Molecular heterogeneity of EmaA, an oligomeric autotransporter adhesin of *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*

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Adhesion of *Aggregatibacter actinomycetemcomitans* to extracellular matrix proteins is mediated by antennae-like surface structures composed of EmaA oligomers. EmaA is an outer-membrane protein orthologous to the autotransporter YadA, a virulence determinant of *Yersinia*. *emaA* was present in the 27 strains examined, covering the six serotypes of *A. actinomycetemcomitans*. Ten individual genotypes and three different forms of the protein (full-length, intermediate and truncated) were predicted. The prototypic, full-length EmaA (202 kDa) was only associated with serotypes b and c, which displayed antennae-like surface structures. These strains bound to collagen embedded in a 3D matrix. The intermediate form of EmaA (173 kDa) was exclusively associated with serotypes d and a, which contained a 279 aa in-frame deletion, as well as a different N-terminal head domain sequence. These differences modified the appearance of the EmaA structures on the cell surface but maintained collagen-binding activity. Strains containing the truncated form of EmaA had single or multiple substitutions, deletions or insertions in the sequences, which resulted in the absence of EmaA molecules on the outer membrane and loss of collagen-binding activity. Population structure analyses of this organism, based on *emaA*, indicated that serotypes b and c belonged to one subpopulation, which was independent of the other serotypes. The main divergence was found in the functional head domain. The conserved *emaA* genotype within serotypes suggests a stable clonal linkage between this autotransporter protein and other virulence determinants.

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INTRODUCTION

Actinobacillus actinomycetemcomitans, which has been reclassified as *Aggregatibacter actinomycetemcomitans* (Nørskov-Lauritsen & Kilian, 2006), is a non-motile, Gram-negative, capnophilic bacterium associated with periodontal disease. This bacterium is strongly implicated in localized aggressive periodontitis (LAP) (Haubek *et al.*, 2002; Yang *et al.*, 2004) that is characterized by unexpected, rapid periodontal attachment loss (Paju *et al.*, 2000), bone destruction (Haubek *et al.*, 2002) and neutrophil dysfunction. LAP occurs mainly in young individuals under 35 years of age, and is particularly common in ethnic Africans (Albandar *et al.*, 2002; Haubek *et al.*, 2002; Paju *et al.*, 2000). In addition to oral infections, *A. actinomycetemcomitans* is an opportunistic pathogen that causes serious systemic infections, such as endocarditis (Paju *et al.*, 2000; Patrel *et al.*, 2004), pulmonary infections and

osteitis (Paju *et al.*, 2000). Recent studies also link this micro-organism with coronary heart disease (Pussinen *et al.*, 2005; Spahr *et al.*, 2006).

The pathogenesis of *A. actinomycetemcomitans* involves multiple virulence determinants, which contribute to the development of disease. These virulence molecules include LPS (Dixon & Darveau, 2005), leukotoxin (Kolodrubetz *et al.*, 1989; Lally *et al.*, 1994), bundle fimbriae (Kachlany *et al.*, 2001), as well as afimbrial adhesins. These adhesins include an epithelial cell adhesin, Aae (Rose *et al.*, 2003), a multifunctional protein involved in cell adhesion and invasion, Omp100 (Asakawa *et al.*, 2003), and the extracellular matrix protein adhesin A (EmaA) which mediates the interaction of *A. actinomycetemcomitans* with collagen (Mintz, 2004; Ruiz *et al.*, 2006).

EmaA was described initially as an outer-membrane protein (202 kDa), encoded by a 5898 bp ORF (Mintz, 2004). Recently, we have demonstrated that *emaA* is essential for the formation of antennae-like surface structures, composed of multimeric EmaA molecules, which are required for collagen binding (Ruiz *et al.*,

Abbreviations: LAP, localized aggressive periodontitis; MCMC, Markov chain Monte Carlo; O-PS, O polysaccharide; TEM, transmission electron microscopy.

2006). EmaA is an orthologue of the well-characterized, trimeric autotransporter and collagen-binding adhesin YadA of *Yersinia enterocolitica* (Hoiczky *et al.*, 2000; Skurnik & Wolf-Watz, 1989). EmaA may be grouped with the oligomeric coiled-coil adhesin (Oca) family. The Oca family of adhesins shares common structural characteristics, including an N-terminal globular head domain that contains active motifs, a coiled-coil pillar-like intermediate stalk, and a C-terminal transmembrane anchor domain (or pore-forming translocator domain) that inserts into the outer membrane and facilitates the localization of the adhesin on the bacterial surface (Cotter *et al.*, 2006; Hoiczky *et al.*, 2000; Roggenkamp *et al.*, 2003).

LPS is an important outer-membrane molecule of Gram-negative bacteria, and covers 75 % of the cell surface (Caroff & Karibian, 2003). The O polysaccharide (O-PS) component of LPS, which extends from the outer membrane, is the major antigen that stimulates the host immune response. According to distinct O-PSs, *A. actinomycetemcomitans* is categorized into six serotypes: a, b, c, d, e and f (Kaplan *et al.*, 2001; Nakano *et al.*, 2000, 1998; Suzuki *et al.*, 2000; Yoshida *et al.*, 1999, 1998). Serotype b is strongly implicated in aggressive periodontitis (Dogan *et al.*, 1999; Haubek *et al.*, 2002; Yang *et al.*, 2004). Serotype c is predominant in most populations, including ethnic Asians (Yoshida *et al.*, 2003), Caucasians (Dogan *et al.*, 1999; Yang *et al.*, 2004) and Hispanics (Teixeira *et al.*, 2006). Both b and c serotypes are found in extra-oral infections more frequently than other serotypes (Paju *et al.*, 2000).

In this study, *emaA* was found to be present in all 27 strains of *A. actinomycetemcomitans* investigated, covering six serotypes. Genetic variations appear to be serotype-related, and translate into three different forms of the protein (full-length, intermediate and truncated), which results in both

structural and biological diversity. Only the full-length and intermediate proteins form antennae-like structures associated with the outer membrane. The strong linkage between EmaA and serotype suggests an association between this autotransporter and OP-S that determines the serotype of this organism.

