Microevolution and Patterns of Dissemination of the JP2 Clone of 

*Aggregatibacter (Actinobacillus) actinomycetemcomitans*

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Running head: Microevolution of the JP2 clone

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ABSTRACT

The natural history, microevolution, and patterns of inter-individual transmission and global dissemination of the JP2 clone of *Aggregatibacter (Actinobacillus) actinomycetemcomitans* were studied by population genetic analysis. The JP2 clone is strongly associated with aggressive periodontitis in adolescents of African descent and differs from other clones of the species by several genetic peculiarities including a 530-bp deletion in the promoter region of the leukotoxin gene operon, which results in increased leukotoxic activity. Multilocus sequence analysis of 82 *A. actinomycetemcomitans* strains, of which 66 were JP2 clone strains collected over a period of more than 20 years, confirmed a clonal population structure with evolutionary lineages corresponding to serotypes. Although genetically highly conserved, as evidenced from alignment of sequences of eight housekeeping genes, the JP2 clone showed a number of point mutations particularly in the pseudogenes Ψ-hbpA and Ψ-tbpA. Characteristic mutations allowed distinction of isolates from individuals from the Mediterranean area and from West Africa including the Cape Verde Islands. The patterns of mutations indicate that the JP2 clone initially emerged as a distinct genotype in the Mediterranean part of Africa approximately 2,400 years ago and subsequently spread to West Africa from where it was transferred to the American continents during the transatlantic slave trade. Sustained exclusive colonization of individuals of African descent despite geographical separation for centuries suggests a distinct host tropism of the JP2 clone. The colonization of family members by JP2 clone strains with unique point mutations provides strong evidence of intrafamilial transmission, and suggests that dissemination of the JP2 clone is restricted to close contacts.
INTRODUCTION

Aggressive periodontitis is a major clinical problem eventually resulting in loss of the supporting tissues of teeth at an early age. It is an infectious disease but the complexity of the microflora that constitutes the biofilm in the affected periodontal pockets makes it difficult to identify the exact etiology (44). Early studies demonstrated an association between disease and the presence of bacteria belonging to the species Actinobacillus actinomycetemcomitans (51), which recently was reclassified in the new genus Aggregatibacter together with its close relatives Aggregatibacter (Haemophilus) aphrophilus and Aggregatibacter (Haemophilus) segnis (42). A. actinomycetemcomitans has its primary habitat in dental plaques and may be isolated also from part of the healthy population (32, 51). It is a recognized opportunistic pathogen occasionally causing endocarditis and abscesses at various body sites and internal organs (43, 45).

A. actinomycetemcomitans isolates from healthy individuals, patients with periodontitis, and from cases of systemic infections show significant genetic diversity in agreement with its status as an opportunistic pathogen. However, the majority of isolates from aggressive periodontitis in adolescents of African descent living in different parts of the world are genetically homogeneous and belong to a single clone termed the JP2 clone (19, 21, 22, 25). Studies of adolescents in Morocco demonstrate that the JP2 clone is endemic to that population and that it is strongly associated with disease in contrast to other clones of A. actinomycetemcomitans (24, 25). The concept of the high pathogenic potential of the JP2 clone was also supported in a longitudinal study of African-American families with aggressive periodontitis (9). The association between the JP2 clone and periodontitis in adolescents was demonstrated in several study populations living geographically widespread, however all supporting the association of the JP2 clone with patients of African descents (10, 11, 18-22, 31, 35). Isolation of the JP2 clone from occasional periodontitis patients of other ethnic origin was mentioned recently but without detailed information (18). The
possible spread of the JP2 clone to other ethnic groups requires further study and should take into account the inherent complexity of defining genetic background in some human populations (3, 6).

The primary association of the JP2 clone with patients of various African descents and its putative unusual virulence may contribute to observed differences in prevalence of aggressive periodontitis in different countries (2).

The JP2 clone is named after the first recognized isolate belonging to the clone, which was obtained from an 8-yr old African-American child with prepubertal periodontitis (56). It was first noted because of its enhanced leukotoxic activity caused by a specific deletion of 530 base pairs (bp) in the promoter region of the leukotoxin gene operon (8). The clone belongs to serotype b and is further characterized by a distinct profile of alleles of intracellular metabolic enzymes as revealed by multilocus enzyme electrophoresis, a distinct MspI DNA fingerprint, and by clear β-hemolytic zones on blood agar caused by the enhanced leukotoxic activity (5, 21, 22). Besides, as a result of a mutational event affecting the gene encoding the hemoglobin-binding protein (Ψ-hbpA), members of the JP2 clone are unable to use human hemoglobin as an iron source (26).

