Identification of an extracellular matrix protein adhesin, EmaA, which mediates the adhesion of *Actinobacillus actinomycetemcomitans* to collagen

Keith P. Mintz

Department of Microbiology and Molecular Genetics, Rm 110 Stafford Hall, University of Vermont, Burlington, VT 05405, USA

*Actinobacillus actinomycetemcomitans* is an aetiological agent in the development of periodontal and some systemic diseases in humans. This pathogen localizes to the underlying connective tissue of the oral cavity in individuals with periodontal disease. The adhesion of *A. actinomycetemcomitans* to extracellular matrix components of the connective tissue prompted this study to identify gene products mediating the interaction of *A. actinomycetemcomitans* to these molecules. A transposon mutagenesis system was optimized for use in *A. actinomycetemcomitans* and used to generate an insertional mutant library. A total of 2300 individual insertion transposon mutants were screened for changes in the adhesion to collagen and fibronectin. Mutants were identified which exhibited the following phenotypes: a decrease in collagen binding; a decrease in fibronectin binding; a decrease in binding to both proteins; and an increase in binding to both collagen and fibronectin. The identification of mutants defective in adhesion to the individual proteins indicates that distinct adhesins are expressed by this organism. Molecular analysis of these mutants implicated 11 independent loci in protein adhesion. One gene, emaA, is likely to encode a direct mediator of collagen adhesion, based on predicted protein features homologous to the collagen-binding protein YadA of *Yersinia enterocolitica*. EmaA was localized to the outer membrane, as expected for an adhesin. Reduction in fibronectin adhesion appeared to be influenced by abrogation of proteins involved in molybdenum-cofactor biosynthesis. Several other loci identified as reducing or increasing adhesion to both collagen and fibronectin are suggested to be involved in regulatory cascades that promote or repress expression of collagen and fibronectin adhesins. Collectively, the results support the hypothesis that *A. actinomycetemcomitans* host colonization involves afimbrial adhesins for extracellular matrix proteins, and that the expression of adhesion is modulated by global regulatory mechanisms.

**INTRODUCTION**

Periodontal diseases are a group of chronic inflammatory diseases of the gingiva and the supporting structures of the periodontium. These diseases are associated with the colonization of the subgingival crevice with specific anaerobic Gram-negative bacteria (Socransky & Haffajee, 1992). Activation of host immune-surveillance cells by these bacteria, or bacterial products, results in cell activation and the subsequent release of effector molecules that cause tissue damage. In concert with bacterial factors, the net result is the degradation of connective tissue and the destruction of the supporting bone, which leads to tooth loss (Baker, 2000).

**Abbreviation:** ECM, extracellular matrix.

The GenBank/EMBL/DDBJ accession number for the complete *A. actinomycetemcomitans* emaA sequence reported in this paper is AY344064.
adhesion to specific host macromolecules under stringent or hostile conditions (Finlay, 1990). These molecules include the proteins, secreted by host cells, that form the extracellular matrix (ECM). The ECM is a biologically active tissue composed of a complex mixture of macromolecules, including multiple collagen types, fibronectin, laminin and glycosaminoglycans. The ECM not only serves a structural function but also affects a number of cellular activities, including migration, proliferation and differentiation. ECM proteins that have been described to act as a substrate for bacterial adhesion include collagens, laminin, fibronectin, fibrinogen, vitronectin and heparan sulfate (Patti et al., 1994). A large number of intracellular and extracellular human pathogens adhere to mammalian ECM proteins and have been shown to contribute to the virulence of the micro-organism (Patti et al., 1994; Westerlund & Korhonen, 1993).

A. actinomycetemcomitans invades and migrates through epithelial cells (Fives-Taylor et al., 1999), and is eventually found in contact with collagen fibres of connective tissue (Carranza et al., 1983; Gillett & Johnson, 1982). Previously, we have demonstrated the adhesion of A. actinomycetemcomitans to fibrillar collagens, with the greatest affinity for type V collagen and fibronectin. In the present study, we describe the generation and screening of a transposon mutant library to isolate variants of A. actinomycetemcomitans with altered binding to collagen and fibronectin. Mutants deficient for adhesion to each ECM protein were identified, showing that independent adhesins mediate the binding of this bacterium to collagen and fibronectin. A mediator required for collagen adhesion, EmaA (extracellular matrix protein adhesin), was identified and described. Based on the protein sequence, EmaA is predicted to be structurally related to the collagen-binding protein YadA of Yersinia enterocolitica (Roggenkamp et al., 2003), and is likely to directly mediate adhesion to collagen.

