Differences in iron acquisition from human haemoglobin among strains of *Actinobacillus actinomycetemcomitans*

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To get a better insight into the physiology of the high-toxic JP2 clone of *Actinobacillus actinomycetemcomitans* serotype b, which is strongly associated with juvenile periodontitis in adolescents of African descent, the modes of iron acquisition in this clone were examined and compared to those of other strains of the species. None of the strains examined could utilize human transferrin as a source of iron. This was in accordance with the presence of a non-functional *tbpA* gene, which normally encodes the A subunit of the transferrin-binding-protein complex. Southern blot analysis indicated that functional duplications of *tbpA* were not present in the genome. Thus, *A. actinomycetemcomitans* seems to be in a process of evolution, in which iron acquisition from host transferrin is not essential as in many other members of the *Pasteurellaceae*. All strains could utilize haem as a source of iron. All 11 *A. actinomycetemcomitans* strains examined harboured a single genomic sequence with homology to the *hgpA* gene encoding haemoglobin-binding protein A in *Haemophilus influenzae*. However, in all three strains belonging to the JP2 clone and in one serotype e strain *hgpA* was a pseudogene. Seven other strains possessed a functional *hgpA* gene which, according to insertion mutagenesis experiments, was responsible for the ability of these strains to utilize haemoglobin as a source of iron. Thus, the presence of an *hgpA* pseudogene and the inability to use human haemoglobin as an iron source discriminate the high-toxic JP2 clone from low-toxic serotype b strains and most other strains of *A. actinomycetemcomitans*.

Keywords: pseudogenes, transferrin-binding protein, haemoglobin-binding protein, haem

INTRODUCTION

Actinobacillus actinomycetemcomitans has been implicated in the pathogenesis of different forms of early-onset periodontal diseases, including localized juvenile periodontitis (Slots *et al.*, 1980; Zambon, 1985). Differences in pathogenic potential appear to exist between different evolutionary lineages of the species. A particularly virulent clone of *A. actinomycetemcomitans*

serotype b was identified by its widespread occurrence, strong association with disease and a characteristic 530 nt deletion in the promoter region of the leukotoxin gene operon (*ltx*) that resulted in enhanced leukotoxic activity (Brogan *et al.*, 1994; Haubek *et al.*, 1996, 1997). This clone, termed the high-toxic JP2 clone, shows a pronounced racial tropism, as it has been almost exclusively isolated from subjects of African descent (Contreras *et al.*, 2000; Haubek *et al.*, 1996, 1997). Recent studies performed in Morocco reveal that endemic occurrence of the JP2 clone is associated with significantly increased prevalence of juvenile periodontitis (Haubek *et al.*, 2001). A representative of the high-toxic JP2 clone, strain HK1651, is presently subject to complete-genome sequencing at the Advanced Center for Genome Technology (ACGT) at Oklahoma University (<http://www.genome.ou.edu/act.html>).

The GenBank accession numbers for the *tbpA* homologue sequences reported in this paper are AY028441 (strain HK1119), AF359437 (HK912), AF359438 (HK989), AF359439 (HK1002), AF359440 (HK988) and AF359441 (HK961); the GenBank accession numbers for the *hgpA* homologue sequences reported in this paper are AF359442 (HK989), AF359443 (HK988), AF359444 (HK981), AF359445 (HK961), AF359446 (JP2), AF359447 (HK912), AF359448 (HK1605), AF359449 (HK1604), AF359450 (HK1199) and HK359451 (HK1002).

Iron is an essential nutrient to most bacteria. In animal hosts, the vast majority of iron is found intracellularly where it is bound to ferritin or haem compounds, whereas in serum and secretions it is bound to transferrin and lactoferrin. The latter two proteins maintain levels of free extracellular iron at an extremely low level, which is far below that required for the optimal growth of micro-organisms. Consequently, bacteria that successfully colonize humans have developed specific systems to acquire iron (reviewed by Gray-Owen & Schryvers, 1996; Guerinot, 1994; Mietzner & Morse, 1994; Otto *et al.*, 1992; Ratledge & Dover, 2000). Many bacteria rely on siderophores, which are small inorganic chelators with a high affinity for ferric iron. The expression of such siderophores allows bacteria to acquire iron from a variety of environmental and biological sources other than transferrin and lactoferrin. Members of the families *Pasteurellaceae* and *Neisseriaceae* do not produce siderophores; instead, they produce surface-exposed proteins that bind haemoglobin, transferrin and, in the case of the *Neisseriaceae*, lactoferrin, thereby facilitating iron acquisition from these host proteins. Notably, these bacterial receptors are highly specific for iron-binding proteins of the host species (Gray-Owen & Schryvers, 1996; Ogunnariwo & Schryvers, 1990; Otto *et al.*, 1992).