According to Maynard Smith (37) a bacterial clone is defined as "a set of genetically similar cells, recently derived from a common ancestor, without chromosomal recombination". This definition takes into account the minor evolutionary diversification that inevitably occurs in the long-term perspective as a result of accumulation of mutations. In A. actinomycetemcomitans genomic reorganizations facilitated by intra-genomic homologous recombination between the multicopy rRNA operons and IS150-like elements are unusual contributors to this genetic diversification process (15), which, even among members of the JP2 clone, result in differences in ribotyping patterns and in PFGE patterns of restriction fragments after cleavage of genomic DNA with rare-cutting endonucleases (e.g. XhoI) (15, 21, 22).
In the study reported here we have exploited the markers of genetic diversification within the JP2 clone of *A. actinomycetemcomitans* to obtain information about its natural history and patterns of inter-individual transmission and global dissemination.

**MATERIAL AND METHODS**

**Bacterial strains and growth conditions.** A total of 82 *A. actinomycetemcomitans* strains, 66 with a 530-bp deletion in the promoter region of the leukotoxin (*ltx*) gene operon (JP2 strains) and 16 without this deletion (non-JP2 strains) were included in the study. The strains without the 530-bp deletion included 4, 5, 2, 2, 2, and 1 strains of serotypes a, b, c, d, e, and non-serotypeable, respectively, representing the major evolutionary lineages observed by Poulsen and coworkers (48). Among non-JP2 serotype b strains were HK1605 (UP14), which belongs to the RFLP II group of strains described by DiRienzo and coworkers (12, 13), and strain HK975 (Y4) of Tsai et al. (55). JP2 strains with the 530-bp deletion were isolated from 66 subjects living geographically widespread and span a period of isolation of more than 20 years. Strains were obtained from individuals with an origin in Morocco, Algeria, Israel, Turkey, Portugal, and the Cape Verde Islands, of which some had migrated to Northern European countries (Denmark, Sweden, Switzerland, and the Netherlands), and from individuals now living in Brazil and the US but of African, and Hispanic ethnicity. It is generally accepted that Hispanics have a mixture of native American, African, and European ancestry (6). Five of the strains assigned to the JP2 clone were obtained from laboratories in the Netherlands (two strains) and the US (three strains) and were isolated from periodontitis patients of unspecified ethnic background. Among the 66 JP2 strains 17 strains were obtained from individuals belonging to 7 families.
Leukotoxin promoter subtyping by PCR. Differentiation of the two promoter region types with and without the 530-bp deletion upstream of the \textit{ltx} gene operon was determined by PCR as previously described using primers ltx3 and ltx4 (46).

\textit{MspI DNA fingerprinting.} Whole-cell DNA was prepared as described (47). Approximately 10 µg was digested with \textit{MspI}, electrophoresed in a 1% agarose gel overnight at 1.5 V cm\(^{-1}\) in TAE buffer, and visualized by staining with ethidium bromide (50).

\textit{XhoI DNA fingerprinting.} \textit{XhoI} DNA fingerprinting was performed as previously described (15). Briefly, bacteria were grown on chocolate agar plates and washed off the plates in buffer. Most strains were of the adherent rough phenotype, and to resolve aggregation mediated by proteins the bacterial suspension was treated with proteinase K. After preparing the agarose plugs the proteinase K was inactivated by incubation with PMSF. After washing, the plugs were treated with lysozyme. The plugs were digested with proteinase K followed by treatment with PMSF. DNA in the plugs was digested with \textit{XhoI} in the buffer supplied with the enzyme by adding the restriction enzyme three times with 20 h intervals. \textit{XhoI} was added three consecutive times because we found it difficult to obtain a complete digest of the genomic DNA in the plugs. The resulting DNA fragments were resolved by PFGE.

