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Evidence for Absence in Northern Europe of Especially Virulent Clonal Types of *Actinobacillus actinomycetemcomitans*

DORTE HAUBEK,[1,2] KNUD POULSEN,[1] SIRKKA ASIKAINEN,[3] AND MOGENS KILIAN[1]*

Institute of Medical Microbiology[1] and Department of Oral Biology,[2] Faculty of Health Sciences, University of Aarhus, DK-8000 Aarhus C, Denmark, and Department of Periodontology, University of Helsinki, Helsinki, Finland[3]

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Genetic analysis of an *Actinobacillus actinomycetemcomitans* population consisting of 88 clinically well characterized Finnish isolates performed by multilocus enzyme electrophoresis confirmed that the five serotypes divide into two phylogenetic lineages, one comprising serotypes b and c and one comprising serotypes a, d, and e. There was no association between any subpopulation and the periodontal health status of the subject from whom the isolates originated, suggesting that the role of *A. actinomycetemcomitans* in periodontitis is largely opportunistic in the population examined. Southern blot analyses of genomic DNA digested with each of the restriction endonucleases *Msp*I, *Rsa*I, and *Taq*I revealed extremely limited genetic polymorphism of the structural leukotoxin gene, *ltxA*, and its associated promoter. All isolates hybridized to a 530-bp DNA fragment derived from the promoter region of the leukotoxin gene operon of a minimally leukotoxic *A. actinomycetemcomitans* strain. Deletion of the 530-bp sequence has been associated with significantly increased toxin production detected among isolates from patients with juvenile periodontitis in North America but was detected neither among the 88 isolates in the present collection analyzed nor among more than 60 strains in another population of northern European *A. actinomycetemcomitans* isolates analyzed previously.

Actinobacillus actinomycetemcomitans has been implicated in the pathogenesis of localized juvenile periodontitis (LJP) and severe adult periodontitis (AP) (31). This gram-negative bacterium colonizes both periodontally healthy subjects and patients with periodontal disease, although a significantly higher prevalence and proportions of *A. actinomycetemcomitans* are found in plaques from diseased individuals (31). The role of *A. actinomycetemcomitans* in pathogenesis is not well understood, and it is unclear whether specific genetic variants of *A. actinomycetemcomitans* are correlated with the onset and progression of periodontitis.

We have previously analyzed the population structure of a temporally and geographically diverse collection of *A. actinomycetemcomitans* isolates from periodontally healthy carriers and from patients with periodontal disease as well as various systemic infections (18). The population genetic study was based on multilocus enzyme electrophoresis (MEE), fingerprinting of whole genomic DNA, and restriction fragment length polymorphism (RFLP) analysis and revealed that the population consists of a large number of clones and that each of the serotypes a through e comprises genetically isolated subpopulations. Disease-related strains were found scattered throughout the population, but the sampling method did not allow firm conclusions concerning the pathogenic potential of the different genotypes to be drawn.

A. actinomycetemcomitans produces a number of putative virulence factors including a potent leukotoxin (31). This membrane-bound toxin belongs to the RTX (repeat-in-toxin) family of bacterial cytotoxins (12, 14, 29) and has a specific cytotoxic effect on human polymorphonuclear leukocytes and monocytes (3, 25–28). Leukotoxin expression is encoded by a gene operon consisting of four genes. The structural gene, termed *ltxA*, encodes the toxin, whereas the *ltxB*, *ltxC*, and *ltxD* genes are involved in the translocation and activation of the toxin (11, 12). Significant differences in the level of leukotoxin expression among different *A. actinomycetemcomitans* strains have been demonstrated (3, 5, 24), although all strains carry the complete toxin gene operon (5, 18, 24). Genetic analyses have shown that leukotoxin production is regulated at the level of transcription. The highly toxic strains contain a second *ltx* promoter and are characterized by a 530-bp deletion in the *ltx* promoter region (5), which is also reflected in a *Hin*dIII restriction site polymorphism in this region (5, 7, 8, 11). It is unclear whether variation in the quantity or quality of leukotoxin among *A. actinomycetemcomitans* strains might be correlated with periodontal disease.

In the study described here we analyzed the population structure of a clinically well characterized collection of Finnish *A. actinomycetemcomitans* isolates spanning a period of 8 years in isolation time and originating from patients with LJP or AP and from periodontally healthy subjects. On the basis of MEE analysis of the population and RFLP analysis of the structural leukotoxin gene, *ltxA*, and its associated promoter region, our aim was to evaluate if particular clonal types of *A. actinomycetemcomitans* or alleles of the leukotoxin gene are associated with periodontal disease.

