Phylogenetic associations of ISAa1 and IS150-like insertion sequences in *Actinobacillus actinomycetemcomitans*

Hideaki Hayashida,^{1,2} Knud Poulsen,¹ Okiuji Takagi² and Mogens Kilian¹

Author for correspondence: Mogens Kilian. Tel: +45 89421735. Fax: +45 86176128.
e-mail: kilian@microbiology.au.dk

¹ Department of Medical Microbiology and Immunology, University of Aarhus, The Bartholin Building, DK-8000 Aarhus C, Denmark

² Department of Preventive Dentistry, Nagasaki University School of Dentistry, 1-7-1 Sakamoto, Nagasaki 852-8588, Japan

The distribution and number of two insertion sequences (ISs), ISAa1 and an IS150-like element, in the genomes of a collection of *Actinobacillus actinomycetemcomitans* strains previously subjected to population genetic analysis were determined to obtain information about their stability and biological significance. The hybridization patterns revealed that these IS elements are widespread in the genome of *A. actinomycetemcomitans* strains and that their occurrence agrees with the overall population structure of the species. While the patterns of ISAa1 showed significant evolutionary stability, the IS150-like element showed evidence of intra-genomic variability even within members of the previously identified high-toxicity JP2 clone. Searching of the available genome sequence of strain HK1651 of the JP2 clone (www.genome.ou.edu/act.html) revealed close proximity of the IS elements to housekeeping genes, but no evidence of structural disruption of genes or integrations that may be presumed to influence pathogenic potential.

Keywords: *Actinobacillus actinomycetemcomitans*, insertion sequence, population structure, intra-strain recombination

INTRODUCTION

Numerous studies of juvenile and other forms of rapidly progressive periodontitis have strongly implicated members of the species *Actinobacillus actinomycetemcomitans* in the pathogenesis of these diseases (Slots *et al.*, 1980; Zambon, 1985). Pioneering studies reported by DiRienzo *et al.* (1994) revealed differences in disease association of particular genotypes of *A. actinomycetemcomitans* identified by RFLP analysis. Population genetic studies of this species indicate that it is basically clonal and consists of genetically distinct subpopulations that generally correlate with the five serotypes, a–e (Haubek *et al.*, 1995; Poulsen *et al.*, 1994). The few exceptions may be explained by occasional horizontal transfer or, less likely, by convergent evolution of genes encoding carbohydrate serotype antigens in genetically distinct evolutionary lineages.

Combined with information on origin of the isolates, the clonal population structure of the bacterial collection

studied led us to propose that localized juvenile periodontitis may represent two different types of disease with distinct aetiologies and epidemiologies: one in which a wide variety of geno- and serotypes of *A. actinomycetemcomitans* may act as opportunistic pathogens and another, apparently more rapidly progressive type of disease associated with a particular and disseminating serotype b clone, termed JP2, of *A. actinomycetemcomitans*. Relative to other members of the species, the latter clone shows significantly enhanced leukotoxic activity and has haemolytic activity (Haubek *et al.*, 1996, 1997; Poulsen *et al.*, 1994). The enhanced leukotoxic activity in members of the JP2 clone is due to a 530 bp deletion in the promoter region of the toxin gene operon (Brogan *et al.*, 1994; Kolodrubetz *et al.*, 1996). We have previously shown that the high-toxicity strains, characterized by this deletion, constitute a single clone of serotype b that is homogeneous by multilocus enzyme electrophoresis (MLEE) typing as well as by genomic restriction enzyme analysis using the enzyme *MspI* but shows some heterogeneity by ribotyping (Haubek *et al.*, 1996, 1997). The characteristic deletion is found in the majority of isolates of the RFLP type II, which showed an exclusive association with disease in the study reported by DiRienzo *et al.* (1994) and

Abbreviations: IS, insertion sequence; MLEE, multilocus enzyme electrophoresis.

subsequently has been shown to be strongly associated with conversion from a healthy periodontal status to disease (Bueno *et al.*, 1998). Disease associated with the JP2 clone has been identified in patients living on several continents but so far exclusively in juveniles and adolescents with a genetic background on the African continent (Haubek *et al.*, 1995, 1996, 1997). Further epidemiological studies of disease associated with the JP2 clone of *A. actinomycetemcomitans* and of the clone itself are needed to confirm our hypothesis that it has enhanced pathogenic potential compared to other *A. actinomycetemcomitans* clones and to assess the evolutionary origin of this clone. A representative of the high-toxicity JP2 clone (strain HK1651) is currently being subjected to complete genome sequencing at the University of Oklahoma Center for Advanced Genome Technology (www.genome.ou.edu/act.html).