A. actinomycetemcomitans, like other members of the *Pasteurellaceae*, does not produce siderophores (Winston *et al.*, 1993) and little is known about the mechanisms of iron acquisition in this species. Human lactoferrin is bactericidal for the bacterium (Kalmar & Arnold, 1988). A transferrin-binding protein A gene homologue was detected by PCR in members of the *Pasteurellaceae*, but its sequence was not characterized further (Ogunnariwo & Schryvers, 1996). Neither transferrin nor lactoferrin is bound by *A. actinomycetemcomitans* under iron-restricted conditions (Winston *et al.*, 1993). However, periplasmic proteins, AfuA and FbpA, with putative iron transport functions have been described, suggesting that an uptake system for iron is actually present on the cell surface of *A. actinomycetemcomitans* (Graber *et al.*, 1998; Willemsen *et al.*, 1997). Finally, Grenier *et al.* (1997) reported that *A. actinomycetemcomitans* can use human haemoglobin as a source of iron. The aim of the present study was to examine further the acquisition of iron by *A. actinomycetemcomitans*.

METHODS

Bacterial strains and culture conditions. The 11 *A. actinomycetemcomitans* strains examined here included one serotype a strain (HK989), four low-toxic serotype b strains (HK912, HK988, HK1604 and HK1605), three high-toxic serotype b strains characterized by the 530 bp deletion of the promoter region in the leukotoxin gene (JP2, HK1199 and HK1651), one serotype c strain (HK981), one serotype d strain (HK1002) and one serotype e strain (HK961). For DNA extraction, the *A. actinomycetemcomitans* strains were grown in 20 ml brain-heart infusion broth (BHI; Difco Laboratories) supplemented with 0.05% (w/v) L-cysteine (Merck) and 0.5%

(w/v) yeast extract (Difco Laboratories) at 37 °C in air plus 5% CO₂ for 2 days. The iron-restricted growth experiments were performed as described by Ogunnariwo & Schryvers (1990) with some modifications. Strains were grown in BHI medium in air plus 5% CO₂ at 37 °C for 2 days, washed once with an equal volume of 0.9% NaCl and then resuspended in 10 volumes of 0.9% NaCl. Samples of the diluted culture were spread onto the surface of BHI plates containing 800 µM deferoxamine mesylate. Circular discs (5 mm diameter) of Whatman 3 mm Chr paper (Whatman International) supplemented with 5 µl of human transferrin (2 mg ml⁻¹), human lactoferrin (2 mg ml⁻¹), human haemoglobin A (1 mg ml⁻¹), human sickle-cell haemoglobin (1 mg ml⁻¹), FeCl₃ (20 mM and 2 mM) or distilled water were placed onto the surface of the agar. The human iron-binding proteins were purchased from Sigma. The amount of each iron-binding protein applied to the discs corresponded to a binding capacity of approximately 25 mM iron. Growth around the discs was scored after incubation for 2 days at 37 °C in air plus 5% CO₂. To assay for the utilization of iron complexed in haem, we used the plate assay described above with Factor X diagnostic tablets (Rosco) as a source of haem.