MATERIALS AND METHODS

Bacterial isolates. A collection of 88 clinical isolates of *A. actinomycetemcomitans* was examined; the collection comprised 56 isolates from patients with adult periodontitis, 16 isolates from patients with localized juvenile periodontitis, and 16 from subjects with healthy periodontia or mild gingivitis (see Fig. 1 and Table 1). The diagnosis of periodontitis was based on clinical and radiographic examination of the dentition. LJP was defined according to the disease characteristics reported by Baer (4). Subjects with AP included patients exhibiting clinical and radiographic destruction of periodontal tissue not resembling that in LJP. The AP patients did not exhibit a history of a previous diagnosis or treatment of LJP. Periodontal health was defined as the absence of periodontal breakdown, although localized bleeding after probing was frequently seen.

A. actinomycetemcomitans was isolated from subgingival plaque samples cultivated on TSBV agar (23) incubated in 5% CO_2 in air at 36°C for 4 to 5 days. Species identification was based on typical colony morphology, positive catalase

* Corresponding author. Mailing address: Institute of Medical Microbiology, Bartholin Building, University of Aarhus, DK-8000 Aarhus C, Denmark. Phone: 045 89421735. Fax: 45 86196128.

reaction, and negative lactose fermentation. *A. actinomycetemcomitans* subcultures originating from a single colony were preserved at −70°C in skim milk.

The collection comprised *A. actinomycetemcomitans* isolates recovered during the years 1984 to 1992 in our studies on the occurrence and characteristics of *A. actinomycetemcomitans* in 81 periodontally healthy and diseased Finnish individuals. Among these, three people were from one family and two people each from 12 families were included, whereas 54 of the subjects were unrelated. Initially 2 to 35 (mean ± standard deviation, 6.0 ± 5.6) *A. actinomycetemcomitans* isolates from each subject were subcultured and serotyped. Unless multiple serotypes were found only one isolate from each subject was included in the material used in the present study.

Serotyping of strains. Five reference strains, OMZ 300, OMZ 295 (B. Guggenheim, Zürich, Switzerland), NCTC 9710 (National Collection of Type Cultures, Colindale, London, United Kingdom), and IDH 781 and IDH 1705 (our own isolates) representing the five serotypes a through e, respectively, were used for the preparation of serotype-specific antisera (2, 18, 19). Serotyping was performed by double immunodiffusion in a 1% (wt/vol) agarose gel by using autoclave extracts of bacteria harvested from a broth culture and washed in phosphate-buffered saline.

MEE. *A. actinomycetemcomitans* isolates were grown in 150 ml of brain heart infusion broth (Difco, Detroit, Mich.) supplemented with cysteine and yeast extract (37 g of brain heart infusion broth, 0.5 g of cysteine, 5.0 g of yeast extract per liter). After incubation for 2 to 3 days at 37°C in air plus 5% CO_2 the bacteria were harvested by centrifugation at 6,000 × *g* for 20 min at 4°C. The bacteria were frozen, thawed, and sonicated on ice in 2.0 ml of a 50 mM Tris-HCl buffer containing 5 mM EDTA (pH 7.5) to lyse the cells, and after centrifugation the supernatant was stored at −70°C. Bacterial lysates were electrophoresed on starch gels and selectively stained for 10 metabolic enzymes as described by Selander et al. (22). The following enzymes were assayed: malate dehydrogenase (MDH), 6-phosphogluconate dehydrogenase (6PG), glucose 6-phosphate dehydrogenase (G6P), glyceraldehyde-3-phosphate dehydrogenase (G3P), adenylate kinase (ADK), phosphoglucomutase (PGM), alkaline phosphatase (ALK), phosphoglucose isomerase (PGI), mannose phosphate isomerase (MPI), and carbamate kinase (CDK). For staining for ADK and CDK the lysates were electrophoresed in a Tris-maleate buffer system (pH 7.4), for staining for PGI they were electrophoresed in lithium hydroxide buffer (pH 8.1), and for staining for the remaining seven enzymes they were electrophoresed in Tris-citrate buffer (pH 8.0) as described by Selander et al. (22). Mobility variants of each enzyme were equated with the alleles of the corresponding structural gene locus. Isolates with identical combinations of alleles at the 10 enzyme loci, corresponding to unique multilocus genotypes, were designated multilocus electrophoretic types (ETs). The absence of activity for an enzyme was treated as missing data.

Genetic diversity at an enzyme locus (*h*) among strains was calculated from allele frequencies as $h = 1 - \Sigma x_i^2 [n/(n-1)]$, where x_i is the frequency of the *i*th allele of the locus and *n* is the number of strains. Mean genetic diversity per locus (*H*) is the arithmetic average of *h* values over all loci. Genetic distance between ETs was expressed as the proportion of enzyme loci at which dissimilar electrophoretic mobilities occurred. The dendrogram was constructed from computerized cluster analysis performed on the genetic distances between ETs by the average-linkage method by using the program ETCLUS kindly provided by T. S. Whittam, Institute of Molecular Evolutionary Genetics, University of Pennsylvania.