Several types of mobile genetic elements are known to affect the evolutionary pattern of bacterial genomes. Among them are insertion sequences (ISs), which are small (<2.5 kb) segments of DNA with a simple genetic organization generally encoding no functions other than those involved in their mobility and with the capacity to insert at multiple sites in a target DNA molecule (Mahillon & Chandler, 1998). Upon integration IS elements may modulate the expression of neighbouring genes and, thereby, influence the phenotype of a bacterium. This phenomenon is of particular medical interest when it involves antibiotic resistance or pathogenic potential (Granlund *et al.*, 1998; He *et al.*, 1999; Hernandez-Alles *et al.*, 1999; Preston *et al.*, 1999; Sawada *et al.*, 1999; Simpson *et al.*, 1999; Ziebuhr *et al.*, 1999). Furthermore, two copies of an IS element may act in concert, rendering the intervening region mobile as has been found for certain pathogenicity islands (Hacker *et al.*, 1997). As several copies of an IS element are often present within the same chromosome they may be targets for intra-genomic recombination resulting in chromosomal rearrangements such as inversions (Daveran-Mingot *et al.*, 1998; Hu *et al.*, 1998). Despite the potential to translocate within the chromosome, IS element integrations are often relatively stable over time and therefore they can be used as molecular markers of the bacterial genome in short-term epidemiological studies. IS fingerprinting, i.e. RFLP analyses of genomic DNA using IS elements as probes, has been extensively used with success in molecular epidemiological typing and evolutionary analyses of *Salmonella*, *Mycobacterium*, *Bordetella*, *Staphylococcus aureus* and *Vibrio cholerae* (Arpin *et al.*, 1996; Bik *et al.*, 1996; Morvan *et al.*, 1997; Stanley & Saunders, 1996; van der Zee *et al.*, 1997), for example.

In this study we examined the distribution and number of two IS-related elements in the genomes of a population of *A. actinomycetemcomitans* previously subjected to population genetic analysis to obtain information about their stability and biological significance. We have taken advantage of the available genome sequence for strain HK1651 to further identify the sites of inserts in a member of the high-toxicity JP2 clone.

METHODS

Bacterial strains. A total of 77 strains of *A. actinomycetemcomitans* were included in this study. Of these, 47 were from our previous study on the population structure of *A. actinomycetemcomitans* (Poulsen *et al.*, 1994). That study, based on MLEE analysis of a diverse collection of strains, revealed that the population of *A. actinomycetemcomitans* could be divided into six clusters (ET divisions) correlating with serotype. Thus, ET divisions I, II, III, IV, V and VI encompassed strains of serotypes d and a, e, b and c, c, a, and e, respectively (Poulsen *et al.*, 1994). The 47 strains included in the present study were selected to represent each of these six ET divisions and included 14, 14, 13, 2 and 3 strains of each of the serotypes a, b, c, d and e, respectively, and 1 non-typeable strain. The 14 serotype b strains included the high-toxicity strain JP2. The strains were isolated in the USA and Europe over a period of 5 decades. Twenty-one were from patients with different forms of periodontitis and 16 were from other infectious diseases. For 10 strains the clinical origin was unknown. The remaining 30 strains were from the study by Haubek *et al.* (1997). Twenty-eight of these are members of the JP2 clone and were isolated in the 1990s from geographically widespread patients of African descent with localized juvenile periodontitis or their relatives, whereas two isolates were genetically closely related strains of the RFLP type II without the characteristic deletion in the leukotoxin gene promoter region (DiRienzo *et al.*, 1994; Haubek *et al.*, 1997). All strains included were previously characterized by MLEE.