METHODS

Strain selection. Twenty-seven strains of *A. actinomycetemcomitans* belonging to six serotypes were chosen for this study (Table 1). These strains included three American Type Culture Collection (ATCC) reference strains, four laboratory strains and 20 clinical strains isolated from subgingival plaque of a Caucasian cohort, except for two strains (BL0293 and CU1000N). BL0293 was isolated from the blood sample of a Caucasian male with endocarditis; CU1000N was originally isolated from a 13-year-old female of African descent with LAP (Kaplan *et al.*, 2001). The serotype of all strains examined was determined by serological tests (Zambon *et al.*, 1983), as well as analysis of partial serotype-specific sequences (Kaplan *et al.*, 2001). All *A. actinomycetemcomitans* strains were stored at -80°C , and recovered on TSBYE agar plates (3 % trypticase soy broth, 0.6 % yeast extract, 1.5 % agar) (Becton Dickinson) at 37°C with 10 % humidified CO_2 . The same growth conditions were used in all the assays mentioned in this study. The original rough-form colonies (1 mm diameter, translucent, with internal star shape) from clinical samples were used for purification of genomic DNA and sequencing of the *emaA* gene. The smooth-colony variants (round and opaque) of clinical strains without fimbriae were obtained after five to eight subcultures in TSBYE broth (Inouye *et al.*, 1990). In order to clearly expose these fine structures and test the collagen-binding activity without the influence of fimbriae, the smooth phenotype was used for transmission electron microscopy (TEM) and collagen-binding assays.

Amplification and sequencing of *emaA*. The genomic DNA was extracted by using a commercial DNA isolation kit (Puregene). The *emaA* gene including upstream and downstream sequences was amplified as two individual amplicons, according to the prototypic

Table 1. *A. actinomycetemcomitans* strains used in this study

VT1169 is a spontaneous rifampicin- (Rif) and nalidixic acid (Nal)-resistant strain derived from the clinical strain SUNY465 (Mintz, 2004).

Serotype	Strain						Source or reference
a	ATCC 29523	IDH1062*	IDH1445*	IDH2303*	IDH3812*	IDH4139a*	ATCC and IDH† (M. Saarela) VT1169; Mintz (2004)
b	VT1169 (SUNY465)	HK 1651	Y4	JP2	ATCC 29522		
c	ATCC33384	PM73*	IDH2681*	IDH84Aa*	BL0293*‡		IDH; PM73: Dr C. I. Hoover, University of California, San Francisco; BL0293: Credit Valley Hospital, Mississauga, ON, Canada
d	IDH269*	IDH1344*	IDH3196*	IDH3863*			IDH
e	IDH1147*	IDH3095*	IDHd-85*	IDH2149*	IDHd13Ba*		IDH
f	IDHp474a*	CU1000N					IDH, CU1000N; Kaplan <i>et al.</i> (2001)

*Clinical strains were isolated from subgingival plaque of human oral cavity.

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‡BL0293 was isolated from the blood sample of a patient with endocarditis and bacteraemia.

emaA sequence reported previously (Mintz, 2004). The PCR reactions were performed using an Expand High-Fidelity PCR system (Roche). The amplicons were purified using a QIAquick gel extraction kit (Qiagen). All DNA sequence analyses were performed using an ABI 3130xl genetic analyser (Applied Biosystems) in the DNA Analysis Core Facility, Vermont Cancer Center, University of Vermont.

Prediction of protein sequences. Protein sequences were deduced based on the nucleotide sequences using the Expert Protein Analysis System (ExPASy) of the Swiss Institute of Bioinformatics (SIB) (<http://www.expasy.org>). If the deduced protein sequences were truncated due to substitution, deletion or insertion mutation, the longest ORF was chosen for phylogenetic analyses. The nucleotide sequences and the deduced protein sequences were aligned using Needleman–Wunsch global alignment through the European Molecular Biology Laboratory, the European Bioinformatics Institute (EMBL-EBI) (<http://www.ebi.ac.uk/emboss/align/>).

Subtyping of *emaA* DNA sequences and corresponding EmaA proteins. The *emaA* sequences were subgrouped into genotypes based on the aligned DNA sequences. In addition, different forms of EmaA were predicted at the protein level, according to the deduced amino acid sequences and homology with the prototypic structural domains of EmaA reported in our previous work (Mintz, 2004).

Development of mAbs against the stalk domain of EmaA. mAbs targeting the stalk domain of the prototypic full-length EmaA were developed. A glutathione-S-transferase (GST) fusion protein was synthesized by amplification of a 2172 bp fragment from the prototype *emaA* sequence (*emaA-L1*, Fig. 1) using primers engineered with *Sma*I and *Sal*I sites: *emaA*1892*Sma*I (5'-CCCCCGGGCAGT-CGTGTAGAACA-3') and *emaA*4144*Sal*I (5'-ATGTCGACTATC-TGCACCACCCACAA-3'). The amplicon was cloned into the pGEX-6P1 vector (Amersham). The generated GST fusion protein included a fragment of EmaA between amino acids 631 and 1354

Long *emaA* genotypes

Short *emaA* genotypes

Fig. 1. Genotyping of *emaA*. The percentages represent identity when compared with *emaA-L1* or *emaA-L3* (marked with an asterisk in parentheses). The numbers on the right represent the corresponding serotypes, e.g. for *emaA-L1*, 'Serotype b (5/5); serotype c (2/5)' represents five of five examined serotype b strains and two of five serotype c strains belonging to *emaA-L1*. *emaA* was arbitrarily divided into two groups: long *emaA* (>5890 bp) and short *emaA* (≤5040 bp). The long *emaA* sequences were subdivided into six genotypes: *emaA-L1* to *-L6*. The short *emaA* sequences were composed of four genotypes: *emaA-S1* to *-S4*. The transmembrane anchor domain sequence was conserved in different genotypes. *emaA-L1* and *-L2* had the prototypic head sequence that showed 25% variation in the DNA sequence when compared with the non-prototypic head sequence found in the others.

(Fig. 3). The fusion protein was expressed in *Escherichia coli* (strain BL21 pLysS), and purified using glutathione Sepharose according to the manufacturer's instructions. The GST tag was cleaved by incubation with Prescission Protease (Amersham) before injection into mice for generation of the anti-EmaA mAb.