Southern blot analysis. Whole-cell DNA from *A. actinomycetemcomitans* strains was extracted from 10-ml fluid cultures. The cells were harvested by centrifugation, resuspended in 0.5 ml of 0.1 M NaCl–0.05 M Tris-HCl–0.05 M EDTA (pH 8), and lysed by adding 20 μl of 10% sodium dodecyl sulfate (SDS). DNA was prepared by proteinase K digestion (1 mg/ml for 16 h at 37°C); this was followed by several extractions with phenol-chloroform, one chloroform extraction, and ethanol precipitation, and finally, the DNA was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7.6]). The quality and concentration of DNA were assayed by agarose gel electrophoresis, which also revealed the presence of plasmids migrating at a different rate than chromosomal DNA.

For Southern blot analysis 1 to 2 μg of whole-cell DNA was digested with 10 U of restriction endonuclease (Boehringer, Mannheim, Germany) for at least 2 h as recommended by the manufacturer, and DNA samples were treated with 0.05 μg of RNase (Boehringer) for 15 min. Fragments of the digested DNA samples were separated according to size by 1% agarose gel electrophoresis in 1× TAE buffer (21) for 18 h at 1 V/cm. The gel was denatured and neutralized, and the DNA fragments were transferred to nylon membranes (Schleicher & Schuell, Dassel, Germany) as described previously (21) and were subsequently cross-linked by UV irradiation (UV-Stratalinker 1800).

A hybridization probe, termed LtxA-3.1, of 3.1 kb was prepared by PCR amplification of genomic DNA from strain JP2 with oligonucleotide primers 5′-TATGGCAACTACTACACTGCCAAAT and 5′-GGATAACGCTTTACC GGATGAAC. The LtxA-3.1 probe represents the *ltxA* gene, which has been sequenced by Lally et al. (13) and Kraig et al. (12). The PCR product was electrophoresed in an agarose gel, and the LtxA-3.1 fragment was purified by the Geneclean procedure (Bio 101, La Jolla, Calif.). As a second probe we used the 530-bp PCR-amplified DNA fragment from *A. actinomycetemcomitans* 652, termed Δ530, which is deleted in the *ltx* promoter of strain JP2 (5). The fragment was cloned into the pCR II vector by using the TA cloning kit (Invitrogen, San Diego, Calif.) and was subsequently purified by the Geneclean procedure from plasmid DNA restricted with *Eco*RI and electrophoresed in an agarose gel. The DNA fragments used as probes in the hybridizations were labelled with [^{32}P]dATP by nick translation as described previously (21). Hybridizations were performed as described by Sambrook et al. (21) except that 0.01% Na-PP_i was included in all solutions. The final posthybridization wash was at 65°C in 0.5× SET (1× SET is 0.15 M NaCl-0.02 M Tris base-0.5 mM EDTA-1 ml of HCl per liter) buffer, and the hybridizing fragments were visualized by autoradiography. The filters were hybridized with each of the two probes, and previous hybridizing fragments were stripped off by soaking the filter in boiling 0.01% SDS before the subsequent hybridization.

TABLE 1. Serotype distributions of 88 *A. actinomycetemcomitans* isolates from periodontally healthy and diseased sites

Periodontal health status	No. of strains of serotype:					
	a	b	c	d	e	nt[a]
LJP	6	5	3	1	0	1
AP	12	14	13	4	8	5
H[b]	4	2	9	1	0	0
Total	22	21	25	6	8	6

[a] nt, nonserotypeable.
[b] H, healthy periodontium or mild gingivitis.

RESULTS

A. actinomycetemcomitans isolates were isolated from 81 subjects with defined periodontal disease status. Seven subjects were found to be colonized by two different serotypes of *A. actinomycetemcomitans*, whereas from each of the remaining 74 subjects all isolates initially screened were of the same serotype, suggesting that the subjects were colonized by a single clonal type of *A. actinomycetemcomitans*. A single representative of each type was included in the study, adding up to a total of 88 strains.

Serotyping. The distributions of the 88 strains among the five known serotypes a through e are provided in Table 1. Autoclave extracts of six isolates did not react with any of the five antisera, and these were categorized as nonserotypeable. We found no strong relationship between serotype and periodontal disease status, although serotype c may be more prevalent among healthy carriers (Table 1).

Genetic diversity and relationships revealed by MEE. For the MEE analysis we determined the electrophoretic mobilities of 10 metabolic enzymes. The detailed data are available upon request. Our previous population structure analysis of *A. actinomycetemcomitans* revealed that the enzymes nucleoside phosphorylase, peptidase, and malic enzyme are monomorphic (18), and accordingly, they were not included in the present study. It should be noted that by excluding these three monomorphic loci the genetic diversity is overestimated. The enzyme leucine aminopeptidase was excluded because some strains could not be unambiguously scored. Among the 88 strains all remaining 10 enzyme loci analyzed were polymorphic, with two to four alleles per locus. The average number of alleles per locus was 3.1 (Table 2).