Southern blotting and hybridization. Whole-cell DNA of *A. actinomycetemcomitans* was extracted by the method described by Poulsen *et al.* (1988). The quality and concentration of DNA was assayed by agarose gel electrophoresis. Approximately 2 µg of whole-cell DNA was digested with *EcoRI* and electrophoresed in a 1% agarose gel overnight at 1.5 V cm⁻¹ in TAE buffer and visualized by staining with ethidium bromide (Sambrook *et al.*, 1989). The DNA in the gel was blotted and fixed onto Nytran nylon membranes (Schleicher & Schuell) and hybridized as previously described with a final post-hybridization wash in 1 × SET (0.15 M NaCl, 0.5 mM EDTA, 20 mM Tris/HCl, pH 7.0), 0.1% SDS and 0.1% sodium pyrophosphate at 60 °C for medium-stringency conditions and 0.5 × SET, 0.1% SDS and 0.1% sodium pyrophosphate at 68 °C for high-stringency conditions (Poulsen *et al.*, 1994). Probes for hybridization were prepared by PCR. Two primer sets were designed based on the sequence of ORFs of IS*Aa1* as previously described (Hayashida *et al.*, 1996) and of an IS150-like element identified by searching the preliminary *A. actinomycetemcomitans* HK1651 genome sequences released by the Advanced Center for Genome Technology (ACGT) at the University of Oklahoma (www.genome.ou.edu/act.html). The two primer sets were: 5'-ATGGAAGTGTAAAGTATC-ACATCGTC-3' combined with 5'-TACACTACCTTTGT-ATTAGCCCTA-3' for IS*Aa1* and 5'-ATTTCCGCCTGT-AATTCGGCAATCG-3' combined with 5'-ACATCGCA-TATTGCCCCGAATGTG-3' for the IS150-like element, resulting in amplicons of 0.3 and 0.4 kb, respectively, when using whole-cell DNA from strain HK1651 as template in the PCR. The temperature profile for the PCR was 30 cycles at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min, with an initial denaturation at 94 °C for 5 min and a final extension at 72 °C for 8 min. The two PCR products were purified from agarose gels upon electrophoresis and labelled with [³²P]dATP using the Random Primed DNA Labelling Kit (Boehringer Mannheim), according to the manufacturer's instructions. The same membranes were used for hybridization with each

Fig. 1. RFLP patterns of ISAA1 and IS150-like elements in *A. actinomycetemcomitans* strains. A Southern blot of whole-cell DNA digested with *Eco*RI was probed with a PCR product of a partial ISAA1 element (a) or of an IS150-like element (b). Lanes: 1, ATCC 29523 (serotype a); 2, HG1232 (serotype b); 3, PH150/50-51 (serotype b); 4, HK1001 (serotype b); 5, JP2 (serotype b); 6, HK1199 (serotype b); 7, HK982 (serotype b); 8, PH417/47-48 (serotype c); 9, PH24/48-49 (serotype c); 10, NCTC 9709 (serotype c); 11, TSA1651 (serotype c); 12, EF12604 (serotype d); 13, SA1075 (serotype e); 14, PHA300/61 (serotype e); 15, PH791/56 (serotype e). The positions and sizes of DNA markers are indicated on the left (the higher intensity of the bands in lanes 9, 10 and 11 is caused by the larger amount of DNA applied to these lanes).

of the two probes after stripping by soaking in boiling 0.1% SDS.

RESULTS

Occurrence of ISAA1 among *A. actinomycetemcomitans*

A 0.3 kb fragment of ISAA1 from strain HK1651 was used as probe in Southern blotting analysis of *Eco*RI-digested whole-cell DNA (Fig. 1a). Using medium-stringency conditions in the post-hybridization washings, one to six hybridizing fragments were detected in the Southern blot patterns of the 77 strains examined and a total of 16 different RFLP patterns were identified (Fig. 2). Fifteen RFLP patterns were found among the 47 strains selected from our previous study of the population structure of *A. actinomycetemcomitans* (Poulsen *et al.*, 1994). The ISAA1 RFLP types correlated with serotypes of the strains as well as with the population structure of *A. actinomycetemcomitans*

revealed by MLEE typing (Fig. 2). All strains of serotypes a and d belonging to ET division I, and the two serotype a and the non-typeable strains from ET division V showed a single *Eco*RI fragment of identical size with homology to the ISAA1 probe. Two hybridizing fragments were detected for the one serotype e strain belonging to ET division II, and two and three hybridizing fragments, respectively, all distinct from the former, were detected for two serotype e strains belonging to ET division VI. Ten different RFLP patterns containing three to six hybridizing bands, two of which were common to all 10 patterns, were found among serotype b and c strains belonging to ET division III. Four hybridizing fragments, three of which were identical in size to those of several ISAA1 RFLP types found in strains belonging to ET division III, were detected for the one serotype c strain of ET division IV.

