Glycosylation of the Collagen Adhesin EmaA of *Aggregatibacter actinomycetemcomitans* Is Dependent upon the Lipopolysaccharide Biosynthetic Pathway

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The human oropharyngeal pathogen *Aggregatibacter actinomycetemcomitans* synthesizes multiple adhesins, including the nonfimbrial extracellular matrix protein adhesin A (EmaA). EmaA monomers trimerize to form antennae-like structures on the surface of the bacterium, which are required for collagen binding. Two forms of the protein have been identified, which are suggested to be linked with the type of O-polysaccharide (O-PS) of the lipopolysaccharide (LPS) synthesized (G. Tang et al., Microbiology 153:2447–2457, 2007). This association was investigated by generating individual mutants for a rhamnose sugar biosynthetic enzyme (*rmlC*; TDP-4-keto-6-deoxy-d-glucose 3,5-epimerase), the ATP binding cassette (ABC) sugar transport protein (*wzt*), and the O-antigen ligase (*waaL*). All three mutants produced reduced amounts of O-PS, and the EmaA monomers in these mutants displayed a change in their electrophoretic mobility and aggregation state, as observed in sodium dodecyl sulfate (SDS)-polyacrylamide gels. The modification of EmaA with O-PS sugars was suggested by lectin blots, using the fucose-specific *Lens culinaris* agglutinin (LCA). Fucose is one of the glycan components of serotype b O-PS. The *rmlC* mutant strain expressing the modified EmaA protein demonstrated reduced collagen adhesion using an *in vitro* rabbit heart valve model, suggesting a role for the glycoconjugant in collagen binding. These data provide experimental evidence for the glycosylation of an oligomeric, coiled-coil adhesin and for the dependence of the posttranslational modification of EmaA on the LPS biosynthetic machinery in *A. actinomycetemcomitans*.

The human oropharyngeal pathogen *Aggregatibacter actinomycetemcomitans* preferentially colonizes the subgingival region of the human oral cavity. This microorganism is implicated as the etiological agent of localized aggressive periodontitis (9, 13) and causes extraoral infections, including pneumonia, osteitis (30), and infective endocarditis (6). Recent studies also link this periodontal pathogen to cardiovascular diseases, such as atherosclerosis (20).

Typical of Gram-negative bacteria, the outer membrane of *A. actinomycetemcomitans* possesses an asymmetric lipid-protein bilayer. The inner leaflet of the outer membrane is mainly phospholipids, and the outer leaflet consists of lipopolysaccharide (LPS), phospholipids, and proteins (4). LPS molecules are ubiquitously distributed on the outer membrane and are essential for maintaining the membrane integrity (3). Intact LPS molecules are also required for the assembly of some large outer membrane proteins (3, 18, 41). A typical LPS molecule is composed of hydrophobic lipid A, a nonrepeat core oligosaccharide, and a repeating O-antigen or O-polysaccharide (O-PS). The distal O-PS is a major antigen, stimulating the host immune response, and the basis for serotyping Gram-negative bacteria (36), including *A. actinomycetemcomitans* (32, 50).

Six different serotypes (a to f) and the corresponding genetic loci have been identified for *A. actinomycetemcomitans* (19, 22, 27, 44, 50, 54, 55). Serotype b remains one of the common serotypes found in the human oral cavity (9, 13, 51). The serotype b O-PS of *A. actinomycetemcomitans* is encoded by an operon composed of 21 genes, which are responsible for the biosynthesis of the repeating trisaccharide unit of this particular serotype (53, 55). Each O-PS unit of serotype b contains a disaccharide backbone composed of L-rhamnose (L-Rha), linked by a non-reducing D-N-acetylglactosamine (D-GalNAc) at the O-3 position of L-Rha (33) (Fig. 1A).