Sequence analysis of genes encoding housekeeping enzymes and pseudogenes for \textit{hemoglobin-binding and transferrin-binding proteins}. Fragments of genes for six housekeeping enzymes were selected for the MLST analysis: \textit{pgi} encoding glucose-6-phosphate isomerase, \textit{recA} encoding RecA protein, \textit{adk} encoding adenylate kinase, \textit{frdB} encoding fumarate reductase, \textit{atpG} encoding the gamma subunit of ATP synthase F1, and \textit{mdh} encoding malate dehydrogenase. These housekeeping genes were selected among the seven genes included in the \textit{Haemophilus influenzae} MLST scheme (http://haemophilus.mlst.net). One copy of each of the six housekeeping genes was found in the \textit{A. actinomycetemcomitans} HK1651 genome (http://www.genome.ou.edu/act.html).
from which the primers were designed (Table 1). The seventh *H. influenzae* gene (*fucK*) was not
found in *A. actinomycetemcomitans*. The six genes were located widespread in the 2.1-Mb genome
of HK1651 (Table 1) and not in close proximity to any virulence genes that might be expected to be
under selection pressure. In addition, two fragments of the hemoglobin-binding protein gene *hbpA*
(which is a pseudogene, Ψ-hbpA, in JP2 strains) and one fragment of the pseudogene for
transferring-binding protein, Ψ-tbpA, were sequenced (Table 1). The PCR and DNA sequencing
were performed as previously described (15). The regions sequenced are shown in Table 1. The
selected fragments from serotype a, c, d, and e strains were sequenced in both directions. Fragments
from strains belonging to the serotype b cluster of strains were only sequenced in one direction
except if polymorphic sites were revealed. Fragments with polymorphic sites were sequenced in
both directions for confirmation.

For alignments of the sequences we used the program ClustalX (54). Cluster analysis of the 82
isolates, based on a concatenated sequences consisting of 9 sequenced DNA fragments in the order
*adk, atpG, frdB, mdh, pgi, recA, hbpA-1, hbpA-2, and tbpA*, was conducted by the Minimum
Evolution algorithm in MEGA version 3.0 (33).

**Sequence accession numbers.** The DNA sequences determined in this study were deposited in
the GenBank database (GenBank, National Center for Biotechnology Information,
http://www.ncbi.nlm.nih.gov). The accession numbers for the sequences are as follows: *adk* alleles
alleles EF142363-EF142443, *hbpA-2* alleles EF142444-EF142524, *mdh* alleles EF142525-
EF142606, *pgi* alleles EF142607-EF142688, *recA* alleles EF142689-EF142770, and *tbpA* alleles
EF142771-EF142852.
RESULTS

Genomic variation as determined by *Msp* I fingerprinting of whole-cell DNA. A total of 16 different RFLP patterns were found among the 82 isolates after cleavage of whole-cell DNA with the restriction enzyme *Msp* I and analyzed by conventional 1% agarose gel electrophoresis. All 66 JP2 strains had an identical DNA fingerprint, except for strain HK1986 which deviated slightly due to loss of two bands (Fig. 1, lane 12). Different *Msp* I fingerprints were found among the 16 non-JP2 strains without the 530-bp deletion and with different serotypes, except for two strains belonging to serotype d with a unique and identical pattern (Fig. 1, lane 3). Among non-JP2 strains of serotype b strain HK911 had a DNA fingerprint identical to the characteristic DNA cleavage pattern for JP2 strains (Fig. 1, lane 8), strains HK912 and HK1605 (UP14) had very similar but distinct fingerprints (Fig. 1, lanes 7 and 13), and strains HK908, HK913, and HK975 also had patterns similar to the JP2 clone strains (Fig. 1, lanes 9, 10, and 11). Thus, the JP2 clone is closely related to other non-JP2 serotype b strains. As previously shown by DiRienzo and coworkers (12, 13) strain HK1605 (UP14) constituted an exception in their study as it belonged to the RFLP II group, showing the strongest correlation with disease and conversion to disease, but HK1605 (UP14) did not have the 530-bp deletion in the promoter region of the leukotoxin gene operon that is characteristic to the JP2 clone (9).