In addition to the high-toxicity strain JP2, this study also included 28 other high-toxicity strains belonging to the JP2 clone and two genetically very closely related low-

ISAA1 RFLP type	No. of strains	ET division	Serotype	No. of 'JP2 clone' strains	23.1 kb ▼	9.4 kb ▼	6.6 kb ▼	4.4 kb ▼	2.3 kb ▼	2.0 kb ▼
1	17	I, V	a(14), d(2), nt(1)	0						
2	33	III	b	28						
3	1	III	b	1						
4	5	III	b	0						
5	2	III	b	0						
6	1	III	b	0						
7	1	III	b	0						
8	2	III	b(1), c(1)	0						
9	6	III	c	0						
10	2	III, IV	c	0						
11	2	III	c	0						
12	1	III	c	0						
13	1	III	c	0						
14	1	II	e	0						
15	1	VI	e	0						
16	1	VI	e	0						

Fig. 2. Diagram illustrating the ISAA1 RFLP types detected among 77 strains of *A. actinomycetemcomitans* and their correlation with serotype and ET division.

IS150 RFLP type	No. of strains	ET division	Serotype	No. of 'JP2 clone' strains	ISAA1 RFLP type	23.1 kb ▼	9.4 kb ▼	6.6 kb ▼	4.4 kb ▼	2.3 kb ▼	2.0 kb ▼
1	12	I	a(10), d(2)b	0	1						
2	2	I	a	0	1						
3	1	V	a	0	1						
4	1	V	a	0	1						
5	15	III	b	14	2						
6	5	III	b	1	2						
7	4	III	b	4	2						
8	2	III	b	2	2						
9	2	III	b	2	2						
10	2	III	b	2	2						
11	1	III	b	1	2						
12	1	III	b	1	3						
13	1	III	b	1	2						
14	1	III	b	1	2						
15	5	III	b	0	4						
16	1	III	b	0	7						
17	1	III	b	0	5						
18	1	III	b	0	5						
19	1	III	b	0	6						
20	1	III	b	0	8						
21	5	III	c	0	9, 12						
22	4	III	c	0	9, 10, 11						
23	1	III	c	0	11						
24	1	III	c	0	13						
25	1	III	c	0	8						
26	1	IV	c	0	10						
27	1	II	e	0	14						
28	1	V	nt	0	1						

Fig. 3. Diagram illustrating the IS150-like RFLP types detected among 77 strains of *A. actinomycetemcomitans* and their correlation with serotype and ET division.

Fig. 4. Distribution of ISaA1 and IS150-like types, and serotypes among the six ET divisions in the phylogenetic tree constructed on the basis of MLEE typing. The bar indicates genetic distance.

toxicity serotype b strains. The ISaA1 RFLP patterns of 27 of the 28 high-toxicity strains and the two low-toxicity strains were all identical to that of strain JP2. The same pattern was observed in five other serotype b strains each representing distinct ETs within ET division III (Fig. 2). A unique RFLP pattern differing in one band was identified for the remaining high-toxicity strain which was isolated in the USA.

For most patterns, the bands were of equal intensity, suggesting that each band represents an integration of the ISaA1 element (Fig. 1). Differences in band intensity seen in some strains may be due to several circumstances, e.g. more than one hybridizing fragment may be present in the strong bands, the fragments in the weak bands may include only part of the DNA sequences present in the probe or they have a lower degree of homology to the probe used. To examine the latter possibility we used high-stringency washing of the filter upon hybridization. The relative intensity of most of the bands was not affected by this, although the upper band

in ISaA1 RFLP type 14 and the single band in type 1 disappeared, indicating that the DNA fragment in these represents an ISaA1-like element different in sequence from the one used as probe.