The mean genetic diversity per locus for the 10 loci analyzed was 0.50. Among the 88 strains we identified 45 ETs, each of which was characterized by a distinct combination of electrophoretic mobilities of the 10 enzymes. Thirty-one of the ETs were represented by a single strain, whereas the remaining 14 ETs were represented by from 2 to 10 isolates (Fig. 1). None of the ETs contained strains of different serotypes. The nontypeable strains were scattered throughout the dendrogram and were often found to be of the same ET as strains of the known serotypes. This confirms our previous notion that the nontypeable strains are serotype antigen-deficient variants originating

TABLE 2. Number of electrophoretic alleles and genetic diversity per enzyme locus for 88 isolates of *A. actinomycetemcomitans*

Enzyme locus[a]	Frequency of indicated allele				Genetic diversity[b]
	1	2	3	4	
ADK	0.39	0.37	0.24		0.65
CDK	0.83	0.17			0.27
PGM	0.07	0.86	0.07		0.24
P6G	0.06	0.73	0.17	0.05	0.43
G6P	0.07	0.68	0.25		0.47
MDH	0.03	0.57	0.40		0.51
ALK	0.40	0.33	0.26		0.65
PGI	0.56	0.41	0.02		0.51
G3P	0.43	0.25	0.32		0.65
MPI	0.57	0.01	0.25	0.17	0.58

[a] Abbreviations are given in Materials and Methods.
[b] The mean genetic diversity per locus was 0.50.

from strains of the known serotypes (18). Among the ETs containing more than one strain, ET 16 and ET 43 each contained one pair of strains originating from related subjects and ET 24 and ET 28 each contained two such pairs of strains. Two of these pairs of strains originated from the same family. For 8 of the 13 families we found that strains isolated from related individuals were of different ETs. That approximately half of the strains isolated from related individuals were of the same ET supports the fact that intrafamilial transmission of *A. actinomycetemcomitans* takes place.

On the basis of MEE typing data a dendrogram was constructed from a computerized cluster analysis performed by the average-linkage clustering method from a matrix of pairwise genetic distances between ETs. The dendrogram shown in Fig. 1 demonstrates the genetic relationships of the 45 ETs on the basis of the allelic variations at the 10 enzyme loci. Corresponding to a single-locus difference between ETs, the smallest genetic distance was 0.1. The population was divided into two main evolutionary lineages, lineages A and B, including four major clusters, termed ET divisions I through IV, joining at a mean genetic distance of 0.50. Notably, alleles for the enzyme MPI were not shared between the two lineages. The dendrogram shows a strong correlation between ET and serotype. Thus, all strains of serotypes a, d, and e were found in lineage A, whereas all strains of serotypes b and c were found in lineage B. Within the two lineages ET division I was represented by 4 strains belonging to serotypes a and e, ET division II included 32 strains belonging to serotypes a, d, and e and 2 nonserotypeable strains, ET division III included 29 strains belonging to serotypes b and c and 3 nonserotypeable strains, and ET division IV included 17 serotype b strains and a single nonserotypeable strain. This strong correlation supports our previous conclusion that the *A. actinomycetemcomitans* population consists of genetically isolated subpopulations (18).

Some ETs included several strains. These ETs may represent strains with a random association of frequent alleles because of extensive recombination between strains, or they may represent truly identical strains reflecting a clonal population structure characterized by a strong linkage disequilibrium. In ET 9, ET 28, and ET 44 the observed numbers of strains were 4, 7, and 6, respectively, whereas the corresponding expected numbers calculated on the basis of allele frequencies were 0.12, 0.32, and 0.16, respectively, when assuming a random association of alleles within the ET divisions. Thus, these ETs most likely encompass multiple isolates of the same clonal type. In contrast, ET 16, ET 17, ET 22, and ET 24 were characterized by strains with combinations of very frequent alleles of the