Southern blot analysis. Whole-cell DNA was extracted as described previously (Poulsen *et al.*, 1988). Briefly, bacterial cells were suspended in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7.5), lysed with 1% SDS under high salt conditions, treated with proteinase K, extracted with phenol/chloroform (1:1) and then precipitated with cold 96% ethanol. After rinsing once with cold 70% ethanol, the DNA was dried and dissolved in TE buffer. Approximately 1 µg whole-cell DNA was digested with *EcoRI*, electrophoresed overnight through a 1% agarose gel at 1.5 V cm⁻¹ in TAE buffer (0.04 M Tris/acetate, 1 mM EDTA) and visualized by staining with ethidium bromide (Sambrook *et al.*, 1989). The separated DNA fragments in the gel were transferred onto a Nytran nylon membrane (Protran; Schleicher & Schuell) and fixed by UV irradiation. The transferred DNA was hybridized as 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 (Poulsen *et al.*, 1994). The DNA fragments used as probes were prepared by PCR using Ready To Go PCR Beads (Amersham Pharmacia Biotech). Two primer sets were designed, based on the sequence of the transferrin-binding protein A gene (*tbpA*) and the haemoglobin-binding protein gene (*hgpA*) homologues identified by searching the preliminary *A. actinomycetemcomitans* HK1651 genome sequences released by the Advanced Center for Genome Technology (ACGT) at Oklahoma University (<http://www.genome.ou.edu/act.html>). The primer pair for *tbpA* was 5'-GGTTATCACTTTTCCGAGCAAC-3' and 5'-CCAGATTAGCCGAAACCTGATG-3', resulting in an amplicon of 531 nt, and the primer pair for *hgpA* was 5'-CATTGAAAATCACCTATTCCTAAC-3' and 5'-ATAGGACAGATCATGTTCTGTACC-3', which amplified a product of 753 nt. As template in the PCR, we used approximately 1 ng whole-cell DNA from strain HK1651, and the temperature profile for the PCR included an initial denaturation at 94 °C for 5 min followed by 30 cycles at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min, with a final extension at 72 °C for 8 min. The PCR products were purified using Wizard Minicolumns (Promega) and labelled with [α -³²P]dATP using the Random Primed DNA labelling Kit (Boehringer Mannheim) according to the manufacturer's instructions.

Sequence analysis of the *tbpA* and *hgpA* homologues. For amplification of the *tbpA* homologue from *A. actino-*

mycetemcomitans strains, we used two primer pairs: 5'-CAACACAAATACTGCTTAAGATGC-3' with 5'-ATGCA-GGTAATGTCATATCGCG-3', and 5'-GGTTATCACTTT-TCCGAGCAAC-3' with 5'-CACTTACGCGTGTGCAACA-ATC-3'. Amplification using these primer pairs resulted in two overlapping DNA fragments of 1.1 and 1.8 kb, respectively, which covered the putative 2.7 kb *tbpA* gene. For amplification of *hgpA*, we used the four pairs of primers: 5'-CTATTCCACCGATGAAGTTTATTTTC-3' with 5'-TCGA-ATGGAATATCAACCGGTGTTCC-3', 5'-CATTGAAAA-TCCCTATTCCCAAC-3' with 5'-ATACAGATTAGTTAC-CAGATCATC-3', 5'-CCGGAATATATCCCCGGCGTCA-CC-3' with 5'-TTGTACCAAATGGGCGGATTGATC-3', and 5'-CACATATATTAAGCTGTGATGC-3' with 5'-CC-GCTTCGGTATTGAACCTGTC-3'. Amplification using these primer pairs resulted in amplicons of 1.7, 1.1, 1.0 and 1.9 kb, respectively, which covered the 3.2 kb *hgpA* gene. PCR and purification of the subsequent products were carried out as described above. For DNA sequencing, we used the same primers as for the PCR and additional primers designed on the basis of the preliminary genome sequence of strain HK1651. Sequencing of both strands of the amplified fragments was achieved by using the Thermo Sequenase Dye Terminator Cycle Sequencing Kit (Amersham Life Science), and the resulting products were analysed on an automated DNA sequencer (model 377; Applied Biosystems). Sequence data were analysed with programs included in the GCG package (Genetics Computer Group, University of Wisconsin, Madison, WI, USA).