METHODS

Bacterial strains and plasmids. The A. actinomycetemcomitans strains developed in this study are derived from the clinical strain SUNY 465. A spontaneous rifampicin- and nalidixic acid-resistant strain, VT 1169, of A. actinomycetemcomitans (Mintz & Fives-Taylor, 2000) was utilized as the recipient strain in all conjugation experiments. All A. actinomycetemcomitans strains were grown statically in trypticase soy broth supplemented with 0-6 % yeast extract (TSBYE) in a humidified 10 % CO₂ atmosphere at 37 °C. All Escherichia coli strains were grown in Luria–Bertani (LB) broth at 37 °C with aeration. The Tn1O-based transposon vector pLOF/Km was a gift from Dr V. de Lorenzo, Centor de Investigaciones Biológicas, Madrid, Spain and Dr K. Timmis, GFB-National Research Centre for Biotechnology, Braunschweig, Germany. The bacterial strains and plasmids used in this study are listed in Table 1.

Transposon mutagenesis in A. actinomycetemcomitans. The pLOF/Km transposon mutagenesis system (Tascon et al., 1993) was optimized for A. actinomycetemcomitans by replacing the aminoglycoside phosphotransferase gene (kan) within the transposon with spectinomycin adenyltransferase, ada9 (accession no. M69221), from Enterococcus faecalis (LeBlanc et al., 1991). The pLOF/Km vector was incubated with NotI to excise kan, gel purified, and used for ligation with ada9 purified as follows. The ada9 gene was isolated from pDL269 (LeBlanc et al., 1991) by incubation with NdeI and HindIII, followed by treatment with Klenow, and cloned into the EcoRV site of Bluescript SK⁺ (Stratagene). ada9 was amplified from pBS::ada9 with primers based on the Bluescript vector sequence, which included an internal NotI restriction site (5′-CACCAGGGTCGGCGGCGTCTTAG-3′) and a sequence which contained a NotI restriction site engineered on the 5′ end of the sequence (5′-GATAAAGATT-GCGCCGCGTTGACGGTGATCGAAGC-3′). The PCR product was restricted with NotI, gel purified and ligated with the pLOF digest. DH5α λ pir transformants were selected on LB agar plates containing 50 μg spectinomycin ml⁻¹. The pLOF/Sp (VT1542) construct was verified by restriction using 250 μl DNEYE and incubated plasmid containing the transposon was transformed into electrocompetent S17-1 λ pir cells for conjugation with A. actinomycetemcomitans strain VT1169. Conjugation was performed as previously described (Mintz & Fives-Taylor, 2000) for 6 h on TSBYE plates containing 0-1 mM IPTG to induce transposition, and plated on selective media containing nalidixic acid and rifampicin to counterselect the E. coli donor strain, and spectinomycin to counter-select the recipient strain. Individual colonies were inoculated into wells of 96-well sterile tissue-culture plastic microtitre plates containing TSBYE/Spc (50 μg spectinomycin ml⁻¹) and grown as described above. DMSO was added to a final concentration of 10 % to the wells, which were stored at −80 °C. The frequency of transposition was calculated by the number of spectinomycin-resistant cells divided by the total number of cells used in the conjugation mixture.

Detection of A. actinomycetemcomitans mutants altered in binding to ECM proteins. The transposon library was replica plated on microtitre plates containing TBSYE. The plates were kept at 37 °C for 3 days. The bacteria were dispersed by pipetting and the bacterial growth determined by measurement of OD₄₉₅. Bacterial suspension (100 μl) was transferred to microtitre plates previously coated with either human type V collagen (Sigma) or fibronectin (ICN Pharmaceuticals) and blocked with BSA (1 %) in Tris-buffered saline (TBS: 20 mM Tris, 150 mM NaCl, pH 7-4). Bacterial adhesion was detected by ELISA, as described previously (Mintz & Fives-Taylor, 1999). Following incubation of the bacteria for 2 h at 37 °C, the wells were washed with TBS, and purified polyclonal antiserum, raised against the whole bacterium, in TBS containing 1 % BSA, were added to detect bound bacteria. The antibodies were incubated for 1 h at room temperature and the wells washed with TBS containing 0-1 % Tween 20 (TBST). The immune complexes were located by the addition of horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulins (Jackson Laboratories) in TBS containing 1 % BSA. Following incubation for 1 h, the wells were washed with TBST, and the immune complexes were detected by the addition of hydrogen peroxide and o-phenylenediamine in 80 mM citrate phosphate buffer, pH 5-0. The reaction was allowed to proceed and stopped by the addition of 4 M sulfuric acid. The absorbance values were recorded using an EL211s microplate reader (Bio-Tek). The ratio of the individual absorption reading divided by the mean absorption reading of all the wells was used to assess bacterial binding in the initial screens.