The generated hybridomas (Green Mountain Antibodies) were grown in RPMI S10 HT [RPMI-1640 cell culture medium, supplemented with 10 % fetal bovine serum, 0.5 % hybridoma cloning factor (HCF; Bioveris), hypoxanthine-thymidine (Gibco-BRL), penicillin (100 IU ml⁻¹) and streptomycin (100 µg ml⁻¹)]. After the cells had grown to ~80 % confluency, the supernatant was removed and screened against the purified target protein using an ELISA. The culture supernatants were screened for antibody activity by ELISA using the antigen purified as a maltose-conjugated fusion protein. The fusion protein was expressed in the pMal system (New England) by using the primers StalkSalIF (5'-GTCGACAGTCGTGTAGAACAAAGG-3') and StalkHindIIIR (5'-AAGCTTTATCTGCACCACCCACAA-3'). Proteins were purified following the manufacturer's instructions. Positive-reacted hybridomas were subcloned by limiting dilution in 96-well plates in 100 µl RPMI S10 HT with 1 % HCF. After two rounds of subcloning and screening by ELISA, positive hybridomas were expanded and injected into mice for ascites production. Antibody was purified from ascites fluid using protein affinity chromatography (Green Mountain Antibodies).

Preparation of membrane proteins. The total membrane protein content of *A. actinomycetemcomitans* was prepared as described previously (Mintz, 2004). Briefly, 200 ml stationary-phase cells was harvested and resuspended in 3.0 ml 10 mM HEPES, pH 7.4, with 1 mM PMSF and 1 × Complete Protease Inhibitor Cocktail (Roche). The cells were lysed using a French press at 9000 Pa three times. The lysate was centrifuged at 100 000 g for 40 min, and the pellet was resuspended in HEPES with 2 % (w/v) SDS as the membrane fraction.

Analysis using SDS-PAGE. Membrane protein (250 µg) from each sample was prepared in a loading buffer containing 10 mM HEPES, 2 % SDS, 5 % (v/v) β-mercaptoethanol, 2 % (v/v) glycerol and 0.05 % (w/v) bromophenyl blue, boiled for 5 min, and loaded in a 4–15 % gradient polyacrylamide Tris/HCl ready gel (Bio-Rad) and run in Laemmli buffer (Laemmli, 1970). The separated proteins were transferred to a nitrocellulose membrane, probed with the anti-EmaA mAb, and detected with horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich). Signals were detected using the SuperSignal West Pico chemiluminescent substrate (Pierce), and visualized by exposure to film (Kodak).

TEM. The EmaA structures were visualized using TEM of whole-cell mount preparations, as described by Ruiz *et al.* (2006).

Collagen-binding activity of *A. actinomycetemcomitans* expressing different forms of EmaA. Collagen-binding activity was evaluated using Bornstein & Traub (1979) type V collagen from human placenta (Sigma type IX, C3657, Sigma-Aldrich) incorporated into a 3D gel matrix (Matrigel, BD Biosciences). A final volume of 50 µl, including 25 µl Matrigel, 40 µg type V collagen and TSBYE, was added to individual wells of sterile 96-well tissue-culture plates. Following polymerization at 37 °C for 2.5 h, 200 µl TSBYE was added to each well and incubated at 37 °C overnight. Mid-exponential-phase cells (5 × 10⁷) were added to each well and incubated at 37 °C with 10 % CO₂ for 3 h. The matrix was washed with PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4), immersed in 200 µl TSBYE, and incubated at 4 °C overnight, which resulted in the depolymerization of the gel. A pilot study proved that all the *A. actinomycetemcomitans* strains used in this study survived during the overnight depolymerization at 4 °C without significant changes in cell numbers. The bacterial suspension was removed from each well,

diluted and enumerated on TSBYE agar plates. Quadruplicate individual experiments were performed, with duplicate assays for each strain examined in each individual experiment. An unpaired *t* test was performed (GraphPad InStat version 3.05), and *P* < 0.05 was considered statistically significant.

Phylogenetic analysis. The *emaA* nucleotide sequences from different strains, as well as the translated protein sequences, were aligned using both T-Coffee (Notredame *et al.*, 2000) and MUSCLE (Edgar, 2004) programs, with default parameters. Phylogenetic analyses were performed using three different approaches: Mr Bayes 3.1 for the Bayesian method (Huelsenbeck & Ronquist, 2001), and PROTPARS/DNAPARS and PROTDIST/DNADIST-NEIGHBOR from the Phylogeny Inference Package (PHYLIP, version 3.6) for maximum-parsimony and neighbour-joining methods, respectively (Felsenstein, 2005). The SEQBOOT and CONSENSE programs of the PHYLIP package were used for the generation of bootstrapped datasets and consensus tree reconstructions, respectively. Phylogenetic analyses were performed using both nucleotide sequences and the deduced protein sequences. YdaA of *Y. enterocolitica* (GenBank accession no. X13882), an orthologue of EmaA, was included as an outgroup for localization of the root of *emaA*.

Genotyping of *aae*. Aae, another *A. actinomycetemcomitans* outer-membrane protein, was chosen as a control to determine if the serotype-related variations found in *emaA* also exist in other outer-membrane autotransporters. The *aae* amplicon was generated using primers AAE5 and AAE3 under identical conditions, as described by Rose *et al.* (2003). The genotyping of the *aae* gene was based on the sizes of generated amplicons.

GenBank accession numbers. The GenBank accession numbers for *emaA* of the strains used in this study are as follows. Serotype a: ATCC 29523 (DQ991438), IDH1062 (DQ991439), IDH2303 (DQ991440), IDH3812 (DQ991441), IDH1445 (DQ991442), IDH4139a (DQ991443); serotype b: VT1169 (AY344064), Y4 (DQ991444), JP2 (DQ991445), ATCC 29522 (DQ991446); serotype c: ATCC 33384 (DQ991447), PM73 (DQ991448), IDH2681 (DQ991449), IDH84Aa (DQ991450), BL0293 (DQ991451); serotype d: IDH1344 (DQ991452), IDH3863 (DQ991453), IDH269 (DQ991454), IDH3196 (DQ991455); serotype e: IDHd-85 (DQ991456), IDHD13Ba (DQ991457), IDH2149 (DQ991458), IDH1147 (DQ991459), IDH3095 (DQ991460); serotype f: IDHp474a (DQ991461), CU1000N (DQ991462). The *emaA* nucleotide sequence of HK1651 was provided by the Los Alamos Oral Pathogen Sequence Database (<http://www.oralgen.lanl.gov/>).