Insertional mutagenesis of *hgpA*. A fragment of *hgpA* (positions 285–2647 in the ORF of the *hgpA* gene from strain HK912) was amplified by PCR from 1 ng whole-cell DNA from *A. actinomycetemcomitans* HK912 using the primers 5'-GGCTTTGCTGTGCGTGGTGTGGA-3' and 5'-ATCG-GAATATCGCCATCCATTC-3'. The amplicons were electrophoresed through a 1% agarose gel, excised, purified using the GeneClean II kit (BIO 101) and cloned into the pGEM-T Easy vector (Promega) using *Escherichia coli* XL-1 Blue for propagation. The resulting plasmid was termed pGEM-*hgpA*. The kanamycin-resistance gene from the 1.7 kb transposon in pGPS1.1 was inserted into the *hgpA* gene fragment in pGEM-*hgpA* using TnsABC Transposase as recommended by the manufacturer (New England Biolabs). The integration was shown by sequencing to be at position 1814 with a five nucleotide duplication of the target sequence. DNA from the resulting plasmid was linearized by digestion with *SphI*, extracted with phenol and introduced into *A. actinomycetemcomitans* by electroporation as described (Sreenivasan *et al.*, 1991; Suzuki *et al.*, 2000). The cells were plated onto TSBYE agar containing 25 mg kanamycin l⁻¹ to select for transformants (Sreenivasan *et al.*, 1991). Insertion of the kanamycin-resistance gene into the genomic *hgpA* gene was confirmed by PCR using primers 5'-CATTGAAAATC-ACCTATTCCCAAC-3' and 5'-ATACAGATTAGTTACC-AGATCAT-3', which flanked the insertion and yielded a 1088 nt amplicon from the original *hgpA* gene.

Accession numbers. The *tbpA* homologues have been deposited in GenBank under accession numbers AY028441 (strain HK1199), AF359437 (HK912), AF359438 (HK989), AF359439 (HK1002), AF359440 (HK988) and AF359441 (HK961). The *hgpA* homologues have been deposited in GenBank under accession numbers AF359442 (HK989), AF359443 (HK988), AF359444 (HK981), AF359445 (HK961), AF359446 (JP2), AF359447 (HK912), AF359448 (HK1605), AF359449 (HK1604), AF359450 (HK1199) and AF359451 (HK1002).

RESULTS

Iron-restricted-growth experiments

Preliminary experiments showed that 800 µM of the iron-binding compound deferoxamine mesylate completely inhibited growth of all 11 strains of *A. actinomycetemcomitans* on BHI agar. When supplied with discs containing 20 mM FeCl₃, haemoglobin A or sickle-cell haemoglobin growth of colonies was observed around the discs after incubation for 2 days for all four low-toxic serotype b strains and for the single repre-

Fig. 1. Plate assay for growth stimulation of *A. actinomycetemcomitans* strains HK1002 (A) and JP2 (B) by various iron compounds. Bacteria were plated onto BHI agar containing deferoxamine mesylate, which binds free ferric iron. Discs containing the iron sources human lactoferrin (LF), human transferrin (TF), human sickle-cell haemoglobin (HB-S), human haemoglobin A (HB-A), FeCl₃ (20 mM) and FeCl₃ (2 mM) were applied to the plates and the cultures were incubated. Distilled water was used as a negative control (the disc in the middle).

Table 1. Iron-restricted growth of *A. actinomycetemcomitans* around discs supplemented with different sources of iron

Strain	Serotype	Iron source*						
		FeCl ₃ (20 mM)	FeCl ₃ (2 mM)	HB-A	HB-S	TF	LF	Haemin
HK989	a	+	—	+	+	—	—	+
HK912	b (LT)†	+	—	+	+	—	—	+
HK988	b (LT)	+	—	+	+	—	—	+
HK1604	b (LT)	+	—	+	+	—	—	+
HK1605	b (LT)	+	—	+	+	—	—	+
JP2	b (HT)	+	—	—	—	—	—	+
HK1651	b (HT)	+	—	—	—	—	—	+
HK981	b (HT)	+	—	—	—	—	—	+
HK1002	d	+	—	+	+	—	—	+
HK961	e	+	—	—	—	—	—	+

* HB-A, human haemoglobin; HB-S, human sickle-cell haemoglobin; TF, human transferrin; LF, human lactoferrin.

† For serotype b, high-toxic strains are indicated by HT and low-toxic strains are indicated by LT.

Fig. 2. Southern blot analysis of *hgpA* homologues in *A. actinomycetemcomitans*. The blot shown in Fig. 5 was re-hybridized with a 0.75 kb fragment of the HK1651 *hgpA* homologue. Lanes: 1, HK989; 2, HK912; 3, HK988; 4, HK1604; 5, HK1605; 6, JP2; 7, HK1199; 8, HK1651; 9, HK981; 10, HK1002; 11, HK961. Molecular size markers (kb) are shown at the right of the image.

sentatives of each of the serotypes a, c and d. In contrast, all three high-toxic serotype b strains and the serotype e strain failed to grow on either of the two forms of haemoglobin as a source of iron, but they did grow on FeCl₃ (Fig. 1). None of the 11 strains was capable of utilizing iron-saturated transferrin or lactoferrin (Table 1).