Genomic variation as revealed by *Xho* I DNA fingerprinting. We have previously shown that PFGE using the rare-cutting restriction enzyme *Xho* I can demonstrate genomic rearrangements in strains of the JP2 clone (15). By use of this method 34 different *Xho* I DNA fingerprints were revealed among the 82 *A. actinomycetemcomitans* strains studied. Representative examples of *Xho* I DNA fingerprints are illustrated in Fig. 2A. Fifteen different *Xho* I DNA fingerprints were found among the 16 non-JP2 strains, meaning that each of the non-JP2 strains had a unique *Xho* I DNA fingerprint, except for two serotype b strains, HK911 and HK1605 (UP14), with an identical pattern.
(Fig. 2A, lanes 9 and 11). Among the 66 JP2 clone strains 19 different patterns were identified. One XhoI DNA fingerprint was dominating (demonstrated in 33 out of 66 strains), and was found among JP2 clone strains only (Fig. 2A, lane 13 and Fig. 2B, lanes 1 and 2). Five different XhoI DNA fingerprints among the remaining 33 JP2 strains were each represented by 6, 5, 4, 3, and 2 strains, respectively, and the remaining 13 strains each had a unique pattern. The second and third most dominating XhoI DNA fingerprint among JP2 clone strains were very similar to XhoI DNA fingerprints found among serotype b non-JP2 strains, represented by one (HK913) and two (HK911, HK1605 (UP14)) strains, respectively (Fig. 2A, lanes 9, 10, 11, 14, and 15). Twelve out of 17 isolates from members belonging to seven families showed the most frequent XhoI DNA fingerprint including all isolates from family 1, 2, and 4, HK1507, HK1512, HK1514 from family 3, HK1520 from family 5, and HK1613 from family 7 (Fig. 2A, lane 13 and Fig. 2B, lanes 1 and 2). XhoI DNA fingerprints of isolates from family 3, 5, 6, and 7 showed two different patterns in each of the families.

**Overall population structure of A. actinomycetemcomitans and microevolution in the serotype b cluster of strains as revealed by multilocus sequence analysis.** The multilocus sequence analysis was based on fragments of genes encoding six house keeping enzymes, two fragments of the hbpA gene which is a pseudogene (Ψ-hbpA) in the JP2 strains, and a fragment of the tbpA pseudogene (Ψ-tbpA). Concatenated sequences of these nine gene fragments adding up to 4143 nucleotides was used to index the overall genetic similarity among strains. The PCR using the primer sets for hbpA did not result in amplification of a product for strain HK975 (Y4). Strain HK929 had an insertion sequence in the hbpA-2 fragment leaving only 187 bp (position 525072 to 525258 in the HK1651 genome) of this fragment to be included in the concatenated sequence of this strain.
Cluster analysis demonstrated a close relationship to serotypes (Fig. 3). The serotype b strains including JP2 clone strains constituted a separate cluster within the dendrogram, and this close genetic relatedness among JP2 and non-JP2 strains of serotype b is in agreement with their very similar MspI fingerprints.

The 66 JP2 strains were genetically very homogeneous and only 11 sequence types (STs) with minor differences were identified (Table 2). In the sequenced DNA fragments from the six housekeeping enzymes a total of five point mutations were found among JP2 clone strains in the sequenced fragments of adk, atpG, frdB, and recA, whereas no variation was found in the fragments of mdh and pgi. Thus, the frequency of polymorphic sites in the six functional housekeeping gene fragments varied from 0 (mdg and pgi) to 1:250 nucleotides (atpG). Among the five mutations three were transitions and two were transversions, and all were silent mutations. The frequency of mutations detected in JP2 clone strains in the three fragments of the two non-functional pseudogenes was higher (1:165 – 1:220) than in the sequenced fragments from housekeeping enzyme genes in agreement with the lack of functional constraints on sequences from pseudogenes.

The genetic diversity among non-JP2 strains of serotype b has previously been shown to be much higher than among JP2 strains. Thus, among 26 serotype b non-JP2 strains 18 distinct ETs were revealed by MLEE (48), whereas 38 JP2 clone strains except for one showed an identical ET (22). Based on this difference in diversity and because all JP2 clone strains have a unique deleterious mutation in the hbpA gene not found in other strains of the species, it is conceivable that the JP2 clone is relatively new and that a non-JP2 strain of serotype b is the progenitor of the JP2 clone. Assuming that the sequence variation observed in the serotype b cluster (Table 2) is caused by single site mutations and that each mutation has occurred only once, it is possible to deduce the evolutionary scenario of the 72 strains in the serotype b cluster as outlined in Fig. 4. The cluster of serotype b strains consisted of two evolutionary lineages of which all six non-JP2 strains clustered
separately primarily due to one polymorphic site (position 525077 in the HK1651 genome) in the 
*hbpA* gene. In addition, a specific mutation in the non-functional $\Psi$-*hbpA* in the JP2 strains
(position 525285 in the HK1651 genome) divided the group of JP2 strains further into two
 evolutionary lineages (Table 2, Fig. 4). The vast majority of isolates in one cluster of JP2 clone
 strains (ST1 to ST4) were from individuals with an origin in the Mediterranean area (32 out of 34
 individuals with known origin (94%)), whereas in the other cluster (ST5 to ST11) 23 out of 27
 isolates (85%) were from individuals with known origin in West Africa or were West African
descendants living in the Cape Verde Islands, in Brazil, or in the U.S.A. (Fig.4). In addition to this
 mutation, which resulted in separation of JP2 clone strains into two major clusters, 15 of the JP2
 clone strains had a single mutation, and one strain, HK1651, had two mutations within the 4143
 nucleotides analyzed.