Occurrence of the IS150-like element among *A. actinomycetemcomitans*

A 0.4 kb fragment of the IS150-like element present in the genome of strain HK1651 was used as a probe to rehybridize the same membranes used for hybridization with the ISaA1 probe (Fig. 1b). Twenty different RFLP patterns were found among the 47 strains selected from our previous study on the population structure of *A. actinomycetemcomitans* (Fig. 3). Most bands in the fingerprints were of equal intensity and, presumably, each represents an integration of the IS150-like element, whereas the stronger bands most likely represent two integrations contained within fragments migrating together in the agarose gel. There was a correlation between ET division and IS150-like *EcoRI* fingerprint, but strains of ET division III were highly diverse. Except for two strains, all serotype a and d strains belonging to ET division I produced the same IS150-like fingerprint containing seven hybridizing fragments. The two exceptional serotype a strains of ET division I also showed seven hybridizing fragments with a pattern very similar to the majority of serotype a strains. In contrast, the two serotype a strains belonging to ET division V only showed a single hybridizing *EcoRI* fragment. Thirteen IS150-like RFLP patterns with four to seven bands were detected among serotype b and c strains belonging to ET division III and four bands were present in the pattern of a serotype c strain of ET division IV. Five hybridizing fragments were detected for one serotype e strain of ET division II, whereas the probe did not hybridize with the genomic DNA of the two serotype e strains belonging to ET division VI. Except for strains of serotype c, those of the same IS150-like RFLP type also shared the same ISaA1 RFLP type (Fig. 3).

Ten IS150-like RFLP patterns with six to nine hybridizing fragments, many of which were shared by the different patterns, were detected among the 28 high-toxicity JP2 clone strains and the two genetically closely related low-toxicity strains of serotype b (Fig. 3).

Among the 10 patterns, one shared by 14 high-toxicity strains was observed also in one of the low-toxicity serotype b strains belonging to ET division III. Another pattern observed in one high-toxicity and the two closely related low-toxicity RFLP II serotype b strains was identical to that of two other serotype b strains from ET division III. The remaining eight patterns were unique to the high-toxicity strains. The results of IS fingerprinting in relation to MLEE typing are summarized in Fig. 4.

A search of the unfinished genome sequence for *A. actinomycetemcomitans* strain HK1651, which is a high-toxicity member of the JP2 clone, identified five copies of ISaA1 and seven copies of the IS150-like element, one of which was only partial. This is in complete agreement

Fig. 5. Positions of ISAA1 and IS150-like elements in relation to putative genes in the *A. actinomycetemcomitans* HK1651 genome as revealed by searching of the unfinished genomic sequence released by ACGT at the University of Oklahoma. For some integrations the flanking sequences were not available because the IS element constituted the terminus of the corresponding sequence contig.

with the results of our RFLP analyses. Inspection of the sequences flanking the individual ISs revealed that the integrations were between rather than within genes (Fig. 5). No apparent putative virulence factor genes were coupled to, and thereby potentially influenced by, the IS elements. Notably, two integrations of the ISAA1 element in HK1651 were located between genes encoding ribosomal proteins and a non-ribosomal protein gene.

DISCUSSION

The population structure of bacteria depends on selection combined with the relative rates of two processes: (1) the divergence of evolutionary lineages through the accumulation of mutations; and (2) the transfer of genes between lineages by recombination. Our previous

studies of the genetic structure of various populations of *A. actinomycetemcomitans* demonstrated that accumulation of mutations is the predominant driving factor in the evolution of that species (Haubek *et al.*, 1995; Poulsen *et al.*, 1994), although occasional exceptions from the overall pattern of linkage disequilibrium suggest that recombination may take place.

Various types of mobile genetic elements such as bacteriophages, plasmids, transposons and IS elements may move across evolutionary lineages of bacteria and may significantly affect the phenotype of otherwise genetically identical strains. Among these genetic elements, the simplest and smallest are IS elements which occur in a variety of bacterial species, including *A. actinomycetemcomitans* (Hayashida *et al.*, 1996,

1998; Mahillon & Chandler, 1998). The *EcoRI* RFLP typing in this study of 77 *A. actinomycetemcomitans* strains using an *ISAa1* probe revealed one or more bands in all strains, confirming the widespread occurrence and variable numbers of *ISAa1*-like sequences in the species (Hayashida *et al.*, 1996, 1998). However, the finding that high-stringency washing of the membrane resulted in the disappearance of some fragments (the single hybridizing fragment of *ISAa1* RFLP type 1 and one of three hybridizing fragments of *ISAa1* RFLP type 15 in Fig. 2) suggests that these strains harbour sequences related to but different from *ISAa1*. If this assumption is correct, it indicates that strains of ET division I are devoid of *ISAa1* itself. Likewise, serotype e strains of ET division VI appeared to lack the *IS150*-like element in contrast to all other strains, the majority of which contained multiple copies.