RESULTS

Genotyping of *emaA* and serotype-specific heterogeneity

The *emaA* gene was present in all examined strains covering six serotypes and showed serotype-specific variations. The gene was conserved within the serotype, and the apparent diversity within serotypes a and e was due to one particular strain in each of the serotypes (Fig. 1). The genotyping was based on the aligned nucleotide sequences and the homology to the prototypic *emaA* reported in our previous work (Mintz, 2004). *emaA* was arbitrarily divided into two groups based on the sizes of the nucleotide sequences: long *emaA* (>5890 bp; 17 strains) and short *emaA* (≤5040 bp; 10 strains). The major difference

between these two groups was an 837 bp in-frame deletion, corresponding to the region between nucleotides 1540 and 2378 of the prototypic sequence, which was downstream of the head domain.

The long *emaA* group was subdivided into six genotypes, *emaA-L1* to *emaA-L6*, based on nucleotide sequence (Fig. 1). *emaA-L1*, the prototype (5898 bp) of *emaA*, corresponded to the intact ORF that encodes the full-length protein (1965 aa, 202 kDa). *emaA-L1* was found in all serotype b strains examined, which shared 99 % sequence identity. Two serotype c strains were also included within this genotype. The remaining three serotype c strains were grouped as *emaA-L2*, which was the result of a 4 bp insert (5'-TTAA-3') at nucleotide 154. This frame-shift mutation generated a premature stop codon within the signal sequence (Fig. 2). Excluding the insertion mutation, *emaA-L1* and *emaA-L2* had an almost identical sequence.

The remaining four long *emaA* genotypes (*emaA-L3*, -*L4*, -*L5* and -*L6*) were differentiated from *emaA-L1* and *emaA-L2* in the non-prototypic head sequence, which shared only 75 % identity with the corresponding region of the

prototype gene (Fig. 1). *emaA-L3*, -*L4* and -*L6* genotypes were associated with serotype e only. *emaA-L3* (5907 bp), found in one serotype e strain, was a single ORF encoding a protein (1968 aa, 202 kDa) with a size similar to that of the prototypic EmaA. *emaA-L4*, found in a single serotype e strain, contained an 888 bp insertion element (IS888) within the stalk domain. This insertion element disrupted the ORF of *emaA* (Fig. 2). Excluding the insertion IS888, *emaA-L4* was identical to *emaA-L3*. *emaA-L6* comprised the remaining three serotype e strains. The 8 and 6 % sequence variation in the stalk and transmembrane anchor domains differentiated *emaA-L6* from *emaA-L3*, as well as *emaA-L5*. However, all of them shared the identical non-prototypic head sequence (Fig. 1). In addition, a point mutation at nucleotide 389 (C→A) of *emaA-L6* resulted in a premature stop codon close to the N terminus (Fig. 2).

The *emaA-L5* genotype was exclusively found in serotype f, and shared 99.8 % similarity with *emaA-L3*. Unlike *emaA-L3*, which encodes an intact full-length EmaA, a single base deletion at nucleotide 479 of *emaA-L5* resulted in a premature stop codon within the head domain (Fig. 2). These two serotype f strains were isolated from distinct

Fig. 2. Subtyping of EmaA proteins. The percentages represent identity values when compared with the deduced protein sequences of either *emaA-L1* or *emaA-L3* (marked by an asterisk in parentheses). Three forms of EmaA were predicted: full-length, intermediate and truncated, based on the predicted protein sequences of 10 genotypes. The full-length EmaA was found mainly in *emaA-L1* (serotypes b and c) and occasionally in *emaA-L3* (serotype e), while the intermediate EmaA was only present in *emaA-S1* (serotypes d and a).

sources (a 13-year-old African-American female and a Scandinavian Caucasian), but the *emaA* sequences were 99.9% identical.

The short *emaA* genotypes (*emaA-S1*, -S2, -S3 and -S4), containing the non-prototypic head sequence, were associated with serotypes a and d. Only *emaA-S1*, found in all serotype d and 33% of serotype a strains, encoded an intact ORF (Fig. 2). *emaA-S2*, present in 33% of serotype a strains, contained point mutations that created a premature stop codon close to the C terminus (Fig. 2), otherwise *emaA-S2* was identical to *emaA-S1*. *emaA-S3* was identical to *emaA-S1* and -S2, except for an 8 bp deletion within the signal sequence, which generated a premature stop codon within the signal sequence (Figs 1 and 2). *emaA-S4* was different from the other genotypes. The signal peptide, the head domain and the first half of the stalk region were completely missing in this genotype. However, the C terminus was highly conserved throughout all genotypes (Fig. 1).

Subtyping of EmaA proteins

Three forms of the EmaA protein were deduced: full-length, intermediate and truncated (Fig. 2). The full-length EmaA was predicted to be a 202 kDa protein, which was associated with 100% of serotype b strains, 40% of serotype c strains, and 20% of serotype e strains investigated. The full-length EmaA of serotype e was

homologous to the prototypic EmaA of serotypes b and c in the stalk and anchor domains, but differed in the head sequence. However, the putative collagen-binding motifs were invariant within the head domain sequence (data not shown).

The intermediate EmaA contained a 279 aa deletion between the head domain and the stalk region, and was predicted to be a 173 kDa (1679 aa) protein. The predicted protein was identical to the full-length EmaA found in serotype e, excluding the deletion. One hundred per cent of serotype d and 33% of serotype a strains were associated with the intermediate EmaA. The truncated EmaA was found in 100% of serotype f strains, 80% of e strains, 67% of a strains and 60% of c strains. The deduced protein sequences indicated premature termination of translation in the signal sequence, the head or the stalk region.