Comparison of JP2 clone strains isolated from family members as revealed by multilocus
sequence analysis. Among the 66 JP2 clone strains included in this study 17 originated from seven
families with two to four individuals representing each family (Fig. 4). Two pairs of related
individuals, a sister and a brother from Algeria living in Denmark (family 2), and a mother and her
daughter from Morocco (family 1), were all colonized by the dominating ST 1 (Fig. 4, Table 2).
Two individuals from each of two families were colonized by ST8 (family 5 and 6). Each of the
remaining three families was colonized by a unique type (ST4, ST9, and ST11) of the JP2 clone
(Fig. 4, Table 2). Strains isolated from three related siblings (family 4) living in Denmark and
originating from Morocco had a single unique point mutation in the sequenced fragment of *frdB*
(ST4). Isolates from three siblings originating from the Cape Verde islands and living in Sweden
(family 3) carried a unique type of the JP2 clone with a point mutation in the *atpG* gene (ST9),
whereas the fourth sibling in this family carried *A. actinomycetemcomitans* of the prevailing ST1.
Finally, two African-American family members (family 7) were colonized by a strain with a unique point mutation in the sequenced recA gene fragment (ST11).

DISCUSSION

Identification of bacterial clones clearly associated with disease often leads to better understanding of decisive virulence properties, routes of dissemination, and potential specific host adaptations (39, 41, 52). In this study we examined the natural history, microevolution, and patterns of global dissemination and inter-individual transmission of the JP2 clone of A. actinomycetemcomitans, which is strongly associated with aggressive periodontitis in adolescents of African descent (9, 24, 25). Our previous studies demonstrated that the JP2 clone differs from other clones of the species by several genetic peculiarities including a 530-bp deletion in the promoter region of the leukotoxin gene operon resulting in increased leukotoxic activity (8, 31). Our study was based on 66 JP2 clone strains collected over a period of more than 20 years and isolated from individuals living on five continents plus 16 non-JP2 strains representing the different evolutionary lineages of the A. actinomycetemcomitans population (48). The number of JP2 clone strains from each continent and country clearly does not provide a complete picture of the global distribution of the clone. The collection is to a significant degree affected by availability of isolates and the strains that we have been able to collect during our long-term interest in this species. In spite of geographic biases of the strain collection, the study reveals interesting new information about the JP2 clone.

The population genetic analysis performed in this study was based on sequences in eight loci. MLSA of the basically selectively neutral sequences provides a quantitative measure of the similarity between strains and is suited for inferring phylogeny. Although it is conceivable that examination of additional loci would reveal additional polymorphic sites, seven loci is considered a
reasonable sample of the genome for estimating the overall population structure of bacteria (36). As

*Actinobacillus actinomycetemcomitans* has a clonal population structure, there is no reason to assume that

inclusion of additional loci would affect the observed structure.

According to the observed sequence polymorphism, which is quite compatible with the current
definition of bacterial clones (37), the 66 JP2 strains were divided into two major clusters, which
provide important epidemiological information (Fig. 4). A mutation in Ψ-hbpA divided the JP2
clone into strains that originated in two groups of human hosts that belong to distinct ethnic groups
(i.e. Arabs and Africans) and differ by culture, religion, and history (Fig. 4). Thus, the characteristic
mutation distinguished isolates from individuals from the Northern Mediterranean part of Africa
from isolates from West Africa including the Cape Verde Islands. Isolates from patients in Brazil
and the United States of America were indistinguishable from isolates from West Africa in support
of the assumption that the JP2 clone was disseminated to the American continents by the
transatlantic slave trade.