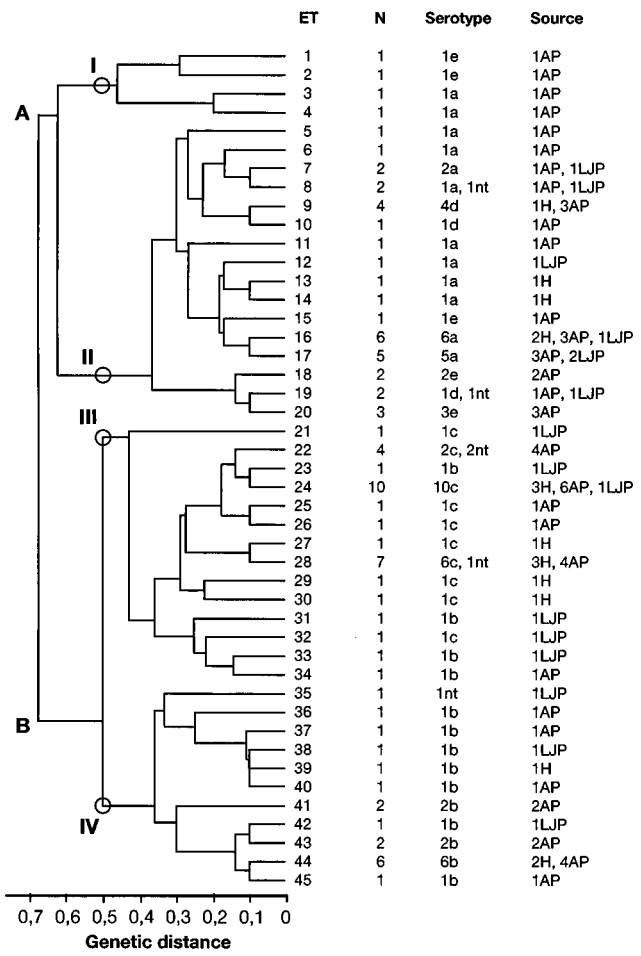

FIG. 1. Genetic relationship among 88 *A. actinomycetemcomitans* strains on the basis of allele profiles at 10 enzyme loci. The dendrogram was constructed from the ET typing data. ETs are numbered sequentially from top to bottom. Two major evolutionary lineages, lineages A and B, are indicated to the left, and four major divisions, termed I, II, III, and IV, are marked by circles in the dendrogram. Nonserotypeable strains are indicated by nt. The *A. actinomycetemcomitans* isolates originated from patients with AP and LJP and from patients with healthy periodontia (H).

gene loci examined and may represent strains with a random association of these alleles. The observed numbers of strains in ET 16, ET 17, ET 22, and ET 24 were 6, 5, 4, and 10, respectively, which were of the same order of magnitude as the expected numbers of 2.8, 1.4, 1.3, and 2.6, respectively.

No particular ET division of *A. actinomycetemcomitans* was associated with periodontal disease. All of the ETs containing several strains included isolates from healthy as well as periodontally diseased subjects except for ET 17 and ET 22, which included strains from periodontally diseased subjects only. However, as described above it is likely that these two ETs represent a random association of frequent alleles rather than representatives of a single clonal type. As shown in Fig. 1 there was no strong correlation between ET division and periodontal disease status except for ET division I, which, while very small, had an absolute association with AP.

Polymorphism of the structural leukotoxin gene and the *ltx* promoter region. Whole-cell DNA was prepared from each of the 88 strains. Only one strain, which constituted ET 29 (Fig. 1), harbored plasmid DNA, as revealed by agarose gel electrophoresis. To evaluate the degree of diversity of the leukotoxins

