other isolates, represented by the isolate at the upper right. The isolates were cultivated for 3 days in Levinthal broth added to holes cut in the agar.

nistic pathogenic member of the resident oral microflora. The same conclusion has been drawn on the basis of population genetic analyses of several suspected pathogens of adult periodontitis, i.e., *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Prevotella nigrescens* (19, 21, 36). Our findings based on the results of studies with isolates from Caucasian populations lent no support to the concept that LJP is a contagious disease caused by disseminating clones of *A. actinomycetemcomitans*. With this background, our subsequent observation that 16 *A. actinomycetemcomitans* isolates from African LJP patients living in the United States and two European countries belonged to a single clone was highly unexpected (11). This clone was characterized by a 530-bp deletion from the promoter region of the leukotoxin gene operon previously defined in strain JP2 as giving rise to considerably enhanced leukotoxin activity (3).

The geographically widespread but racially restricted occurrence of this JP2 clone is further substantiated by the results of this study. Of a total of 326 *A. actinomycetemcomitans* isolates from 29 countries in Europe, Asia, Africa, and South and North America, 38 showed the identical 530-bp deletion from the leukotoxin gene promoter region. Our findings that all 38 isolates were of serotype b and, in contrast to the *A. actinomycetemcomitans* population in general (10, 25), showed identical DNA fingerprinting and MLEE patterns indicate that they all belong to a single clone. This is further supported by the identical nucleotide sequences of the regions flanking the deletion among the 38 isolates. The latter finding supports the hypothesis that the origin of the deletion in the isolates is a single mutational event in a common ancestor. The polymorphism disclosed by ribotyping of the 38 isolates, however, suggests that, subsequent to the deletion event, the clone has been undergoing further evolution (Fig. 2).

In the collection as a whole, 35% of the strains were from LJP patients. Nevertheless, all 38 JP2 clone isolates were exclusively associated with LJP, although 1 was from a 10-year-old healthy brother of a sister with LJP and one was from a mother (with AP) of a son with LJP. Furthermore, several siblings or one of the parents in at least 12 of the 28 families from which the JP2 clone isolates originated had a history of LJP. Thus, there was a very strong association between the JP2 clone and LJP, indicating that the clone has an enhanced virulence potential for this particular disease. This is supported by the findings recently reported by Zambon et al. (39). Although they did not examine the clonal relationships of the strains, the characteristic 530-bp deletion was detected in isolates from 12 of 21 LJP patients from the United States but not in isolates from 11 healthy individuals and 5 AP patients. Bias in the sampling of our strain collection does not allow us to draw conclusions related to the possibly enhanced transmissibility of the JP2 clone.

Previous studies in the United States of families with a history of LJP identified a distinct RFLP type (group II) of *A. actinomycetemcomitans* exclusively associated with disease (7). Twelve of 14 RFLP group II strains included in this study showed the 530-bp deletion and were otherwise identical to other isolates of the JP2 clone. The two strains without the deletion differed from the 38 isolates by *Msp*I restriction enzyme analysis but were otherwise identical by their serotypes and MLEE patterns and, furthermore, shared the predominant ribotype pattern. Thus, they are closely related to the highly toxic JP2 clone and may represent the ancestor of the deletion genotype. Alternatively, these two strains may have originated from the JP2 clone and may have regained the usual leukotoxin gene promoter type by horizontal gene transfer.

The possibility that the JP2 clone expresses additional virulence factors or possesses more virulent alleles of other determinants than the remaining *A. actinomycetemcomitans* population cannot be excluded. Interestingly, in contrast to the other isolates tested, all JP2 clone isolates showed clear β-hemolytic activity after prolonged incubation (Fig. 3). Although distinct hemolysins have recently been described in strains of *A. actinomycetemcomitans* (14, 16), the hemolytic activity of the JP2 clone isolates observed in this study may be an effect of increased leukotoxin production. Studies of the related leukotoxin of *Pasteurella haemolytica* have supported the notion that RTX leukotoxins may have hemolytic activity (5, 22).