The strains from the initial screen that displayed differences in binding to human collagen and/or fibronectin were isolated, purified and screened again as follows. Mutant and parent strains were streaked for isolation and a single colony was inoculated into broth cultures and incubated overnight. The cultures were diluted 1 : 10 and allowed to grow for an additional 3 h under normal culture conditions. The bacterial cell density was calculated from an established standard curve of the parent strain, correlating OD₄₉₅ with colony forming units.
Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Source</th>
<th>Genotype/relative genotype</th>
<th>Description/relative phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
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<td></td>
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<td>JM109</td>
<td>Mintz et al. (2002)</td>
<td>recA1supE44 endA1 hsdR17 gyrA96 relA1 thi-1 mcrA Δ(lac-proAB)F’[traD36 proAB + lacIq lacZAM15]</td>
<td>Host for routine genetic constructions and cloned PCR products</td>
</tr>
<tr>
<td>S17-1ZIP</td>
<td>This study</td>
<td>Tp’ Sm’ZIP</td>
<td>Permissive host for transposon delivery</td>
</tr>
<tr>
<td>DH5ZIP</td>
<td>This study</td>
<td>endA1 hsdR17(r- m+) recA::RP4-2Tc::Mu Km’::Tn7 ZIP</td>
<td>Host for allelic exchange constructions</td>
</tr>
<tr>
<td>SM10ZIP</td>
<td>This study</td>
<td>thl thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km’ ZIP R6K</td>
<td>Permissive host for allelic exchange</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>Promega</td>
<td>F’, ompT, hsdS(r- b-, m- y) gal, dCM (DE3)</td>
<td>Host for protein expression</td>
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<tr>
<td><strong>A. actinomycetemcomitans</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VT1169</td>
<td>Mintz et al. (2002)</td>
<td>recA1supE44 endA1 hsdR17 gyrA96 relA1 thi-1 mcrA Δ(lac-proAB)F’[traD36 proAB + lacIq lacZAM15]</td>
<td>Host for routine genetic constructions and cloned PCR products</td>
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<tr>
<td>7C12</td>
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<td>This study</td>
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<td>col- fn-</td>
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<td>col+ fn+</td>
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<td>Tn10Sp::?</td>
<td>col+ fn+</td>
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<td>Promega</td>
<td>Ap’</td>
<td>Cloning PCR products</td>
</tr>
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<td>Ap’, Km’</td>
<td>Tn10-based delivery plasmid with Km’</td>
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<tr>
<td>pVT1460</td>
<td>Mintz et al. (2002)</td>
<td>Km’</td>
<td>Plasmid for allelic exchange</td>
</tr>
</tbody>
</table>

Equal numbers of bacteria from each strain were added to the wells in a final volume of 100 μl TSBYE. Assays were performed in triplicate, as described above, and repeated a minimum of three times.

Southern analysis. Strains were grown in TSBYE containing 50 μg spectinomycin ml⁻¹, and DNA was isolated using the Puregene DNA extraction Kit (Gentra Systems). Chromosomal DNA was restricted with EcoRI and the fragments separated on a 0.7% agarose gel in TAE buffer. The DNA fragments were transferred to Hybond nylon membranes (Amersham Life Sciences) and the membranes were treated following the method of Sambrook et al. (1989). The membranes were hybridized with DNA probes conjugated with horseradish peroxidase, using the conditions suggested by the manufacturer (Amersham Life Sciences). Hybridizing fragments were visualized using the ECL detection system (Amersham Life Sciences) and exposure to photographic film (XAR-5, Eastman Kodak).

Determination of transposon integration sites within the chromosome of A. actinomycetemcomitans. The integration site of the transposon within the A. actinomycetemcomitans chromosome was determined by DNA sequencing of inverse PCR products. Total chromosomal DNA was extracted as described above and restricted with AluI or BamHI. The enzymes were inactivated by heating, and the DNA fragments ligated by the addition of T4 DNA ligase. A portion of this material was ligated as the template for PCR. Oligonucleotide primers were synthesized (Sigma-Genosys) based on the published aadA nucleotide sequence (complementary strand, bases 48–67, 5’-CTCTTGGCACTACAGTTACG-3’) and a sequence adjacent to the unique AluI restriction site (bases 563–578, 5’-GGAAATCTATGTATCAAGAAGCAG-3’) or the BamHI restriction sequence within the spc gene (bases 420–441, 5’-AGTCGTCGATCCGTTACG-3’). The PCR products were analysed by agarose gel electrophoresis and the products were either gel purified (Qiagen) and subcloned into the T/A cloning vector pGEM T-Easy (Promega), or purified by QIAquik (Qiagen) and sequenced. DNA sequencing was performed at the University of Vermont Cancer Center DNA Analysis Facility. The generated sequences were used to search the A. actinomycetemcomitans strain HK1651 DNA database at the University of Oklahoma for homologous sequences.