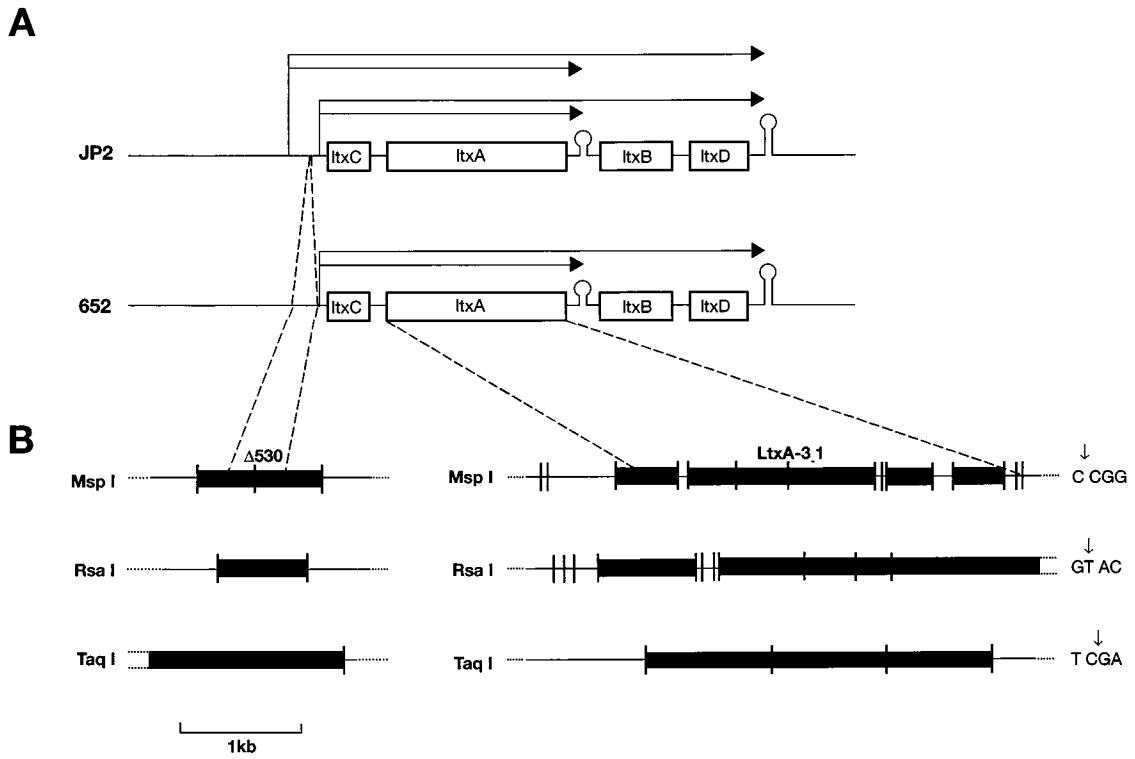

FIG. 2. Physical map of the leukotoxin gene operon. (A) Locations of the four genes in the leukotoxin gene operon and the transcripts, indicated by arrows, found in a highly toxic strain, strain JP2, and a minimally toxic strain, strain 652 (4). (B) Restriction map of the structural gene, *ltxA*, deduced from the published sequence from strain JP2 and map of the promoter region deduced from the sequence of strain 652. The vertical bars indicate sites for the restriction enzymes *Msp*I, *Rsa*I, and *Taq*I. Black boxes show the fragments expected to be visible on Southern blots hybridized with the probes Δ530 (to the left) and LtxA-3.1 (to the right).

in the collection of *A. actinomycetemcomitans* strains, we performed RFLP analysis of the structural gene *ltxA* using the three restriction enzymes *Msp*I, *Rsa*I, and *Taq*I. On the basis of the published *ltxA* sequence (12, 13), genomic DNA from strain JP2 digested with *Msp*I should yield six fragments with molecular sizes of 0.52, 0.40, 0.42, 0.72, 0.36, and 0.44 kb that are large enough to be visible on a Southern blot and with homology to the 3.1-kb *ltxA* probe, LtxA-3.1, derived from strain JP2 (Fig. 2). Similarly, *Rsa*I-restricted genomic DNA from strain JP2 should hybridize to LtxA-3.1 with five fragments of 0.80, 0.73, 0.42, 0.29, and >1.24 kb and four fragments of 1.12, 0.94, 0.86, and >0.42 kb when the DNA is digested with *Taq*I (Fig. 2). Thus, using these three four-base restriction endonucleases, we tested for cleavage at a total of 17 presumptive sites within the *ltxA* gene. As shown in Fig. 3 our results were in agreement with those deduced from the published sequence for strain JP2, although it was difficult to distinguish between some of the fragments in the *Msp*I digestions, because these fragments were very similar in size. The >0.42-kb *Taq*I fragment originating from the 3′ end of the *ltxA* gene presumably migrated together with the 0.94-kb fragment since the intensity of this band is slightly greater than expected.

The *ltxA* gene appeared to be very homogeneous in the *A. actinomycetemcomitans* population examined. The RFLP analyses with *Msp*I and *Taq*I revealed no detectable polymorphism of the structural leukotoxin gene among the 88 strains analyzed, whereas digestion with *Rsa*I revealed that the three serotype b strains included in ET 39 and ET 41 (Fig. 1) within the *ltxA* gene were different from the other 85 strains. In these three exceptional strains the 0.73-kb *Rsa*I fragment was slightly larger and migrated together with the 0.80-kb fragment, which was most likely a consequence of a mutated cleavage site within the *ltxA* gene. Some digestions showed additional larger hybridizing fragments (e.g., Fig. 3, lane 2), which presumably was due to partial digestion of the genomic DNA since these bands disappeared when the restriction was repeated for prolonged periods of time (data not shown).

To assay for variation in the *ltx* promoter region and to test for the presence of JP2-like promoters previously found to be associated with significantly increased toxin activity, the Southern blots were hybridized with the 530-bp fragment, termed Δ530, originating from the *ltx* promoter of strain 652 and deleted from strain JP2. All 88 strains analyzed hybridized to the Δ530 probe. No variation was detected by the enzymes *Rsa*I and *Taq*I, which resulted in fragments of 0.73 and 2.65 kb, respectively, that were recognized by Δ530 (Fig. 3). These sizes are consistent with the published sequence for the *ltx* promoter of strain 652. The restriction enzyme *Msp*I, however, revealed genetic polymorphism in the promoter region hybridizing to Δ530, amounting to a total of three variants among the 88 Finnish strains analyzed (Fig. 3). These different promoter types correlated strongly with serotype and ET division. All 21 serotype b strains examined and the four strains in ET division I (serotypes a and e) were characterized by two *Msp*I fragments that were equal in size (0.60 kb) and that hybridized to Δ530. The remaining 67 strains which constituted ET division II and the serotype c and nonserotypeable strains in ET division III showed, with one exception, two *Msp*I fragments of 0.60 and 0.56 kb with homology to Δ530, although the intensities of the two bands differed in the serotype e strain, which constituted ET 15. The sizes of the corresponding fragments of the excep-