Sharing of a characteristic nucleotide (the G nucleotide in *hbpA* at position 525285 in the
HK1651 genome) among non-JP2 strains of serotype b and JP2 clone strains with ST 1 to ST4 (Fig.
4) strongly suggests that the JP2 clone originated in the Northern Mediterranean part of Africa
where it is still endemically present and associated with an unusually high prevalence of aggressive
periodontitis according to our studies of Moroccan adolescents (25). A strain with the point
mutation changing G to A in Ψ-hbpA (position 525285 in the HK1651 genome) characteristic of the
other cluster of JP2 clone strains (ST 5 to ST11) was subsequently spread to the ethnically different
population of Western Africa and, during the transatlantic slave trade, further to the North and
South American continents.

Identification of strains without the 530-bp deletion that are identical to the JP2 strain in the six
housekeeping genes indicates that this is the allelic composition of the immediate ancestor of the
JP2 clone. This provides an opportunity to estimate the approximate age of the JP2 clone. Among the 56 independent isolates of the JP2 clone a total of 5 synonymous mutations, with a maximum of one per isolate, were found within the combined 3031 nucleotides of six housekeeping genes (Table 2). It is generally assumed that sequence diversity at synonymous sites accumulates by mutations at a relatively constant rate (molecular clock hypothesis). Assuming that the mutation rate in A. actinomycetemcomitans is $1.4 \times 10^{-10}$ as demonstrated in *Escherichia coli*, that 23.7% of the nucleotides in the sequence are at risk of synonymous mutations (14, 34), and that its generation time in biofilms *in vivo* is 24 hours, it can be calculated that the JP2 clone emerged as a distinct genotype some 2,400 years ago. This is fully compatible with the necessary assumption, based on history and our findings here that the characteristic leukotoxin promoter deletion and the divergence into at least two separate lineages now colonizing Arabs and Africans, respectively, took place after that time and prior to the transatlantic slave trades.

We have previously hypothesized that the JP2 clone has developed a distinct host tropism to individuals with a genetic origin in Northern and Western parts of Africa (22). Studies of several other pathogens have revealed geographically structured populations with distinct types or lineages causing infection in distinct populations of human hosts, in some cases discernable also after human migration (1, 16, 17, 29, 39, 40, 58). Such patterns may be due to geographic and/or social separation as an alternative to specific tropisms resulting from coevolution of specific lineages of bacteria with distinct lineages of hosts. Several findings support the conclusion that specific host tropism is the major factor behind the restricted epidemiology of the JP2 clone. First, there is no direct evidence that the clone has been disseminated to Caucasian and Asian populations despite its widespread geographical dissemination for centuries with hosts of African descent (10, 11, 18-20, 28, 35, 38, 48, 53). Secondly, the two lineages of JP2 clone isolates demonstrated in this study have remained separated according to host ethnicity within a restricted geographic area of the African
continent. Thirdly, although social separation of races was the rule in most countries for centuries
there was a long tradition for African-American foster mothers and maids in Caucasian families in
the U.S.A.. If not restricted by host tropism it is likely that this habit would have resulted in transfer
of the JP2 clone to Caucasian children as the majority of the oral microflora is acquired early in life
from the mother or other close contacts.

Only in a few cases has the molecular mechanism of specific tropisms of microorganisms been
identified (7, 49). However, it will be of obvious interest to study the molecular mechanisms of the
host tropism of the JP2 clone in order to be able to evaluate the chances that this pathogen, and the
high prevalence of aggressive periodontitis, eventually may spread to other human populations as a
consequence of changing demographic patterns.

Our demonstration of identical sequence types of the JP2 clone strains in several members of
each of seven families, and in particular, in three families each with a unique sequence type (Fig. 4)
provides strong evidence of transmission of the JP2 clone of A. actinomycetemcomitans within
families and supports earlier reports (4, 57).

PFGE is a frequently used tool in studies of bacterial transmission. We recently demonstrated that
this technique discloses considerable polymorphism, even among members of the JP2 clone, due to
intragenomic inversions resulting from homologous recombination between multicopy sequences in
the genome (15). We therefore tested the applicability of this technique for studies of the local
epidemiology of the JP2 clone. A high number of XhoI fingerprints were found among both JP2 and
non-JP2 strains of A. actinomycetemcomitans. However, due to the possibility of homologous
intragenomic recombination, and the potentially reversible nature of the events, interpretation on
evolutionary issues based on results obtained by PFGE should be performed with precaution. Two
out of three families (families 3 and 7) harbored JP2 clone strains with identical unique point
mutations in the sequenced housekeeping genes, and yet with different XhoI fingerprints in the
PFGE analysis. This finding suggests that the intragenomic inversions leading to changing patterns take place at a very high rate. As a result, the method is applicable only for very short term epidemiological studies.