All 11 strains grew around haemin tablets on the deferoxamine-mesylate-containing BHI agar, indicating that they could scavenge iron from haem (see Fig. 4 later). The growth-supporting effect was observed up to 1 cm from the tablet.

Homologues of the haemoglobin-binding-protein gene *hgpA*

The observed differences in the abilities of the strains to grow on human haemoglobin prompted us to examine the strains for homologues of the gene encoding the

haemoglobin-binding protein (*hgpA*) in several species of the family *Pasteurellaceae*.

Screening of the preliminary genome sequence of *A. actinomycetemcomitans* HK1651, which is a representative of the JP2 clone, revealed that it harbours a stretch of 3.2 kb with 70.9% identity to the *Haemophilus influenzae* *hgpA* gene encoding HgpA. Using a 0.75 kb fragment of this *hgpA* homologue in strain HK1651 as a probe in Southern blot analysis of *Eco*RI-restricted whole-cell DNA, we found that the remaining 10 strains of *A. actinomycetemcomitans* also harboured a single sequence similar to this probe (Fig. 2). The complete *hgpA* homologue was amplified by PCR and sequenced for nine of the 10 strains. In the last strain, HK961, the primers used in the PCR failed to amplify the start of the gene; consequently, the sequence of the first 400 nt of the 3.2 kb *hgpA* homologue was not determined for this strain.

We found a complete correlation between the ability of each strain to utilize human haemoglobin as a source of iron and the presence of an apparently functional *hgpA* homologue. In strains JP2, HK1199 and HK1651 (all of the high-toxic serotype b clone) and strain HK961 (serotype e), which failed to grow on haemoglobin, the 3.2 kb *hgpA* sequence represented a pseudogene as it did not constitute an ORF (Fig. 3). The *hgpA* homologues in two of these strains, HK1199 and JP2, were identical and very similar (99.9% similar) to that found in strain HK1651. All three of these high-toxic strains showed a mutation at position 859 relative to the start codon in the *H. influenzae* *hgpA* gene, which resulted in an in-frame stop codon. In strain HK1651, an additional mutation creating a stop codon was found at position 2722. The 2.8 kb fragment of the *hgpA* homologue sequenced in strain HK961 was the most distinct (96.6% similar to strain HK1651), and a single nucleotide deletion at position 1074 interrupted the ORF.

Fig. 3. (A) Deleterious mutations found in some of the 3.2 kb *hgpA* homologue sequences from 11 strains of *A. actinomycetemcomitans*. The first 400 nt were not determined for strain HK961. In-frame stop codons are shown by asterisks; a frame-shift mutation is shown by ●. The ability to grow (+) or inability to grow (–) on human haemoglobin is indicated on the right-hand side. (B) Proportional similarities among the 11 *hgpA* homologues sequenced. The serotype of each strain is shown; LT, low-toxic; HT, high-toxic.

The seven strains that were capable of utilizing human haemoglobin, HK912, HK981, HK988, HK989, HK1002, HK1604 and HK1605, all harboured an apparently functional *hgpA* homologue with the potential of encoding a protein of 1023 aa. The nucleotides AGG with the features of a ribosome-binding site (Stormo *et al.*, 1982) were present just 5' to the proposed ATG start codon. The sequence TAAAAT, separated from the start codon by 20 nt, may constitute a –10 promoter

region, whereas a –35 region (Hawley & McClure, 1983) was not evident. Fifty-nine nucleotides upstream of the start codon the sequence AGAAATGAATTTC-ATTATC has 13 of 19 positions identical to the consensus Fur box (Escobar *et al.*, 1999), suggesting that expression of *hgpA* is iron-responsively regulated by the Fur protein that has been shown to be functional in *A. actinomycetemcomitans* (Haraszthy *et al.*, 2002). An inverted repeat structure of 46 nt located 44 nt downstream of the TAA stop codon presumably constitutes a transcriptional terminator.