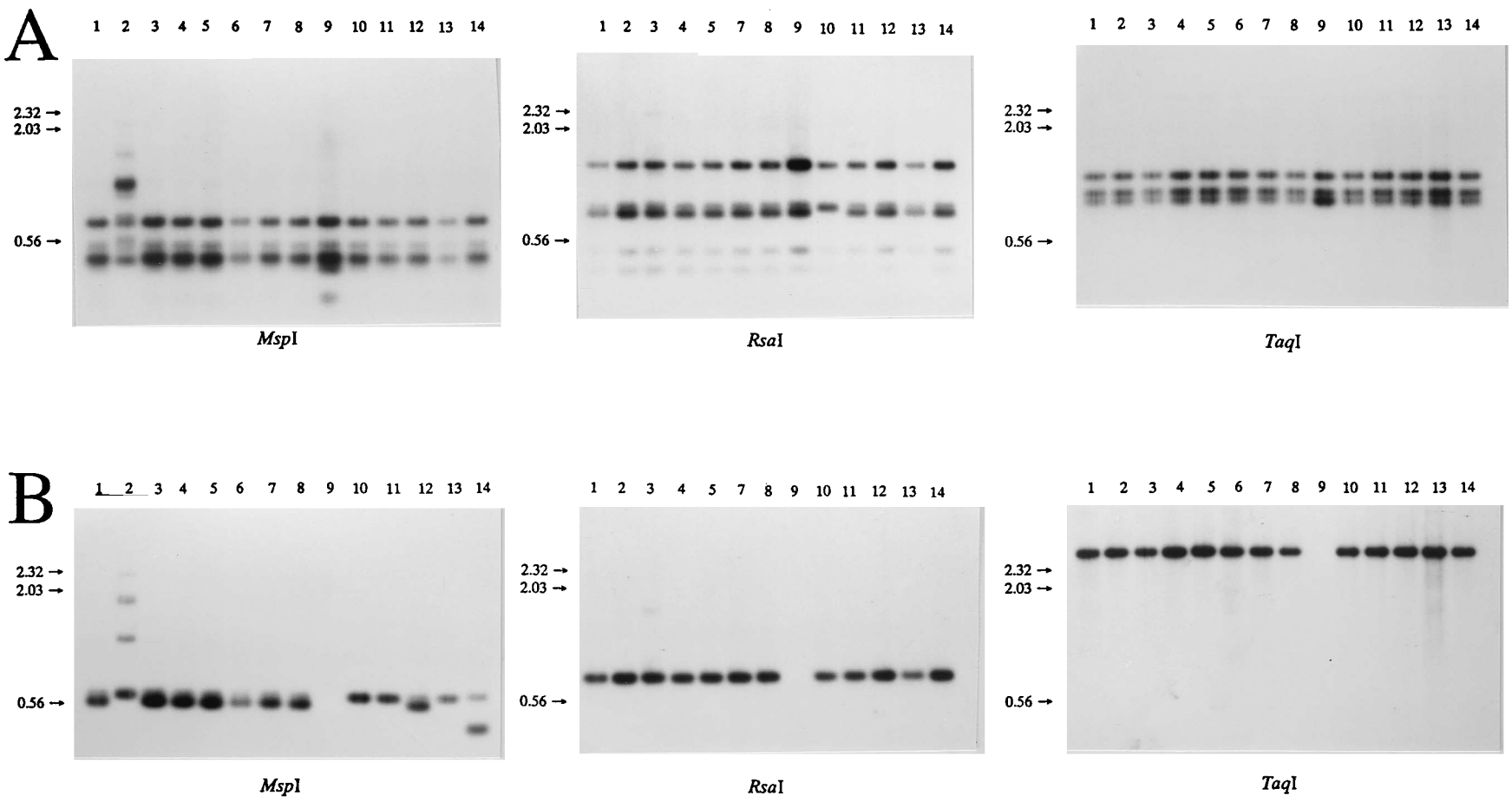

FIG. 3. Representative Southern blots showing genomic DNAs digested with the enzymes *Msp*I, *Rsa*I, and *Taq*I and hybridized with the two probes LtxA-3.1 (A) and Δ530 (B). Lanes: 1, HK1094 (nonserotypeable, ET 22); 2, HK1093 (serotype e, ET 2); 3, HK1091 (serotype e, ET 20); 4, HK1086 (serotype d, ET 9); 5, HK1085 (serotype d, ET 9); 6, HK1079 (serotype c, ET 29); 7, HK1061 (serotype c, ET 22); 8, HK1058 (serotype c, ET 27); 9, JP2 (serotype b); 10, HK1051 (serotype b, ET 41); 11, HK1042 (serotype b, ET 45); 12, HK1033 (serotype a, ET 7); 13, HK1031 (serotype a, ET 4); 14, HK1021 (serotype a, ET 17). Strain HK1079 (lane 6) was not included in the *Rsa*I digestion presented here.

tional serotype a strain belonging to ET 17 were estimated to be 0.60 and 0.44 kb.