Allelic replacement mutagenesis of emaA of A. actinomycetemcomitans. Directed gene mutagenesis was achieved by conjugative transfer of a non-replicating broad-host-range plasmid modified for efficient use in A. actinomycetemcomitans (Mintz et al., 2002). An internal fragment of emaA was generated by PCR using the primers 5’-CCCTTGTACCACTACAGATAACC-3’ (corresponding to bases 80–104) and 5’-CAGTCATCGATCATCATTACGACC-3’.
Taq (Invitrogen). respectively) were used as the primers, and conditions were optimized following the method of Talian et al. (1997). Treatment of the bacteria with 3 ml 10 mM HEPEs, pH 7-4, containing 1 mM phenylmethylsulfonyl fluoride. Bacterial membranes were isolated by disruption of the bacteria using a French pressure mini-cell at 18 000 p.s.i. (124 200 kPa), following the protocol of Nikaido et al. (1997). Intact bacteria and debris were collected by centrifugation at 7650 g for 10 min. The supernatant was carefully removed and centrifuged at 100 000 g for 30 min. The pellet was resuspended in 10 mM HEPEs, pH 7-4, and recentrifuged. The membranes were resuspended in 10 mM HEPEs, pH 7-4, and sodium N-lauroylsarcosine was added to a final concentration of 1% and incubated at room temperature for 30 min. Based on bacterial membrane protein solubility in sarcosinate (Filip et al., 1973; Nikaido, 1997), outer-membrane proteins are insoluble in the detergent. The mixture was centrifuged at 15 600 g for 30 min and the pellet was resuspended in 10 mM HEPEs, pH 7-4, and centrifuged as above. The final pellet was resuspended in 10 mM HEPEs, pH 7-4. Protein concentration was determined by absorbance at 280 nm. The insoluble proteins were solubilized in 1% SDS before determination of the protein concentration.

**Polyacrylamide gel electrophoresis and immunoblot analysis of proteins.** Equal concentrations of proteins were boiled for 10 min in Laemmli sample buffer, before application to 5–15% polyacrylamide-SDS gels. The separated proteins were transferred to nitrocellulose and probed with the affinity-purified antibodies. The immune complexes were detected using horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (Jackson Laboratories) and visualized using the ECL detection system (Amersham Life Sciences) and exposure to photographic film (XAR-5, Eastman Kodak).

**RESULTS**

**Development and characterization of a transposon mutagenesis system for A. actinomycetemcomitans**

During the initial phase of this work, a functional transposon mutagenesis system in this strain of A. actinomycetemcomitans (VT1169) was not readily available. Subsequently, a mutagenesis system was published for A. actinomycetemcomitans (Thomson et al., 1999). Therefore, we employed a transposon system that had been successfully used in the swine pathogen A. pleuroneumoniae (Tascon et al., 1993) to generate transposon mutants in A. actinomycetemcomitans. This system involved the use of a suicide conjugative plasmid, pLOF/Km, carrying a mini-Tn10 with an IPTG-inducible transposase located outside the mobile element (Herrero et al., 1990).

Initial conjugation studies with this plasmid and the recipient A. actinomycetemcomitans strain VT1169 resulted in the region of the membrane corresponding to the protein excised and treated as described previously, and blocked with 5% non-fat dried milk dissolved in TBS. The antiserum was applied to the nitrocellulose and incubated for 2 h at room temperature. The antibodies were dissociated from the protein by incubation with 0-2 M glycine, pH 2-7, and neutralized by the addition of 5 M NaOH.

**Isolation of outer-membrane proteins.** Cells from a 200 ml overnight culture of A. actinomycetemcomitans were collected by centrifugation (5860 g, 15 min) and washed with PBS (10 mM phosphate, 150 mM NaCl, pH 7-4). The resulting cell pellet was resuspended in 3 ml 10 mM HEPEs, pH 7-4, containing 1 mM phenylmethylsulfonyl fluoride. Bacterial membranes were isolated by disruption of the bacteria using a French pressure mini-cell at 18 000 p.s.i. (124 200 kPa), following the protocol of Nikaido et al. (1997). Intact bacteria and debris were collected by centrifugation at 7650 g for 10 min. The supernatant was carefully removed and centrifuged at 100 000 g for 30 min. The pellet was resuspended in 10 mM HEPEs, pH 7-4, and recentrifuged. The membranes were resuspended in 10 mM HEPEs, pH 7-4, and sodium N-lauroylsarcosine was added to a final concentration of 1% and incubated at room temperature for 30 min. Based on bacterial membrane protein solubility in sarcosinate (Filip et al., 1973; Nikaido, 1997), outer-membrane proteins are insoluble in the detergent. The mixture was centrifuged at 15 600 g for 30 min and the pellet was resuspended in 10 mM HEPEs, pH 7-4, and centrifuged as above. The final pellet was resuspended in 10 mM HEPEs, pH 7-4. Protein concentration was determined by absorbance at 280 nm. The insoluble proteins were solubilized in 1% SDS before determination of the protein concentration.

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**RESULTS**
in the generation of thousands of transconjugants. However, the frequency of transposition events decreased with repeated studies. The reason for this phenomenon remains elusive. To generate consistent numbers of transconjugants in the A. actinomycetemcomitans strain used in this study, it was necessary to exchange the antibiotic cassette encoding kanamycin resistance for a cassette encoding spectinomycin resistance. The kanamycin gene in the mini-Tn10 was replaced with the spectinomycin gene (aad9) from Enterococcus faecalis, which has been used extensively in this strain of A. actinomycetemcomitans. This modified plasmid, pLOF/Sp (pVT1542), gave consistent high levels of transposon mutants not obtained with pLOF/Km. Characterization of this transposon system in A. actinomycetemcomitans is described below.