Detection of the full-length and intermediate forms of EmaA

The mAb used in this study interacted with the denatured form of the protein only, and was targeted to an epitope within the stalk region of EmaA. Two individual forms of EmaA molecules were detected from the membrane fraction isolated from the different genotypes (Fig. 3). The full-length EmaA was detected in the *emaA-L1* genotype (VT1169 and ATCC 33384), while the intermediate EmaA was found in the *emaA-S1* genotype

Fig. 3. Detection of EmaA from membrane fractions using mAbs. EmaA was detected using a mAb that targeted the stalk domain of the protein. Total membrane protein (250 µg) from each strain sample was loaded onto a 4–15% gradient polyacrylamide gel, transferred to a nitrocellulose membrane and probed with the antibody. Two forms of EmaA molecules were detected: the full-length (I) and the intermediate (II). However, no product was detected from the membrane of strains with the truncated form of EmaA (III).

(IDH3863). However, no EmaA products were detected in the membrane protein of strains with the truncated EmaA using this mAb (Fig. 3). The four strains with the truncated form of EmaA included three strains with stop codons close to the N terminus: IDH2681 (*emaA-L2*), CU1000N (*emaA-L5*) and IDHd-85 (*emaA-L4*); and one close to the C terminus: ATCC 29523 (*emaA-S1*) (Fig. 3).

Visualization of EmaA structures

Antennae-like appendages were present on the surface of strains containing the full-length and intermediate EmaA only (Fig. 4). The full-length prototypic EmaA structures expressed a rod-like stalk and an ellipsoidal head domain at the end of each stalk. In comparison, the intermediate EmaA structure appeared to be shorter than the prototypic structures. The shorter appearance of the intermediate form was associated with the 279 aa deletion downstream of the head domain. In addition, the ellipsoidal head present on the prototypic structures was not visible on the intermediate EmaA structures containing the non-prototypic head sequence.

Collagen-binding activity of EmaA

Collagen-binding activity was evaluated using a reconstituted basement membrane model, in which human type V collagen was incorporated into the 3D Matrigel matrix. Collagen-binding activity was positively correlated with the presence of EmaA surface structures, composed of either the full-length or the intermediate form of EmaA (Fig. 5). The strain containing the truncated form of EmaA demonstrated little binding activity to either incorporated collagen or Matrigel alone, and in this respect was identical to the *emaA*⁻ mutant.

Phylogeny of the *emaA* gene

The interrelationship between *emaA* genotypes and serotypes was determined by constructing a phylogenetic tree. The confidence value in each clade was determined by three parameters: bootstrap support under maximum-parsimony on 100 samples; bootstrap support using the neighbour-joining algorithm on 100 samples; and the posterior probability obtained from Markov chain Monte

Full-length EmaA (genotype *emaA-L1*)

Intermediate EmaA (genotype *emaA-S1*)

Fig. 4. EmaA surface structures visualized by TEM of whole mount bacterial preparations. Only the full-length and intermediate EmaA proteins formed antenna-like appendages (shown by arrows) on the surface of *A. actinomycetemcomitans*, while the truncated EmaA did not. The ellipsoidal heads (labelled in circles) are shown clearly in the full-length EmaA with the prototypic head domain. The intermediate EmaA that had the non-prototypic head domain and a 279 aa deletion at the neck region appeared to be shorter and the ellipsoidal head absent. Bars, 100 nm.

Fig. 5. Collagen-binding activity of *A. actinomycetemcomitans* strains displaying the three forms of EmaA protein. Error bars represent SEM. Both the full-length (I) and intermediate (II) EmaA showed a greater number of cells binding to collagen V than Matrigel alone ($P < 0.05$). The truncated EmaA (III) showed little binding to either collagen V (black bars) or Matrigel alone (white bars) ($P > 0.05$), which was identical to the *emaA*⁻ mutant.

Carlo (MCMC) simulation using the Mr Bayes program (Fig. 6). The data indicated that the *emaA* genotypes could be grouped into two major clusters: one included *emaA-L1* (serotypes b and c) and *emaA-L2* (serotype c); the other included *emaA-L3* (serotype e), *emaA-L4* (serotype e), *emaA-L5* (serotype f), *emaA-L6* (serotype e), *emaA-S1* (serotypes a and d), *emaA-S2* (serotype a) and *emaA-S3* (serotype a). However, the unique *emaA-S4* (serotype a) appeared to be distinct from the above two clusters (Fig. 6). The main difference between these two lineages was the functional head domain, which contains multiple collagen-binding motifs. *emaA-S4*, however, was divergent from these two lineages. Further analysis, based on the predicted protein sequences of EmaA and YadA, suggested that *emaA-S4* was either an allele generated by multiple mutations or a member of another evolutionary clade.

Serotype-related genetic diversity is not associated with all outer-membrane proteins of *A. actinomycetemcomitans*

Aae, another autotransporter adhesin of this bacterium, was included in this study to determine whether serotype-related

Fig. 6. Phylogeny of *emaA*. The values at the nodes were posterior probability values estimated using Bayesian inference (MCMC) and bootstrapping confidence values (in parentheses) obtained by using maximum-parsimony and neighbour-joining methods. a_IDH4139a (*emaA-S4*, III) represents serotype a, strain IDH4139a, genotype *emaA-S4*, truncated EmaA. The two major clades represent the evolution of two different head domain sequences, prototype (serotypes b and c) and non-prototype (serotypes a, d, e and f). I, full-length EmaA; II, intermediate EmaA; III, truncated EmaA. The genotypes with EmaA structures on the cell surface are in bold type.

heterogeneity is a common phenomenon in genes encoding outer-membrane proteins of *A. actinomycetemcomitans*. The *aae* gene contains multiple copies of a 135 bp repeat, the number of which varies between strains (Rose *et al.*, 2003). To determine whether the number of repeats is linked to a specific serotype, the ~3 kb amplicon of the *aae* gene from different serotypes was determined. Four different genotypes of *aae*, based on difference sizes of the amplicons, were found among 14 strains covering the six serotypes. Multiple *aae* alleles were found associated with the serotype b strains, which contrasted with the findings for *emaA*. Variation in the number of repeats within *aae* was also found in the other five serotypes of *A. actinomycetemcomitans*. However, the number of repeats was not linked to a specific serotype. Therefore, the conserved *emaA* genotypes found associated with specific serotypes did not apply to another outer-membrane protein, Aae.