The results of *ISAa1* and *IS150*-like fingerprinting correlated with serotype of the strains as well as with the population structure of *A. actinomycetemcomitans* determined by MLEE (Fig. 4). Notably, the present results confirm that ET division I is genetically relatively homogeneous, although it includes both serotype d strains and the majority of serotype a strains. Furthermore, *IS150*-like fingerprinting demonstrated that the serotype a strains in ET division V are indeed genetically distinct from those of ET division I. Likewise, the segregation of serotype e strains into two evolutionary lineages is supported by the presence of five copies of the *IS150*-like element in the serotype e strain of ET division II and its absence in serotype e strains of ET division VI. The IS fingerprinting data further support conclusions based on MLEE typing that ET division III is genetically very diverse and includes both serotype b and c strains. The IS fingerprints of the single serotype c strain in division IV was not significantly different from serotype c strains in division III, suggesting that assigning this strain to a distinct division in the phylogenetic tree based on MLEE may be an artefact.

The strong correlation between IS patterns and the phylogenetic tree indicates that the integrations of *ISAa1* are extremely stable over time. This implies that RFLP typing using this element as probe is not suitable for short-term epidemiological studies of *A. actinomycetemcomitans* due to the very limited diversity, particularly among strains of ET division I. However, the extreme stability of the *ISAa1* patterns and their correlation with the major evolutionary lineages within the species make this an excellent tool for delineating major genetic groups among *A. actinomycetemcomitans* isolates.

Interestingly, the hybridization patterns based on the *IS150*-like element showed significant diversity within ET division III and even within members of the JP2 clone, which makes this IS element valuable for short-term epidemiological studies of this particular sub-population of *A. actinomycetemcomitans*. With one exception, the 28 high-toxicity strains all had the same

ISAa1 RFLP type, which they shared with some low-toxicity serotype b strains. This type was very similar to that of other strains of serotype b (Fig. 2). This strongly supports the hypothesis that the high-toxicity JP2 clone originated recently in evolution from a low-toxicity serotype b strain of this *ISAa1* RFLP type.

Conversely, the significant diversity in *IS150*-like fingerprinting patterns among members of the high-toxicity JP2 clone is in agreement with our previous findings using ribotyping (Haubek *et al.*, 1996, 1997). A common feature of the *IS150*-like element and the rRNA gene operon is that multiple copies of these sequences are scattered throughout the genome which makes them potential targets for intra-genomic recombination (Daveran-Mingot *et al.*, 1998; Hu *et al.*, 1998; Ng *et al.*, 1999). The observed high degree of diversity in ribotype and *IS150*-like RFLP type among the JP2 clone strains compared to that obtained by other typing methods may conceivably be explained by such recombinational events leading to genome rearrangements like inversions. Further studies, including genome mapping, are needed to confirm this hypothesis. Differences in recombinational capabilities may explain why serotype a strains of ET division I are not subject to the same variation in *IS150*-like RFLP types. We speculate that the stability of the *ISAa1* integrations compared to those of the *IS150*-like element may be caused by sequences present in the former, which repress, or in the latter, which enhance recombination. Alternatively, genomic rearrangements involving the *ISAa1* integrations may be deleterious to the bacteria.

Although phenotypically cryptic, IS elements may influence the transcription of genes (Mahillon & Chandler, 1998) or directly inactivate genes into which they become inserted (Granlund *et al.*, 1998; Ziebuhr *et al.*, 1999). Our search of the available parts of the genome sequence of JP2 clone strain HK1651 revealed close proximity between each of the two IS elements and several housekeeping genes, but no example of inserts within an identified ORF (Fig. 5). These data do not exclude that these IS elements affect transcription of some of the housekeeping genes. The potential significance of IS elements is emphasized by the recent demonstration of a novel IS element in the promoter region of the leukotoxin gene operon in occasional Japanese isolates of *A. actinomycetemcomitans*, resulting in enhanced expression of leukotoxin, similar to members of the JP2 clone (He *et al.*, 1999).

In conclusion, IS elements are widespread in the genome of *A. actinomycetemcomitans* strains and their occurrence agrees with the overall population structure of the species. While the patterns of *ISAa1* show significant evolutionary stability, the *IS150*-like element shows evidence of intra-genomic variability. The available genome sequence of strain HK1651 of the high-toxicity JP2 clone revealed close proximity of the IS elements to housekeeping genes, but no evidence of structural disruption of genes or integrations that may be presumed to influence pathogenic potential.

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