It is remarkable that 9 of 38 JP2 clone cultures received from other laboratories were contaminated with other clonal types of *A. actinomycetemcomitans*. This finding emphasizes the difficulties in purifying isolates of *A. actinomycetemcomitans* and indicates that JP2 clone-positive LJP patients are often colonized with additional clones. Thus, the possibility that disease in these patients is the result of a synergistic effect of different clones of *A. actinomycetemcomitans* cannot be excluded.

The epidemiological data for the 38 isolates reveal several striking results. All but two isolates, for which information was not available, were isolated from individuals with a genetic background in an African population but were usually physically separated from that continent by one to many generations. Because the JP2 clone belongs to serotype b, it is conceivable that differences in the racial distributions of LJP patients explain different conclusions concerning the predominance of that serotype in studies from different parts of the world (10, 12, 37, 38). Although more detailed epidemiological studies are required to make definitive conclusions, the data in Table 1 combined with the available demographic data suggest that the patients carrying the JP2 clone are descendants of North and West African populations (Table 1). The distribution of the seven observed ribotype patterns (Table 1) shows that 15 of 16 isolates from first- or second-generation African immigrants to European countries belonged to a single ribotype (ribotype a), whereas all seven ribotypes were represented among 17 isolates from African-American patients. This difference in polymorphism probably reflects the more heterogeneous geographic origins of the African-American population. Similar differences in the degree of polymorphism have been observed for *Haemophilus influenzae* serotype b clones from Europe and the United States (23, 24).

Why is the JP2 clone still restricted to descendants of African populations? One possible explanation is that the clone is specific for hosts with a distinct genetic constitution. Previously identified reasons for the specificities of pathogens for particular host species include interaction with species-specific adhesin receptors and iron-binding proteins, species-specific plasminogen activation, and cleavage of host immunoglobulin A (8). Although the activity of the *A. actinomycetemcomitans* leukotoxin shows specificity for primates and especially human polymorphonuclear leukocytes (35), no studies have indicated specificity for cells of particular human races or individuals. Geographic variation in the relative frequencies of different clones has previously been recorded for several pathogenic bacteria (24), but there is not yet any evidence proving that this can be explained by differences in the genetic constitutions of the hosts. An alternative explanation for the restricted epidemiology of the JP2 clone may be that the clone has a very low level of transmissibility and that acquisition is restricted to a short period in childhood, leading primarily to vertical transmission, combined with social separation of the races.

The contrasting nature of the etiology of LJP in Caucasians and patients genetically associated with certain African populations appears to be exceptional. As suggested previously (11), juvenile periodontitis could possibly represent two different

types of disease with distinct etiologies and epidemiologies: one type, found worldwide, in which a diversity of *A. actinomycetemcomitans* clones may act as an opportunistic pathogen, and another type in which a particular clonal type of *A. actinomycetemcomitans* serotype b characterized by the 530-bp deletion acts as an exogenous pathogen. Whether disease in these two populations is characterized by different clinical pictures remains to be elucidated. Likewise, further epidemiological studies of *A. actinomycetemcomitans* isolates from healthy and periodontally diseased individuals in relevant populations are needed to clarify whether the JP2 clone represents a traditional pathogen within a population of otherwise opportunistic pathogenic *A. actinomycetemcomitans* clones.

ACKNOWLEDGMENTS

The technical assistance of Kirsten Holmgren, Lene Friis, Birte Esmann, Karen Halabi, Ella Brandt, and Søren Hempel Thomassen is gratefully acknowledged. We especially thank the following scientists for providing some of the bacterial strains used in this study: Satu Alaluusua, Sirkka Asikainen, Gunnar Dahlén, Birgit Ellegaard, Paula Fives-Taylor, Rudolf Gmür, Peter Holbrook, Edward T. Lally, Andrea Mombelli, Cholticha Amornchat, and Arie Jan van Winkelhoff.

This work was supported by the Danish Medical Research Council (grant 12-1615), the Velux Foundation, a FUT-Calcin grant from the Danish Dental Association, and Public Health Service grant DE/OD 10891 from the National Institutes of Health.

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