DISCUSSION

MEE has successfully been used to elucidate the population structures of bacterial species and, in some cases, to differentiate between virulent and avirulent clones or subpopulations of human pathogens (6, 9, 15, 16, 30). *A. actinomycetemcomitans* has been isolated from subjects with periodontal disease and implicated as a causative agent, although the species may also be detected in the oral cavities of periodontally healthy subjects (10). The objective of the present study was to analyze the phylogenetic relationships in a cross-sectional collection of clinically well characterized *A. actinomycetemcomitans* isolates from Finland and to examine whether especially virulent clones or subpopulations of *A. actinomycetemcomitans* might be identified.

Several isolates from each of 81 subjects were cultured and serotyped. The results supported the general assumption that each individual is colonized by a single or a very few types (1), and the MEE typing supported the conclusion that the bacterium may be transmitted within families (1, 17, 20).

The MEE analysis showed that the structure of the *A. actinomycetemcomitans* population analyzed is very similar to what we have found previously for a temporally and geographically diverse collection of 97 *A. actinomycetemcomitans* strains (18). Thus, the present study confirms that the species consists of genetically separate subpopulations which, to a large extent, correspond to the serotypes of the strains.

Among the 88 Finnish strains some genotypes were more prevalent than expected when assuming a random association of alleles. The fact that the same clonal type apparently has been isolated repeatedly from different subjects suggests that some genotypes are more successful, e.g., in transmission or colonization, than others. Notably, these postulated differences in success were apparently not parallelled in differences in virulence since the clonal types did not associate with the disease status of the subject from whom the strains were isolated.

In general, we observed no strong correlation between periodontal disease status and genetic clustering of the strains. Thus, it appears that there is no significant difference in pathogenic potential among the genetic divisions of *A. actinomycetemcomitans*, although we cannot exclude the possibility that the presence of transmittable DNA elements like plasmids or phages may influence the pathogenic potential. The observed lack of association between specific chromosomal genotypes and disease together with the detection of the bacterium in healthy carriers suggest that the role of *A. actinomycetemcomitans* in periodontitis is largely opportunistic in the population examined. The same conclusion was reached on the basis of our previous study of isolates from other European countries. However, the fact that subjects in general are colonized only by a single clone of the species suggests that *A. actinomycetemcomitans* is not a traditional commensal organism.

Leukotoxin production is a putative virulence factor of *A. actinomycetemcomitans*. It appears that in the Finnish collection of strains included in the present study there are no differences in pathogenic potential caused by differences in the structure or the amount of toxin produced. There was very limited diversity in the primary structure of the leukotoxin since the structural gene, *ltxA*, encoding the toxin was extremely conserved among the 88 strains analyzed. Within the 3.1-kb gene we tested for the presence of a total of 17 4-bp sequences recognized by restriction enzymes, and all strains were identical except for three strains which differed at a single site. This limited polymorphism did not correlate with periodontal disease status. Although it is unlikely, we cannot exclude the presence of a functional or antigenic polymorphism of the leukotoxin as a result of variation in the *ltx* gene not detected by the technique used in the present study. None of the 88 Finnish isolates contained a deletion in the *ltx* promoter region, which has previously been shown to identify strains with highly elevated levels of leukotoxin production (5), nor was this deletion detected among more than 60 isolates from northern Europe which we have analyzed previously (18). An *Msp*I polymorphism detected in the promoter region of the *ltx* gene confirmed the genetic division of the *A. actinomycetemcomitans* population, and like for the other genetic traits analyzed, this polymorphism did not correlate with disease. However, we cannot exclude the possibility that this genetic variant detected by *Msp*I is associated with altered expression of the leukotoxin gene operon.

In contrast to the results of the present study, DiRienzo and McKay (7) analyzed a collection of *A. actinomycetemcomitans* strains from a regional population in Philadelphia, Pa., and found a correlation between families with a history of LJP and specific genetic variants identified by RFLP analysis using an anonymous probe cloned from *A. actinomycetemcomitans*. The reason for these discrepancies is unknown, but it may suggest that the genetic structures of the two bacterial populations studied are different; e.g., there might exist a clone in the collection from Philadelphia with enhanced pathogenic potential. In the Philadelphia collection DiRienzo and McKay (7) found several isolates characterized by that version of a *Hin*dIII polymorphism in the *ltx* gene operon which reflects the deletion in the promoter region identifying the highly toxic type. Interestingly, this *Hin*dIII variant was associated with strains of an RFLP type which was prevalent among strains from subjects with LJP but was not found among those isolated from healthy carriers. In a previous study of a diverse collection of 97 *A. actinomycetemcomitans* strains originating from Europe and the United States (5) we detected only two strains of the highly toxic type, and both strains originated from the United States. This supports the hypothesis presented above that different epidemiological situations may exist in Europe and the United States and that a particularly virulent clone characterized by the high-level production of leukotoxin is present in the United States but is absent from northern Europe.

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