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.

INTRODUCTION

Actinobacillus actinomycetemcomitans, which has been reclassified as Aggregatibacter actinomycetemcomitans (Nørskov-Lauritsen & Kilian, 2006), is a non-motile, Gram-negative, capsulophilic 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; Patel 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.,
A. immune response. According to distinct O-PSs, membrane, is the major antigen that stimulates the host component of LPS, which extends from the outer (Caroff & Karibian, 2003). The O polysaccharide (O-PS) negative bacteria, and covers 75% of the cell surface 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). 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 CO2. 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

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

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Strain</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>ATCC 29523</td>
<td>ATCC and IDH† (M. Saarela) VT1169; Mintz (2004)</td>
</tr>
<tr>
<td>b</td>
<td>VT1169 (SUNY465)</td>
<td>IDH; PM73: Dr C. I. Hoover, University of California, San Francisco; BL0293: Credit Valley Hospital, Mississauga, ON, Canada</td>
</tr>
<tr>
<td>c</td>
<td>ATCC33384</td>
<td>IDH</td>
</tr>
<tr>
<td>d</td>
<td>IDH269*</td>
<td>IDH</td>
</tr>
<tr>
<td>e</td>
<td>IDH1147*</td>
<td>IDH</td>
</tr>
<tr>
<td>f</td>
<td>IDH47a*</td>
<td>IDH, CU1000N; Kaplan et al. (2001)</td>
</tr>
</tbody>
</table>

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

†Institute of Dentistry, Helsinki, Finland.

‡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 Smal and SalI sites: emaA1892Smal (5'-CCCCCGGGGACG- GTGTAGAAC-3') and emaA4144SalI (5'-ATGTCGACTATCGTGCACCACCCA-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.

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![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.](http://mic.sgmjournals.org)
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% hydridoma 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 StalkSacII (5'TCTGACAGTAGTGATAGAAAGGG-3') and Stalk HindIII (5'-AAAGCTTTATCTGCACCACCAAA-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 x 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-EmA mAb, and detected with horseradish peroxidase-conjugated antibody (Amersham) 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). Following polymerization at 37°C overnight, which resulted in the phase cells (5 x 10⁷) were added to each well and incubated at 37°C for 2.5 h, 200 µl TS BYE was added to each well and incubated at 37°C overnight. Mid-exponential-phase cells (5 x 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 TSB YE, 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: MrBayes 3.1 for the Bayesian method (Huelsenbeck & Ronquist, 2001), and PROTPARS/DNAPARS and PROTDIST/DNADIST-NEIGHBOR from the Phylipgeny 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. Yada 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 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

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**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 Carlo simulations.

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**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.
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...
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* (cytotoxic 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 Gene & 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 with the JP2-type of A. actinomycetemcomitans (Cotter et al., 2003), where 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


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