An alignment of the deduced HgpA amino acid sequences from the seven strains that showed an intact ORF revealed >99% mutual identity. The sequences were 76% similar, including 12 gaps, to the *H. influenzae* haemoglobin-binding protein A. A search in the GenBank database revealed no other sequences with a higher degree of homology to the proposed *A. actinomycetemcomitans* HgpA protein. The 25 residues in the N terminus were predicted by the computer program SIGNALP (Nielsen *et al.*, 1997) to constitute a signal peptide resulting in a secreted protein of 113.8 kDa with an isoelectric point of 9.74. As for most outer-membrane proteins, the C-terminal residue is a phenylalanine (Struyve *et al.*, 1991). The sequence NEIVSGSSEG near the N terminus of the protein is homologous to the TonB box found in other TonB-dependent receptors (Elkins *et al.*, 1995; Lundrigan & Kadner, 1986). None of the *A. actinomycetemcomitans hgpA* genes showed an equivalent to the CCAA repeats in the N-terminal part of the *H. influenzae hgpA* gene, which are supposed to be subject to slipped-strand mispairing resulting in the deletion/addition of repeats, thereby potentiating phase variation of the HgpA protein (Ren *et al.*, 1998).

Insertional inactivation of the *hgpA* homologue

We used insertional inactivation of the proposed *hgpA* gene to verify that it was responsible for the ability of the strains to utilize human haemoglobin as a source of iron. A kanamycin-resistance gene was inserted into a 2.4 kb fragment of the *hgpA* gene from strain HK912; this DNA construct was introduced by electroporation into four different strains of *A. actinomycetemcomitans*, each of which was able to grow on haemoglobin and had an apparently functional *hgpA* gene. After plating onto selective media, colonies with possible integrations were analysed by PCR using *hgpA*-specific primers that flanked the inserted kanamycin-resistance gene. In this assay, a 1.1 kb fragment of the parental *hgpA* gene was amplified, whereas the mutated *hgpA* gene resulted in a PCR product of 2.8 kb. For strains HK912, HK988 and HK1002 we did not obtain mutants with the resistance-marker gene integrated into the chromosomal *hgpA* gene, whereas for strain HK989 several such mutants were recovered. Differences in transformability among *A. actinomycetemcomitans* strains have been reported previously (Galli *et al.*, 2002). Notably, in contrast to the parental strain HK989, the mutated version of strain HK989 was unable to grow on human haemoglobin

Fig. 4. Insertional mutagenesis of *hgpA* in strain HK989. (Upper panel) Growth stimulation of strain HK989 by human haemoglobin A and haemoglobin S on iron-restricted medium. (Lower panel) Lack of growth stimulation of mutated strain HK989 by human haemoglobin. The experiments were performed as described in Fig. 1. The discs in the centres of the agar plates contain haemin, which supports the growth of both strains.

(Fig. 4), thus confirming that in strain HK989 the *hgpA* gene is functional in the acquisition of iron from human haemoglobin.

Homologues of the transferrin-binding-protein gene *tbpA*

A search in the preliminary genome sequence of *A. actinomycetemcomitans* HK1651 revealed a sequence with significant homology to the *H. influenzae* *tbpA* gene (Gray-Owen *et al.*, 1995), which encodes the conserved A subunit of the heterodimeric TbpA–TbpB complex that constitutes the transferrin-binding receptor in this species (Gray-Owen & Schryvers, 1996). However, this 2.7 kb sequence represented a non-functional pseudogene, as a deletion of a single nucleotide at position 279 interrupted the ORF and a C to T transition at position 1288 created a stop codon (positions are relative to the ORF of the *H. influenzae* *tbpA* gene, GenBank accession no. U10882). We confirmed this deletion and transition by PCR amplification of the pseudogene and subsequently sequenced the *tbpA* homologue of strain HK1651. In addition, the genomic sequence upstream of this *tbpA* homologue in strain HK1651 did not have the potential of encoding a TbpB protein. For the remaining 10 strains included in this

Fig. 5. Southern blot analysis of *tbpA* homologues from *A. actinomycetemcomitans*. Whole-cell DNA was digested with *EcoRI*, electrophoresed through an agarose gel, transferred to a nylon membrane and hybridized with a 0.53 kb fragment of the HK1651 *tbpA* pseudogene. Lanes: 1, HK989; 2, HK912; 3, HK988; 4, HK1604; 5, HK1605; 6, JP2; 7, HK1199; 8, HK1651; 9, HK981; 10, HK1002; 11, HK961. Molecular mass markers (kb) are shown at the right of the image. The weak band in lane 2 is barely visible in the photographic reproduction of the autoradiogram.