The clonal population structure of *A. actinomycetemcomitans* (20, 27, 30, 48) confirmed by the data obtained in this study makes it possible to trace distinct properties that determine virulence. In line with other bacterial species in the oral flora, it is plausible to consider *A. actinomycetemcomitans* an opportunistic pathogen as phylogenetically diverse and unique strains were recovered from Caucasian individuals with periodontal or various systemic diseases (20, 30, 43, 48). However, the JP2 clone takes up a special position due to its strong association with disease and its global dissemination within particular ethnic groups. It has been argued by Kaplan and coworkers (30) that the JP2 clone simply may be more prevalent in some populations of Arabs and Africans and that the increased prevalence of aggressive periodontitis in these ethnic groups may be due to increased susceptibility intrinsic to the host rather than to a particular pathogenic potential of the JP2 clone. The mechanisms of the increased susceptibility for aggressive periodontitis in hosts of Arabic and African origin needs to be explored further. However, substantial evidence supports the conclusion that the highly toxic JP2 clone constitutes a particular virulent subpopulation of *A. actinomycetemcomitans* (9, 23, 24, 31). Combined with the successful global dissemination of members of the JP2 clone, which implies that horizontal transmission is an important mechanism of its spread in addition to the well-established vertical transmission, these findings sustain the picture of the JP2 clone as having traits similar to that of traditional pathogens.

In conclusion, this study suggests that the JP2 clone of *A. actinomycetemcomitans* originated in the Northern Mediterranean part of Africa and spread to West Africa from where it was transmitted to other continents initially during the transatlantic slave trade in the 16th to 18th century. Distinct lineages of the JP2 clone are still associated with Arabic and African populations, respectively, and
most likely developed tropism for these hosts. With its highly conserved genome as reflected by the MLSA the JP2 clone may be a valuable marker for tracking the population migration due to sustained signatures at the nucleotide level. Results presented in this study can provide a better understanding of the global epidemiology of aggressive periodontitis and may help to anticipate future global trends in the clinical picture of the disease.

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REFERENCES


### TABLE 1. Primers used in multilocus sequence analysis, and length and position of the sequenced fragments

<table>
<thead>
<tr>
<th>Gene</th>
<th>PCR and sequencing primers (5´-3´)</th>
<th>Length of sequenced fragment (bp)</th>
<th>Position of sequenced fragment in the HK1651 genome&lt;sup&gt;a&lt;/sup&gt;</th>
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<sup>a</sup>http://www.genome.ou.edu/act.html
### TABLE 2. Polymorphic sites among 72 serotype b strains

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a ST: multilocus sequence type
b position in the HK1651 genome (http://www.genome.ou.edu/act.html)
c Aa: A. actinomycetemcomitans
FIGURE LEGENDS

FIG. 1. Genotyping of *A. actinomycetemcomitans* strains by restriction of whole-cell DNA with *Msp* I and resolved by 1% agarose gel electrophoresis. Molecular weight markers in kilobases are shown to the left. Since *Msp* I is a frequently cutting enzyme most of the resulting DNA fragments are smaller than 2 kb. These small fragments are seen as a smear in the front of the gel and only few larger fragments are seen as bands in the gel. Thus, the bands of DNA fragments larger than 2 kb represent only a small fraction of the genome (approximately 5%). Lane 1, molecular weight marker; lane 2, HK929; lane 3, HK928; lane 4, HK978; lane 5, HK780; lane 6, HK974; lane 7, HK912; lane 8, HK911; lane 9, HK913; lane 10, HK908; lane 11, HK975 (Y4); lane 12, HK1986; lane 13, HK1605 (UP14); lane 14, HK1630; lane 15, HK921 (JP2); lane 16, HK1651.