The assembly of LPS molecules in Gram-negative bacteria involve diverse enzymes and pathways due to the variation of the O-PS structures among different bacteria (36). *RmlC* (previously RfbD), *Wzt* (previously AbcA or RfbB), and *WaaL* are three enzymes involved in different stages of the LPS synthesis of some Gram-negative bacteria (7, 36, 37). A homologue of *RmlC*, TDP-4-keto-6-deoxy-d-glucose 3,5-epimerase, which is required for L-Rha synthesis, has been identified in *A. actinomycetemcomitans* (53, 55). *Wzt* is an ATP binding cassette (ABC) transporter that exports saccharide polymers from the cytoplasm to the periplasmic space (7, 36). A homologue of *wzt* was originally identified from a serotype b strain of *A. actinomycetemcomitans*, based on protein sequence identity with *Aeromonas salmonicida* (55). Kaplan et al. (19) later showed that a serotype f *wzt* mutant strain of *A. actinomycetemcomitans* produces less O-PS. *WaaL*, an O-antigen ligase found in *Escherichia coli* and *Pseudomonas aeruginosa*, ligates an undecapre-

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nol pyrophosphate-linked oligo- or polysaccharide onto the lipid A-core oligosaccharide in the periplasm (1, 36). A putative O-antigen ligase is located in the chromosome of a serotype b A. actinomycetemcomitans strain (24) (Table 1). Our earlier work suggested a correlation between the type of LPS molecule and the form of EmaA synthesized by A. actinomycetemcomitans strain (HK1651), based on sequence homology (Oralgan, Los Alamos, NM).

Our earlier work suggested a correlation between the type of LPS molecule and the form of EmaA synthesized by A. actinomycetemcomitans (46). The EmaA of serotype b A. actinomycetemcomitans is a 202-kDa protein that forms the antennae-like appendages found on the surface of A. actinomycetemcomitans and is required for collagen binding (40). The appendages are composed of three EmaA monomers that oligomerize to form an ellipsoidal structure required for the collagen binding activity (56, 57). The ellipsoidal structure corresponds to the amino termini of the proteins and is located at the distal end of a long stalk domain that is attached to the outer membrane by the carboxyl termini (57). The carboxyl termini of the proteins assume β-barrel structures required for pore formation and translocation of the molecules through the outer membrane, similar to those of other type Vc autotransporter proteins (14). Recently, we have demonstrated that EmaA is important in the initiation of infective endocarditis in a rabbit model of infectious endocarditis (45).

Two transposon mutant strains (rmlC and wzt) and a waaL mutant strain generated by site-directed insertional mutagenesis have been developed and characterized in this study. The rmlC mutant did not synthesize L-Rha and did not produce detectable O-PS. The wzt and waaL mutant strains synthesized less O-PS than the wild-type strain. Complementation of the mutant strains restored the production of the serotype b O-PS to wild-type levels. An increase in the electrophoretic mobility of the EmaA monomer was observed in all three mutants, which suggests the presence of carbohydrate. The EmaA mobility reverted to wild-type mobility upon complementation. The presence of carbohydrate associated with EmaA was confirmed by lectin blotting, and in vitro collagen binding assessment demonstrated that the glycoconjugant is important for the full function of this adhesin. The experimental data suggest that EmaA contains carbohydrate similar to that present in O-PS and is a substrate for the O-antigen ligase of the LPS biosynthetic pathway of A. actinomycetemcomitans.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** All A. actinomycetemcomitans mutant strains used in this study are based on the nonfimbriated strain VT1169, a spontaneous rifampin- and nalidixic acid-resistant mutant derived from the clinical strain SUNY465, and is referred to as the wild-type strain in this study (24) (Table 1). A. actinomycetemcomitans strains were grown statically in 3% Trypticase soy broth-0.6% yeast extract (TSBYE; Becton, Dickinson and Company) in a 37°C incubator with 10% humidified carbon dioxide. All mutants in this study retained growth characteristics similar to those of the wild-type strain. Escherichia coli strains were grown in 1% Bacto tryptone, 0.1% yeast extract, and 0.5% sodium chloride (Luria-Bertani [LB]) medium at 37°C under aerobic conditions with agitation.