study, we used hybridization analysis to assay for the presence of genomic sequences with homology to the *tbpA* pseudogene. Strain HK1651 was also included in this analysis to detect potential functional duplications of *tbpA*. A Southern blot of *EcoRI*-restricted whole-cell DNA was hybridized with a 0.53 kb fragment of the HK1651 *tbpA* pseudogene. All except one of the 11 strains examined showed a single *EcoRI* fragment hybridizing with the probe, indicating that a single copy of a *tbpA* homologue was present in each of these strains (Fig. 5). Thus, strain HK1651 does not appear to harbour a functional *tbpA* gene in addition to the pseudogene. Genomic DNA of the exceptional serotype c strain HK981 did not hybridize with the probe, indicating that the *tbpA* gene sequences were deleted in this strain.

To further characterize the genomic sequences with homology to *tbpA*, we amplified by PCR and sequenced these *tbpA* homologues from an additional six strains of *A. actinomycetemcomitans*, HK912, HK961, HK988, HK989, HK1002 and HK1199, which represented the different serotypes of this organism. The ORF in all six of the resulting sequences was interrupted by mutations and, therefore, presumably represents a pseudogene. The sequence of the *tbpA* homologue in strains HK1199 and HK1651, both representatives of the JP2 clone, was identical. The *tbpA* sequences of the serotype a, b and d strains HK989, HK988 and HK1002, respectively, were very similar (>99.5% similar) to that of HK1651 and included the single nucleotide deletion at position 279, whereas the mutation at position 1288 that created the stop codon in strain HK1651 was absent in these three strains. In the serotype b strain HK912, the *tbpA* homologue was also very similar to the HK1651 *tbpA* sequence, except for a large out-of-frame deletion of 860 nt at positions 1150–2010. This is in agreement with the relatively weak hybridization observed for strain HK912 in the Southern blot analysis, since a major part of the DNA fragment used as a probe covered this

Fig. 6. Deleterious mutations in the 2.7 kb *tbpA* pseudogene sequences from seven strains of *A. actinomycetemcomitans*. In-frame stop codons are shown by asterisks; frame-shift deletions/insertions of a single nucleotide are shown by ●; deletions marked by black solid bars are indicated by ▲.

deletion. The serotype e strain HK961 *tbpA* sequence was the most distinct (94.9% similar to that of strain HK1651). Compared to the *H. influenzae* *tbpA* gene it showed two out-of-frame mutations, a deletion of 40 nt at positions 617–657 and an insertion of a single nucleotide at position 2421. The deleterious mutations in the different *tbpA* pseudogenes are summarized in Fig. 6.

DISCUSSION

The results described here demonstrate that *A. actinomycetemcomitans* is undergoing evolutionary changes relative to its closest taxonomic relatives with regard to its mechanisms of iron acquisition. While genes encoding proteins used for iron scavenging by other members of the family *Pasteurellaceae* are present in strains of *A. actinomycetemcomitans* some are no longer functional in this organism; in occasional strains of *A. actinomycetemcomitans* these genes appear to be absent. Several members of the family *Pasteurellaceae* express transferrin receptors in the form of heterodimeric TbpA–TbpB complexes (Gray-Owen & Schryvers, 1996). Genomic DNA sequences homologous to *tbpA*, which encodes the most conserved A subunit of this complex, have been reported to be present in *A. actinomycetemcomitans* (Ogunnariwo & Schryvers, 1996). However, our findings demonstrate that only relics of the *tbpA* gene are present as pseudogenes in most strains of this organism and, apparently, the *tbpB* sequences have been completely deleted. This is in full agreement with our observation that none of the 11 strains of *A. actinomycetemcomitans* examined here was capable of utilizing iron-saturated human transferrin as a source of iron. Considerable genetic heterogeneity in the *tbpA* pseudogenes and the apparent loss of the entire *tbpA* gene in one strain (HK981) support the conclusion that a functional selection no longer exists in *A. actinomycetemcomitans*.