FIG. 2. *Xho* I DNA fingerprinting of *A. actinomycetemcomitans* strains. Bacteria in agarose plugs were digested with *Xho* I, and the resulting DNA fragments resolved by PFGE. Molecular weight markers in kilobases are shown to the left. (A) Representative *Xho* I DNA fingerprints of *A. actinomycetemcomitans* strains without and with the 530-bp deletion in the leukotoxin gene operon (non-JP2 and JP2 strains, respectively). Serotype and leukotoxin promoter type of each *A. actinomycetemcomitans* strain are given below the picture. Lane 1, HK892; lane 2, HK907; lane 3, HK978; lane 4, HK928; lane 5, HK1002; lane 6, HK929; lane 7, HK961; lane 8, HK908; lane 9, HK911; lane 10, HK913; lane 11, HK1605; lane 12, HK975; lane 13, HK1507; lane 14, HK1985; lane 15, HK1651; lane 16, HK1607; lane 17, HK1548; lane 18, HK1615; lane 19, HK1659. Lanes 9 and 11 were scored as identical fingerprints whereas the remaining ones were scored as distinct. (B) *Xho* I DNA fingerprints of JP2 clone strains from related and unrelated individuals. Lane 1, HK1536 (family 4); lane 2, HK1631 (family 4); lane 3, HK2006; lane 4, HK1548 (family 6); lane 5, HK1517 (family 3). Lanes 1 and 2 represent the prevailing PFGE pattern of the JP2 clone from two
related individuals. Lane 3 shows an example of a unique PFGE pattern. Lanes 4 and 5 show
identical PFGE pattern from two unrelated individuals, one from an African American family and
one from a family living in Sweden, and originating from the Cape Verde Islands. The upper band
in lanes 3, 4, and 5 represents undigested DNA.

FIG. 3. Phylogenetic relationships among 82 *A. actinomycetemcomitans* strains based on cluster
analysis using the Minimum Evolution algorithm of concatenated sequences of fragments of six
housekeeping enzyme genes, and three DNA fragments from genes encoding haemoglobin-binding
and transferring-binding proteins. A total of 66 strains with the 530-bp deletion in the leukotoxin
gene operon (JP2 clone strains) obtained geographically widespread and 16 without the deletion
(non-JP2 strains) were studied. The scale bar below the dendrogram shows the genetic distance. The
numbers adjacent to the nodes show the percent of bootstrap replicates that support the
corresponding cluster. Electrophoretic type divisions (ET) as determined by multilocus enzyme
electrophoresis in a previous population genetic study of *A. actinomycetemcomitans* are given (48).

actinomycetemcomitans* strains based on genetic variation revealed by MLSA and based on the
assumptions described in the Result section. The cluster of non-JP2 strains is marked by hatched
ellipses, and the JP2 clone strains are marked by black ellipses. Each arrow in the model indicates a
single mutation in the 4143 nucleotides analyzed and each mutation is denoted by position in the
HK1651 genome, name of gene involved, and the nucleotide substitution. The 530-bp deletion in
the *ltx* promoter is marked by ∆530. Bacterial isolates from related family members in seven
families are indicated, as well as country of origin of the individual from whom the JP2 clone
isolates originated. If the individual is living in another country than the country of origin, this is
given after the small arrow. Isolates originating from individuals with an origin in the
Mediterranean area are marked with ●, and isolates from individuals with West African origin,
including African-Americans from the US are marked with ■. No information about origin was
available for five JP2 clone-positive individuals, three living in the US and two living in the
Netherlands. All JP2 strains from the US were obtained from African–Americans, except three
strains isolated from individuals with unknown descent (HK1199, HK1200, HK1202).
FIG. 1

Serotype JP2 type

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

10^- 6^- 4^- 3^- 2^- 1.5^- 2 3 4 6 9 10 11 12 13 14 15 16

e d c a a b b b b b b b b

ACCEPTED on January 12, 2018 by guest
FIG. 2

A

Well of gel -

421 -

229 -

158 -

Seroype
JPZ type

a c d e n t b b b b b b b b

B

Well of gel -

421 -

229 -

158 -
FIG. 3

JP2 clone strains, ET division III

HK975, serotype b
HK913, serotype b
HK908, non-typable
HK1605, serotype b
HK912, serotype b
HK911, serotype b
HK915, serotype c
HK978, serotype c
HK907, serotype c
HK974, serotype c
HK780, serotype a
HK1002, serotype d
HK892, serotype a
HK928, serotype d
HK929, serotype e
HK961, serotype e

0.005

100
100
99
89

ACCEPTED on January 12, 2018 by guest