Transposon insertion was efficient and appeared random. Of the transconjugants, 98% were sensitive to ampicillin (plasmid marker), which indicated that the plasmid was not present in the majority of the transconjugants. Southern analysis of 15 randomly selected transconjugants demonstrated that the transposon integrated randomly into the chromosome of these transconjugants (Fig. 1). In most instances, a single copy of the transposon was present in the chromosome. However, infrequent integration of the plasmid was observed (Fig. 1, lane 12, the faster-migrating hybridizing band corresponded to DNA containing vector sequence following Southern analysis with the vector as the probe). The stability of the transposon within the chromosome of the transconjugants was determined by growing 10 transconjugants in non-selective media for 8 days, followed by replica plating on TSBYE agar plates with or without spectinomycin and Southern blot analysis of the same strains at the start and end of the incubation period. The hybridization pattern of the strains grown in the absence of antibiotic was identical to the same strains grown in the presence of spectinomycin. Collectively, the spc-modified transposon integrates as a stable, single copy genetic element, randomly in the genome. The apparent transposition frequency was calculated to be 2.1 × 10⁻⁴. This is similar to the frequency of transposition determined for another transposon mutagenesis system developed for A. actinomycetemcomitans (Thomson et al., 1999).

Identification of A. actinomycetemcomitans strains altered in binding to collagen and fibronectin

The transposon mutants derived from the library were characterized by ELISA for adhesion to the ECM proteins collagen and fibronectin (Mintz & Fives-Taylor, 1999). Sixteen mutants were chosen for further analysis. The chromosomal DNA was extracted, and the integration site of the transposon was determined by inverse PCR followed by sequencing of the PCR product. Search for homologous DNA sequences in the A. actinomycetemcomitans genome database resulted in high sequence homology with the prototypic strain for 15 of the 16 transposon mutants. The DNA sequence derived from the remaining mutant (1D2) was absent from the genomic database sequence. The ORFs of the affected genes were determined, and 11 individual ORFs were identified. The translated protein sequence of the mutants was compared for homologous sequences in the available protein sequence databases (Table 2). The level of adhesion to collagen (col) and fibronectin (fn) of the mutants was designated as normal (+), decreased (−) or increased (++), as compared with the wild-type control. The mutants were classified into four categories: 1) col⁻ fn⁺, 2) col⁺ fn⁻, 3) col⁻ fn⁻ and 4) col⁺ fn⁻ (Fig. 2).

The existence of mutants that displayed an exclusive fibronectin-adhesion defect (col⁺ fn⁻) was attributed to the insertion of the transposon in two ORFs, moaA and moeA, associated with the biosynthesis of a molybdenum cofactor (MoCo). MoCo is a crucial component of various enzymes catalysing important redox reactions (Kisker et al., 1997). The A. actinomycetemcomitans MoaA (molybdenum cofactor biosynthesis protein A) deduced sequence shares 78–79% sequence identity with the Haemophilus influenzae and Pasteurella multocida proteins. MoA is involved in the first step of MoCo biosynthesis: formation of the sulfur-free pterin precursor Z from a guanosine derivative.
(Rajagopalan, 1996). The MoeA protein of *A. actinomycetemcomitans* also shares a high degree of identity to the proteins of *H. influenzae* and *P. multocida*. Although this protein is reported to be involved in MoCo biosynthesis, the exact function of this protein is unknown (Rajagopalan, 1996).

ECM-binding mutants reduced for both collagen and fibronectin (*col*−*fn*−) were associated with insertion of the transposon in genes transcribing global regulatory proteins most closely related to *P. multocida* and *Haemophilus somnus* cAMP receptor protein (Crp), also known as catabolite activator protein (CAP), and Mlc. These

### Table 2. Protein homology of ECM-protein-adhesion transposon mutants

Percentage binding is relative to the parental strain.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Binding (%)</th>
<th>Protein</th>
<th>Identity (%)*</th>
<th>Accession no.</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Collagen</td>
<td>Fibronectin</td>
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<td></td>
</tr>
<tr>
<td><em>col</em>−<em>fn</em>+</td>
<td>7C12</td>
<td>54 ± 6</td>
<td>91 ± 10</td>
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<tr>
<td></td>
<td><em>fn</em>+</td>
<td>Surface protein</td>
<td>94 (1–1247)</td>
<td>AF316503</td>
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<tr>
<td></td>
<td></td>
<td>Surface protein</td>
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<td>AF316503</td>
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<tr>
<td><em>col</em>+<em>fn</em>−</td>
<td>19D10</td>
<td>102 ± 17</td>
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<td>20G1</td>
<td>82 ± 5</td>
<td>50 ± 4</td>
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<tr>
<td><em>col</em>−<em>fn</em>−</td>
<td>1D2†</td>
<td>51 ± 15</td>
<td>38 ± 6</td>
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<td></td>
<td>21D6</td>
<td>42 ± 14</td>
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<tr>
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<td>21D6</td>
<td>42 ± 14</td>
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†Mutant that does not share any DNA sequence homology with the prototypic *A. actinomycetemcomitans* strain.