Several species in the family *Pasteurellaceae*, including *H. influenzae* and *Haemophilus ducreyi*, express related surface-exposed haemoglobin-binding proteins that facilitate the acquisition of iron from human haemoglobin (Elkins *et al.*, 1995; Jin *et al.*, 1996; Ren *et al.*, 1998). In *H. influenzae* there is even a redundancy in the function of haemoglobin binding as a single strain may express three distinct haemoglobin-binding proteins, termed HgpA, HgpB and HgpC, each of which is subject to phase variation (Morton *et al.*, 1999). *A. actinomycetemcomitans* has been reported to use human haemoglobin as a source of iron for growth, and interactions with lipopolysaccharides have been suggested to mediate the binding of haemoglobin in this organism (Grenier *et al.*, 1997).

The present study demonstrates that sequences homologous to the *H. influenzae* *hgpA* gene are present in *A. actinomycetemcomitans*. The function of the *hgpA* homologue was confirmed by the demonstration that human haemoglobin could be utilized as a source of iron by *A. actinomycetemcomitans*, combined with the observation that mutational inactivation of the *hgpA* gene resulted in a loss of this property (Fig. 4). However, in contrast to all other strains, representatives of the JP2 clone and the single representative of serotype e were unable to grow on human haemoglobin as a source of iron and harboured a *hgpA* pseudogene. Whether this difference is associated with the increased pathogenic potential of the JP2 clone remains to be examined.

We have previously demonstrated that the population structure of *A. actinomycetemcomitans* is basically clonal and that the genetically distinct subpopulations within the species generally correlate with the serotypes a through e (Hayashida *et al.*, 2000; Poulsen *et al.*, 1994). The finding that the proportional similarities among the *hgpA* sequences of the 11 strains of *A. actinomycetemcomitans* studied here correlated with the previously observed genetic relationships within the species is in full agreement with these conclusions (Fig. 3). Thus, the *hgpA* sequences in all seven serotype b strains were very similar, confirming that the high-toxic serotype b strains are evolutionarily closely related to the low-toxic serotype b strains. In addition, the identical *hgpA* gene sequences in the serotype a and d strains are in accordance with the close genetic relatedness of these serotypes. Likewise, the very different *hgpA* sequence of the serotype e strain is in agreement with its distant genetic relationship to the other serotypes.

In the *tbpA* pseudogenes, the out-of-frame mutation at position 279 apparently occurred before serotype diversification and before the 530 nt deletion in the *ltx* promoter that led to the high toxicity of the JP2 clone (Fig. 6). Conversely, the high degree of similarity between *hgpA* sequences of the high- and low-toxic serotype b strains suggests that the mutations rendering *hgpA* inactive in the highly toxic JP2 clone are relatively recent in evolution. The sequence similarities do not provide information on the evolutionary origin of the deleterious mutations in the *tbpA* and *hgpA* genes in the

single serotype e strain, except to infer that they have occurred independently of the evolution of the other *tbpA* and *hgpA* pseudogenes. The presence of evolutionarily unrelated *hgpA* pseudogenes in *A. actinomycetemcomitans* strains suggests that there has been no selection for a functional *hgpA* gene in these strains.

It has been shown that *A. actinomycetemcomitans* Y4 (a low-toxic serotype b strain) binds to and can utilize haem as a source of iron (Graber *et al.*, 1998; Grenier *et al.*, 1997). In agreement with this observation, all strains examined in our study were capable of growing in the presence of haemin as the only source of iron. It is conceivable that free haem may be accessible in the natural environment of *A. actinomycetemcomitans*, the periodontal pocket, as a result of proteolysis of haem-containing proteins by proteases released by other micro-organisms. In this context, it is worth noting that *Porphyromonas gingivalis*, another suspected periodontal pathogen, produces a lysine-specific cysteine proteinase, Kgp, which degrades and releases haem from haemoglobin, haemopexin, haptoglobin and transferrin (Genco & Dixon, 2001). An analogous synergistic cooperation in iron acquisition from haemoglobin has been demonstrated during abscess formation between *E. coli*, which releases a haemoglobin protease, and *Bacteroides fragilis* (Otto *et al.*, 2002).

In conclusion, *A. actinomycetemcomitans* is undergoing evolutionary changes relative to its closest taxonomic relatives with regard to its strategies for acquiring iron. The lack of a functional *tbpA* gene adequately explains the observation that this species cannot utilize human transferrin as a source of iron. The inability to use human haemoglobin as an iron source discriminates strains of the high-toxic JP2 clone from low-toxic serotype b strains and from most other strains of *A. actinomycetemcomitans* that express a functional haemoglobin-binding protein like several other members of the family *Pasteurellaceae*.

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