DISCUSSION

EmaA is an oligomeric adhesin of *A. actinomycetemcomitans* that mediates the interaction between this organism and type V collagen (Mintz, 2004; Ruiz *et al.*, 2006). Type V collagen is ubiquitously distributed in human interstitial tissues, including periodontal ligaments (Becker *et al.*, 1991) and the extracellular matrix of aortic media (Dingemans *et al.*, 2000). This matrix protein is exposed in inflamed or degenerated tissue (Hillmann *et al.*, 1998), and may become a substrate for the colonization of bacteria, consequently causing both oral and extra-oral infections.

Population genetic analyses based on *emaA* suggest that serotypes b and c are closely related, when compared with the remaining four serotypes, which is consistent with previous data (Haubek *et al.*, 1995; Kaplan *et al.*, 2002; Poulsen *et al.*, 1994). The previous work based on DNA fingerprinting, serotypes, 16S rRNA, *lkt* (leukotoxin), *flp-1* (major fimbriae subunit) and *cdt* (cytolethal distending toxin) suggests that serotypes b and c are genetically related, as are a, d, e and f. Among the latter four serotypes, a and d are phylogenetically related, and e and f are closer to each other (Kaplan *et al.*, 2002). The phylogenetic trees based on *emaA* suggest that two lineages diverged during evolution, representing the two different head domains of *emaA*. The serotype-specific genetic heterogeneity found in *emaA* appears to be additional evidence for the hypothesis of the clonal population structure in the evolutionary biology of *A. actinomycetemcomitans* (Poulsen *et al.*, 1994).

However, the serotype-related genetic diversity associated with EmaA is not applicable to another autotransporter adhesin, Aae (Rose *et al.*, 2003). Aae is orthologous to the epithelial adhesin Hap of *Haemophilus influenzae* (St Geme & Cutter, 2000), and mediates the interaction of *A. actinomycetemcomitans* with epithelial cells derived from humans and old-world primates (Fine *et al.*, 2005; Rose

et al., 2003). The allele of the *aae* gene varies due to different numbers of a 135 bp imperfect repeat (Rose *et al.*, 2003). Examination of this gene among the strains investigated in this study indicates that the variation in the number of repeats in *aae* does not segregate with bacterial serotypes, which may be a result of spontaneous mutations stimulated by periodic selection (Spratt *et al.*, 1995). In contrast with *aae*, the conserved *emaA* genotype within each subpopulation of this organism suggests a stable clonal linkage between *emaA* and other virulence determinants within the same descent (Poulsen *et al.*, 1994; Spratt *et al.*, 1995).

Serotyping of *A. actinomycetemcomitans* is based on the O-PS, and these groupings suggest a correlation between the *emaA* and the type of LPS present on the surface of this bacterium. Interestingly, Jain *et al.* (2006) have observed that O-PS stabilizes the membrane structure and is involved in the transmembrane secretion machinery of some large autotransporters in Gram-negative bacilli. Therefore, the O-PS of *A. actinomycetemcomitans* may regulate EmaA assembly or determine the type of EmaA assembled on the surface of the bacterium.

Heterogeneity in the chromosome of *emaA* is recapitulated at the protein level. Three different forms of EmaA are predicted, but only two forms of the protein are associated with the membrane. The diversity in the sequence of these proteins does not interfere with the formation of surface structures or collagen-binding activity. The collagen-binding motifs in the head domain are fully conserved in the full-length and intermediate forms, in spite of the sequence variation in this region. The conservation of these motifs lends support to the hypothesis that these motifs are important for collagen-binding activity. In addition, the higher collagen-binding activity in the strain with the non-prototypic head domain suggests that the amino acid variations may alter the structure of the molecule and consequently affect collagen-binding activity. Studies are under way to determine which sequences are important for the interaction between the structure and collagen.

A sequence of about 20 aa has been identified in YadA that shows high similarity to conserved sequences in other members of the Oca protein family. This region is located between the head and stalk domains and is termed the neck sequence (Roggenkamp *et al.*, 2003). These sequences are important to assemble and maintain the structure of this class of proteins. Three conserved neck sequences are present in the EmaA sequence (Mintz, 2004). The first two are located between the head and the stalk regions, while the third is located close to the anchor domain. In YadA, deletion of this sequence abolishes collagen-binding activity (Roggenkamp *et al.*, 2003). However, the intermediate EmaA protein lacks the second neck sequence and still maintains binding activity. Together, the data suggest that the second neck sequence is not essential for binding activity.

The deletion and the sequence variation within the head domain most likely contribute to the appearance of the

shorter EmaA structures and the apparent absence of the ellipsoidal head (Fig. 4). These structural changes may explain the higher collagen-binding activity of the intermediate EmaA strain. The differences in the proteins lend additional support to the proposal that EmaA is the structural subunit of these surface appendages.

The positive correlation between the presence of EmaA surface structures and collagen-binding activity is also supported by the comparison of strains ATCC 29523 (truncated EmaA) and IDH1062 (intermediate EmaA) (Fig. 5). These two serotype a strains share an identical *emaA* sequence, except for a single thymidine deletion at position 4380 bp in ATCC 29523. The deletion results in a truncated EmaA protein lacking the pore-forming transmembrane domain. The C terminus is essential for translocation, stabilization and full-level function of the trimeric adhesins, such as Hia and YadA (Cotter *et al.*, 2006). ATCC 29523 does not express surface structures and displayed minimal collagen-binding activity. This contrasts sharply with the presence of surface appendages and the 120-fold increase in the number of cells bound to collagen demonstrated with the clinical strain IDH1062. The data imply that the C terminus of the protein is required for the assembly of EmaA structures, as proposed for the orthologous region of YadA (Roggenkamp *et al.*, 2003; Cotter *et al.*, 2006).

In conclusion, two forms of intact EmaA molecules are found in *A. actinomycetemcomitans*, and may mediate the colonization of host tissues by this organism. Incomplete translation or assembly of this oligomeric protein adhesin, as suggested by the truncated forms of EmaA, results in the loss of adhesion activity. However, we cannot exclude the possibility that these forms of the protein have alternative functions. The conservation throughout evolution of such a large protein in the most common serotypes found in human periodontitis and extra-oral infections suggests an important role for this protein or structure in the persistence of this pathogen within the human oral cavity.