*Parentheses show the portion of the derived *A. actinomycetemcomitans* amino-acid sequence used to generate percentage identity with the target protein.
proteins have been thoroughly studied in the regulation of carbohydrate metabolism (Bruckner & Titgemeyer, 2002). However, they may be pleiotropic regulators for other biosynthetic pathways.

Several mutants were identified where insertion resulted in a gain of function: hyper-binding to both substrates (col\(^{+}\) fn\(^{+}\)). These proteins include: 1) the heat-modifiable protein (OMP34) of \textit{A. actinomycetemcomitans} (Wilson, 1991), 2) a protein homologous to the putative membrane protein (YbJ) of \textit{Shigella flexneri}, 3) a protein homologous to a putative N-6-adenine-specific DNA methylase of \textit{Salmonella typhimurium}, and 4) adenylate cyclase (CyaA) of multiple bacterial taxa, which generates cAMP, a common global regulatory molecule in biological systems (Botsford & Harman, 1992).

The mutants that displayed a decrease in collagen binding, without an appreciable effect on fibronectin binding (col\(^{+}\) fn\(^{-}\)), were found to have the transposon inserted in the same ORF. Two distinct integration sites were identified, with one 184 bp downstream to the other, within the amino terminus of the predicted protein. The gene associated with the phenotype of these mutants was designated \textit{emaA} (extracellular matrix protein adhesin A).

The \textit{emaA} sequence from VT1169 was determined to be 99\% identical to the sequence found in the genome of strain HK1651. The gene encodes a putative 201 kDa protein. The protein sequence (Fig. 3) shares homology with the well-characterized collagen-binding protein, YadA, of \textit{Yersinia enterocolitica}. The amino terminus of the \textit{emaA} protein contains sequences that are identical to those required for the binding of YadA to collagen, or contains conserved amino-acid substitutions within these sequences (Tahir \textit{et al.}, 2000). The carboxy terminus of \textit{emaA} contains a conserved domain homologous with the cell-membrane anchor domain of YadA (Tamm \textit{et al.}, 1993). Other conserved features or structural elements, similar to those of the Oca family of adhesins (which includes YadA), are contained in this sequence (Roggenkamp \textit{et al.}, 2003).

The \textit{emaA} ORF identified in SUNY 465 encodes a single protein. In contrast, the sequence from \textit{A. actinomycetemcomitans} strain UT322 (Li \textit{et al.}, 2004) describes two translation products, ApiB and C, from the \textit{emaA} ORF. These two sequences are designated surface proteins which may be involved with the invasion of this pathogen into epithelial cells. One protein (ApiB) is identical to amino acids 1–1247 of \textit{emaA}, but contains an additional 22 amino acids absent from the \textit{emaA} sequence. The second UT322 sequence (ApiC) is identical to amino acids 1245–1932, but \textit{emaA} contains an additional 33 amino acids at the carboxyl terminus.
Characterization of a collagen-binding protein of *A. actinomycetemcomitans*

The loss-of-function phenotype related to *emaA::Tn10dSpc* was confirmed by generating an *emaA*-directed insertion mutant. An allelic replacement mutant was generated and tested for binding to collagen and fibronectin. The *emaA::spc* and *emaA::Tn10dSpc* mutants both displayed a similar decrease in binding to collagen, as compared with the parent strain (51 ± 6 and 46 ± 4 %, respectively), while maintaining wild-type levels of binding to fibronectin (91 ± 10 and 105 ± 15 %, respectively). The binding of the *emaA::spc* mutant to other fibrillar collagen types (I, II and III) was also reduced when compared with the parent strain (data not shown). These data indicate that inactivation of *emaA* is not attributable to pleiotropic effects of the integration of the transposon, and that disruption of this gene results in a general defect for collagen binding.

Putative polar effects of the inactivation of *emaA* on the transcription of upstream and downstream genes were investigated by RT-PCR. Primer sets selected to generate ~400 bp internal fragments were used in these reactions with RNA extracted from *emaA::spc* and the parent strain. Products were generated for the putative long-chain fatty acid CoA ligase (AAC21681), upstream of the start codon of *emaA*, and a putative anaerobic ribonucleoside triphosphate reductase, NrdD (AAK03024), downstream of *emaA*, from RNA extracted from both the *emaA* mutant and the parent strain (Fig. 4). In addition, analysis of the *emaA* sequence indicates the presence of a stem–loop structure (ΔG = −13 kcal mol⁻¹) immediately following the transcriptional stop codon, consistent with a Rho-independent transcriptional termination signal. These data suggest that the disruption of *emaA* does not interfere with the gene expression of the surrounding genes, and that the reduction in the binding to collagen is exclusively associated with the inactivation of *emaA*.