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REFERENCES

- Albandar, J. M., Muranga, M. B. & Rams, T. E. (2002). Prevalence of aggressive periodontitis in school attendees in Uganda. *J Clin Periodontol* **29**, 823–831.
- Asakawa, R., Komatsuzawa, H., Kawai, T., Yamada, S., Goncalves, R. B., Izumi, S., Fujiwara, T., Nakano, Y., Suzuki, N. & other authors (2003). Outer membrane protein 100, a versatile virulence factor of *Actinobacillus actinomycetemcomitans*. *Mol Microbiol* **50**, 1125–1139.
- Becker, J., Schuppan, D., Rabanus, J., Rauch, R., Niechoy, U. & Gelderblom, H. (1991). Immunoelectron microscopic localization of collagens type I, V, VI and of procollagen type III in human periodontal ligament and cementum. *J Histochem Cytochem* **39**, 103–110.
- Bornstein, P. & Traub, W. (1979). The chemistry and biology of collagen. In *The Proteins*, 3rd edn, vol. 4, pp. 411–632. Edited by H. Neurath & R. L. Orlando, FL: Hill Academic Press.
- Caroff, M. & Karibian, D. (2003). Structure of bacterial lipopolysaccharides. *Carbohydr Res* **338**, 2431–2447.
- Cotter, S. E., Surana, N. K., Grass, S. & St. Geme, J. W. (2006). Trimeric autotransporters require trimerization of the passenger domain for stability and adhesive activity. *J Bacteriol* **188**, 5400–5407.
- Dingemans, K. P., Teeling, P., Legendijk, J. H. & Becker, A. E. (2000). Extracellular matrix of the human aortic media: an ultrastructural histochemical and immunohistochemical study of the adult aortic media. *Anat Rec* **258**, 1–14.
- Dixon, D. R. & Darveau, R. P. (2005). Lipopolysaccharide heterogeneity: innate host responses to bacterial modification of lipid A structure. *J Dent Res* **84**, 584–595.
- Dogan, B., Saarela, M., Jousimies-Somer, H., Alaluusua, S. & Asikainen, S. (1999). *Actinobacillus actinomycetemcomitans* serotype e – biotypes, genetic diversity and distribution in relation to periodontal status. *Oral Microbiol Immunol* **14**, 98–103.
- Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* **32**, 1792–1797.
- Felsenstein, J. (2005). PHYLIP (Phylogeny Inference Package) version 3.6. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle.
- Fine, D. H., Velliyagounder, K., Furgang, D. & Kaplan, J. (2005). The *Actinobacillus actinomycetemcomitans* autotransporter adhesin Aae exhibits specificity for buccal epithelial cells from humans and old world primates. *Infect Immun* **73**, 1947–1953.
- Haubek, D., Poulsen, K., Asikainen, S. & Kilian, M. (1995). Evidence for absence in northern Europe of especially virulent clonal types of *Actinobacillus actinomycetemcomitans*. *J Clin Microbiol* **33**, 395–401.
- Haubek, D., Ennibi, O., Abdellaoui, L., Benzarti, N. & Poulsen, S. (2002). Attachment loss in Moroccan early onset periodontitis patients and infection with the JP2-type of *Actinobacillus actinomycetemcomitans*. *J Clin Periodontol* **29**, 657–660.
- Hillmann, G., Dogan, S. & Geurtsen, W. (1998). Histopathological investigation of gingival tissue from patients with rapidly progressive periodontitis. *J Periodontol* **69**, 195–208.
- Hoiczky, E., Roggenkamp, A., Reichenbecher, M., Lupas, A. & Heesemann, J. (2000). Structure and sequence analysis of *Yersinia* YadA and *Moraxella* UspAs reveal a novel class of adhesins. *EMBO J* **19**, 5989–5999.
- Huelsenbeck, J. P. & Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**, 754–755.
- Inouye, T., Ohta, H., Koikeguchi, S., Fukui, K. & Kato, K. (1990). Colonial variation and fimbriation of *Actinobacillus actinomycetemcomitans*. *FEMS Microbiol Lett* **57**, 13–17.
- Jain, S., van Ulsen, P., Benz, I., Schmidt, M. A., Fernandez, R., Tommassen, J. & Goldberg, M. B. (2006). Polar localization of the autotransporter family of large bacterial virulence proteins. *J Bacteriol* **188**, 4841–4850.
- Kachlany, S. C., Planet, P. J., DeSalle, R., Fine, D. H. & Figurski, D. H. (2001). Genes for tight adherence of *Actinobacillus actinomycetemcomitans*: from plaque to plague to pond scum. *Trends Microbiol* **9**, 429–437.

- Kaplan, J. B., Perry, M. B., MacLean, L. L., Furgang, D., Wilson, M. E. & Fine, D. H. (2001). Structural and genetic analyses of O polysaccharide from *Actinobacillus actinomycetemcomitans* serotype f. *Infect Immun* **69**, 5375–5384.
- Kaplan, J. B., Schreiner, H. C., Furgang, D. & Fine, D. H. (2002). Population structure and genetic diversity of *Actinobacillus actinomycetemcomitans* strains isolated from localized juvenile periodontitis patients. *J Clin Microbiol* **40**, 1181–1187.
- Kolodrubetz, D., Dailey, T., Ebersole, J. & Kraig, E. (1989). Cloning and expression of the leukotoxin gene from *Actinobacillus actinomycetemcomitans*. *Infect Immun* **57**, 1465–1469.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lally, E. T., Golub, E. E. & Kieba, I. R. (1994). Identification and immunological characterization of the domain of *Actinobacillus actinomycetemcomitans* leukotoxin that determines its specificity for human target cells. *J Biol Chem* **269**, 31289–31295.
- Mintz, K. P. (2004). Identification of an extracellular matrix protein adhesin, EmaA, which mediates the adhesion of *Actinobacillus actinomycetemcomitans* to collagen. *Microbiology* **150**, 2677–2688.
- Nakano, Y., Yoshida, Y., Yamashita, Y. & Koga, T. (1998). A gene cluster for 6-deoxy-L-talan synthesis in *Actinobacillus actinomycetemcomitans*. *Biochim Biophys Acta* **1442**, 409–414.
- Nakano, Y., Yoshida, Y., Suzuki, N., Yamashita, Y. & Koga, T. (2000). A gene cluster for the synthesis of serotype d-specific polysaccharide antigen in *Actinobacillus actinomycetemcomitans*. *Biochim Biophys Acta* **1493**, 259–263.
- Nørskov-Lauritsen, N. & Kilian, M. (2006). Reclassification of *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, *Haemophilus paraphrophilus* and *Haemophilus segnis* as *Aggregatibacter actinomycetemcomitans* gen. nov., comb. nov., *Aggregatibacter aphrophilus* comb. nov. and *Aggregatibacter segnis* comb. nov., and emended description of *Aggregatibacter aphrophilus* to include V factor-dependent and V factor-independent isolates. *Int J Syst Evol Microbiol* **56**, 2135–2146.
- Notredame, C., Higgins, D. G. & Heringa, J. (2000). T-Coffee: a novel method for fast and accurate multiple sequence alignment. *J Mol Biol* **302**, 205–217.
- Paju, S., Carlson, P., Jousimies-Somer, H. & Asikainen, S. (2000). Heterogeneity of *Actinobacillus actinomycetemcomitans* strains in various human infections and relationships between serotype, genotype, and antimicrobial susceptibility. *J Clin Microbiol* **38**, 79–84.
- Paturel, L., Casalta, J. P., Habib, G., Nezri, M. & Raoult, D. (2004). *Actinobacillus actinomycetemcomitans* endocarditis. *Clin Microbiol Infect* **10**, 98–118.
- Poulsen, K., Theilade, E., Lally, E. T., Demuth, D. R. & Kilian, M. (1994). Population structure of *Actinobacillus actinomycetemcomitans*: a framework for studies of disease-associated properties. *Microbiology* **140**, 2049–2060.
- Pussinen, P. J., Nyyssönen, K., Alfthan, G., Salonen, R., Laukkanen, J. A. & Salonen, J. T. (2005). Serum antibody levels to *Actinobacillus actinomycetemcomitans* predict the risk for coronary heart disease. *Arterioscler Thromb Vasc Biol* **25**, 833–838.
- Roggenkamp, A., Ackermann, N., Jacobi, C., Truelzsch, K., Hoffmann, H. & Heesemann, J. (2003). Molecular analysis of transport and oligomerization of the *Yersinia enterocolitica* adhesin YadA. *J Bacteriol* **185**, 3735–3744.
- Rose, J. E., Meyer, D. H. & Fives-Taylor, P. M. (2003). Aae, an autotransporter involved in adhesion of *Actinobacillus actinomycetemcomitans* to epithelial cells. *Infect Immun* **71**, 2384–2393.
- Ruiz, T., Lenox, C., Radermacher, M. & Mintz, K. P. (2006). Novel surface structures are associated with the adhesion of *Actinobacillus actinomycetemcomitans* to collagen. *Infect Immun* **74**, 6163–6170.
- Skurnik, M. & Wolf-Watz, H. (1989). Analysis of the *yopA* gene encoding the Yop1 virulence determinants of *Yersinia* spp. *Mol Microbiol* **3**, 517–529.
- Spahr, A., Klein, E., Khuseynova, N., Boeckh, C., Muche, R., Kunze, M., Rothenbacher, D., Pezeshki, G., Hoffmeister, A. & Koenig, W. (2006). Periodontal infections and coronary heart disease: role of periodontal bacteria and importance of total pathogen burden in the Coronary Event and Periodontal Disease (CORODONT) study. *Arch Intern Med* **166**, 554–559.
- Spratt, B. G., Smith, N. H., Zhou, J., O'Rourke, M. & Feil, E. (1995). The population genetics of the pathogenic *Neisseria*. In *Population Genetics of Bacteria*, pp. 143–160. Edited by S. Baumberg, J. P. W. Young, E. M. H. Wellington & J. R. Saunders. Cambridge, UK: Press Syndicate of University of Cambridge.
- St Geme, J. W., III & Cutter, D. (2000). The *Haemophilus influenzae* Hia adhesin is an autotransporter protein that remains uncleaved at the C terminus and fully cell associated. *J Bacteriol* **182**, 6005–6013.
- Suzuki, N., Nakano, Y., Yoshida, Y., Nakao, H. & Yamashita, Y. (2000). Genetic analysis of the gene cluster for the synthesis of serotype a-specific polysaccharide antigen in *Actinobacillus actinomycetemcomitans*. *Biochim Biophys Acta* **1517**, 135–138.
- Teixeira, R. E., Mendes, E. N., Rogue de Carvalho, M. A., Nicoli, J. R., Farias Lde, M. & Magalhaes, P. P. (2006). *Actinobacillus actinomycetemcomitans* serotype-specific genotypes and periodontal status in Brazilian subjects. *Can J Microbiol* **52**, 182–188.
- Yang, H. W., Asikainen, S., Dogan, B., Suda, R. & Lai, C. H. (2004). Relationship of *Actinobacillus actinomycetemcomitans* serotype b to aggressive periodontitis: frequency in pure cultured isolates. *J Periodontol* **75**, 592–599.
- Yoshida, Y., Nakano, Y., Yamashita, Y. & Koga, T. (1998). Identification of a genetic locus essential for serotype b-specific antigen synthesis in *Actinobacillus actinomycetemcomitans*. *Infect Immun* **66**, 107–114.
- Yoshida, Y., Nakano, Y., Suzuki, N., Nakao, H., Yamashita, Y. & Koga, T. (1999). Genetic analysis of the gene cluster responsible for synthesis of serotype e-specific polysaccharide antigen in *Actinobacillus actinomycetemcomitans*. *Biochim Biophys Acta* **1489**, 457–461.
- Yoshida, Y., Suzuki, N., Nakano, Y., Shibuya, K., Ogawa, Y. & Koga, T. (2003). Distribution of *Actinobacillus actinomycetemcomitans* serotypes and *Porphyromonas gingivalis* in Japanese adults. *Oral Microbiol Immunol* **18**, 135–139.
- Zambon, J. J., Slots, J. & Genco, R. J. (1983). Serology of oral *Actinobacillus actinomycetemcomitans* and serotype distribution in human periodontal disease. *Infect Immun* **41**, 19–27.

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