The localization of EmaA and verification of the predicted molecular mass was determined by immunoblotting. Polyclonal antibodies were generated against a 21 kDa EmaA internal protein fragment expressed in *E. coli* as a GST fusion protein and cleaved with PreScission protease (Amersham Biosciences) to remove the GST. The product was further purified by SDS-PAGE, and the band corresponding to the EmaA fragment was excised and used as the immunogen in rabbits. Sera were affinity purified and used to detect EmaA in immunoblots.

Membrane fractions of both the wild-type and mutant *emaA* strain were generated and the outer-membrane proteins recovered by differential solubilization in sodium lauroyl-sarcosine. Equivalent amounts of outer-membrane proteins were separated by SDS-PAGE and either stained for protein (Fig. 5, panel A) or transferred to nitrocellulose and probed with affinity-purified antibodies (Fig. 5, panel B). Protein staining (Coomassie Brilliant Blue) revealed very little difference between the profiles of the membrane preparations, which contained few proteins with a molecular mass greater than 90 kDa. However, the immunoblots clearly showed a difference between the two strains. An immunoreactive species greater than 200 kDa was found in the outer-membrane fraction of the parent strain, but was completely absent in the *emaA* mutant. Some cross-reactivity of the anti-EmaA antibodies with other membrane proteins was present. The band between the 29 kDa and 36 kDa markers was the heat-modifiable protein Omp34, which has been demonstrated to bind the Fc portion of immunoglobulins (Mintz & Fives-Taylor, 1994). Collectively, these data suggest that *emaA* encodes a ~200 kDa outer-membrane protein, which mediates the adhesion of *A. actinomycetemcomitans* to collagen.

**DISCUSSION**

In Gram-negative bacteria, specific ECM proteins may be recognized by single or multipurpose adhesins, which in turn may interact with several ECM components. The plasmid-encoded outer-membrane protein YadA mediates the specific binding of enteropathogenic yersiniae to various types of collagen, as well as binding to laminin and the cellular form of fibronectin (Schulze-Koops et al., 1992, 1993; Tamm et al., 1993; Tertti et al., 1992). This is in
contrast to the Dr fimbriae of *E. coli* and the type 3 fimbriae of *Klebsiella pneumoniae*, which bind specifically to one type of collagen (Tarkkanen et al., 1990; Westerlund et al., 1989). The CadF protein of *Campylobacter jejuni* is specific for the binding of this enteric pathogen to fibronectin (Konkel et al., 1997). In addition, bacteria may contain multiple surface proteins that interact with several ECM proteins. The oral spirochaete *Treponema denticola* expresses several proteins that appear to bind to both fibronectin and laminin (Fenno et al., 1996; Umemoto et al., 1993). In this study, we have isolated mutants that display a wild-type level of adhesion to one, but reduced adhesion to another ECM protein (col- fn+, col+ fn-). In our previous study (Mintz & Fives-Taylor, 1999), we demonstrated differences in the binding isotherms to collagen and fibronectin of trypsin-treated bacteria. In addition, differences found in the binding phenotype to these proteins among strains of *A. actinomycetemcomitans* suggest the presence of separate ECM protein adhesins. The biochemical and genetic data strongly suggest that *A. actinomycetemcomitans* expresses separate adhesins to mediate the interaction with individual ECM proteins. However, the presence of residual binding activity in these mutants suggests the existence of additional adhesins for collagen and fibronectin. Therefore, multiple adhesins may exist for each specific ECM protein, but one cannot exclude the possibility that other putative adhesins have broader substrate specificity than the adhesins identified in this study.

Excluding emaA, the insertion of the transposon in the other mutants can have polar effects on the transcription of surrounding genes. Transcriptional analysis must be performed to define the effects of these insertional mutations. With this caveat stated, the ECM protein binding mutants identified by this genetic screen are indicative of structural proteins and proteins which may act directly or indirectly to regulate adhesin gene expression and/or synthesis. The transposon insertions associated with a defect exclusively in fibronectin adhesion were found in distinct, non-contiguous operons associated with molybdenum cofactor (MoCo) biosynthesis. MoCo is an important cofactor of a number of biosynthetic pathways in which the cofactor itself may be required for adhesin synthesis. Alternatively, the metal moiety of the cofactor (Mo2+) may act as a signalling molecule, similar to other metal ions that modulate virulence-associated factors (Groisman, 2001; Hantke, 2001; Hentze & Maguire, 2003; Straley et al., 1993). In addition, MoeA, which is structurally related to the carboxyl-terminal domain of gephyrin (Sola et al., 2001), may serve a structural role in fibronectin adhesion. Gephyrin regulates receptor clustering by forming a subsynaptic protein scaffolding, which anchors the receptors to the cytoskeleton (Kneussel & Betz, 2000). This implies that MoeA may act to stabilize or anchor the fibronectin adhesin or associated molecules.

Loci that reduce or enhance both fibronectin and collagen adhesion (mlc, crp, cyaA) are associated with global regulatory molecules that impact the expression of the actual adhesin. Both Mlc and CRP are usually associated with carbohydrate metabolism, but can activate or repress a large number of promoters outside carbohydrate regulation (Bruckner & Titgemeyer, 2002). The adenylate cyclase and the receptor protein signalling pathway are essential for the global regulation of transcription of the type III secretion systems of both *Pseudomonas aeruginosa* and *Y. enterocolitica* (Petersen & Young, 2002; Wolfgang et al., 2003). The inactivation of this signalling pathway in *Y. enterocolitica* results in a reduction in the virulence of this pathogen (Petersen & Young, 2002).

Several other loci that impacted ECM protein adhesion cannot be readily classified by probable function, but
speculation is still warranted. Increased collagen and fibronectin adhesion was associated with loci encoding outer-membrane proteins. It is speculated that absence of the proteins influences the membrane adhesins or allows more adhesins to be integrated into the outer membrane. These mutants may prove to be of value in identifying additional ECM protein mediators if a greater number of adhesins are expressed.

The collagen adhesin identified in this study is likely a member of a novel class of non-fimbrial oligomeric coiled-coil adhesins (Oca) (Roggenkamp et al., 2003). The members of this family, in addition to YadA, include UsP1 and UsP2 of Moraxella catarrhalis, and the auto-transporter Hia of H. influenzae (Hoiczyk et al., 2000). These proteins share conserved features or structural elements. These include: (i) an amino-terminal Sec-dependent secretion signal, (ii) a head domain consisting of degenerate 14-residue repeats, (iii) a highly conserved neck region(s), (iv) a stalk domain of variable length with a high probability of coiled-coil formation, and (v) a carboxyl-terminal membrane-anchor domain with conserved structural features. In addition, members of this protein class do not contain cysteine residues in the mature polypeptide. The known functions of these adhesins are binding to eukaryotic cells and ECM proteins. The binding domains mediating adherence are usually located in non-conserved regions of the amino terminus of the protein (Roggenkamp et al., 2003).

The EmA protein consists of 1965 amino acids, and there are no cysteine residues in the mature protein. A single cysteine is present in the putative signal sequence. The amino terminus of this protein contains the approximately 14-residue degenerate repeats (alternating pattern of branched aliphatic and small residues, followed by a position consisting mainly of Ala, Gly, Ser or Thr), as found in the head region of YadA (Hoiczyk et al., 2000). Surrounding and included within this region are multiple sequences identical to the collagen-binding consensus sequence NSVAIG-S, or displaying conserved amino-acid substitutions (Tahir et al., 2000). Juxtaposed to the putative collagen-binding domain is the sequence TDAVNAQL, which is identical to the neck sequence of YadA (Hoiczyk et al., 2000). A putative stalk region is present, which contains one region with a high probability of a coiled-coil domain, as determined by COILS version 2.1 (Window = 21 score of 0-62). The carboxyl terminus is separated from the stalk region by another neck sequence followed by a coiled-coil segment (Window = 21 score of 0-75) and a membrane-anchor domain formed by four transmembrane β-strands, as suggested by the A. actinomycetemcomitans sequence alignment with the YadA sequence listed in Hoiczyk et al. (2000).

The emaA sequence predicts a protein with a molecular mass of 201 kDa, and a protein greater than 200 kDa was observed in immunoblots of membrane proteins. Based on the protein staining, EmA is not a significant protein component of the bacterial membrane. Although this may be indicative of the relative abundance of the adhesin within the membrane of the bacterium, it does not reflect the protein’s biological significance. The position of the protein in the immunoblot makes estimation of the molecular mass unreliable. Therefore, EmA may exist as heat-stable aggregates in SDS-PAGE, which is a characteristic of Oca proteins (Roggenkamp et al., 2003). Alternatively, post-translational modification of the protein may alter the protein’s electrophoretic mobility, which implies that the ORF encodes a single protein. Further biophysical and structural studies are warranted to resolve these issues.

Surface ultrastructural analysis of the A. actinomycetemcomitans strain used in this study demonstrates the presence of small vesicles and fibrillar membranous extensions with knob-like ends on the surface of the bacterium (Meyer & Fives-Taylor, 1994). The YadA and UsP adhesins form ‘lollipop’-shaped structures on the outer membrane of the bacterium, as detected by electron microscopy (Hoiczyk et al., 2000). Based on the structural homology of EmA with these proteins, one would predict the presence of similar structures on the surface of A. actinomycetemcomitans. Investigations into the surface structures of the wild-type and emaA mutant strains are being conducted.

Bacterial adhesins that specifically recognize ECM proteins have been characterized for a number of pathogens (Patti et al., 1994). The results of this study are consistent with the existence of distinct mediators for fibronectin and collagen adhesion. However, it is possible that an adhesin that mediates the binding to both ECM proteins is also present. A collagen-specific adhesin, EmA, has been identified which contains protein sequence information and structural characteristics that are analogous to a family of established ECM-binding proteins. In addition, ubiquitous signalling molecules have also been identified that regulate the adhesion of this pathogen to these substrates. Further studies will be required to determine if these pathways are implicated in the regulation of other virulence determinants expressed by this pathogen.

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