The rmlC and wzt mutants described in this study were isolated from a transposon mutant library, and the integration sites of the transposon within the A. actinomycetemcomitans chromosome were determined as described previously.

**TABLE 1. Strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype/remarks</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. actinomycetemcomitans</td>
<td>Wild type, a Rifr Nalr derivative of SUNY465, serotype b</td>
<td>24</td>
</tr>
<tr>
<td>VT1169</td>
<td>VT1169 transformed with plasmid pKM2/emaA (EmaA)</td>
<td>This study</td>
</tr>
<tr>
<td>emaA mutant (emaA::Sp)</td>
<td>Spectinomycin adenyltransferase gene (aad9) inserted into emaA</td>
<td>24</td>
</tr>
<tr>
<td>rmlC mutant (rmlC::pLOF/Sp)</td>
<td>Transposon pLOF/Sp inserted into rmlC (TDP-4-keto-6-deoxy-D-glucose 3,5-epimerase)</td>
<td>This study</td>
</tr>
<tr>
<td>rmlC mutant rmlC</td>
<td>rmlC mutant complemented with plasmid pKM2/itaP/rmlC</td>
<td>This study</td>
</tr>
<tr>
<td>wzt mutant (wzt::pLOF/Sp)</td>
<td>Transposon pLOF/Sp inserted into wzt (ATP binding cassette transporter)</td>
<td>This study</td>
</tr>
<tr>
<td>waaL mutant (waaL::pKM221)</td>
<td>Plasmid pKM221 inserted into waaL (O-antigen ligase)</td>
<td>This study</td>
</tr>
<tr>
<td>waaL mutant waaL</td>
<td>waaL mutant complemented with plasmid pKM2/itaP/waaL</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli</td>
<td>F′ mcrA Δ(mrr-hsdRMS-mcrBC) Δ(φ80lacZΔM15 ΔlacX74 recA1 endA1 araD193 Δ[ampC, A. actinomycetemcomitans] Δ(ara, leu)7697 galU galK λ rpsL supG tonA) endA1 hadR17(“r” m1) supE44 thi-1 recA4 gyrA(Nalr) relA1 Δ[lacIZYA-argF]U1679 droS (φ80lacZΔlacZM15) λ pir</td>
<td>Invitrogen, Carlsbad, CA</td>
</tr>
<tr>
<td>DH5α (λ pir)</td>
<td>ΔmexA Δ(mrr-hsdRMS-mcrBC) Δ(φ80lacZΔM15 ΔlacX74 recA1 endA1 araD193 Δ[ampC, A. actinomycetemcomitans] Δ(ara, leu)7697 galU galK λ rpsL supG tonA) endA1 hadR17(“r” m1) supE44 thi-1 recA4 gyrA(Nalr) relA1 Δ[lacIZYA-argF]U1679 droS (φ80lacZΔlacZM15) λ pir</td>
<td>25</td>
</tr>
<tr>
<td>SM10 (λ pir)</td>
<td>ΔmexA Δ(mrr-hsdRMS-mcrBC) Δ(φ80lacZΔM15 ΔlacX74 recA1 endA1 araD193 Δ[ampC, A. actinomycetemcomitans] Δ(ara, leu)7697 galU galK λ rpsL supG tonA) endA1 hadR17(“r” m1) supE44 thi-1 recA4 gyrA(Nalr) relA1 Δ[lacIZYA-argF]U1679 droS (φ80lacZΔlacZM15) λ pir</td>
<td>25</td>
</tr>
</tbody>
</table>

* Rif, rifampin; Nal, nalidixic acid; Sp, spectinomycin.*
TABLE 2. Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
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<td>pVT1461</td>
<td>A derivative of GPG704, containing the spectinomycin adenylytransferase gene (aad9), Sp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>25</td>
</tr>
<tr>
<td>pKM221</td>
<td>pVT1461 containing 5,545 bp of waaL, Sp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pLOF/Sp</td>
<td>Tn&lt;sub&gt;10&lt;/sub&gt;-based transposon vector; Ap&lt;sup&gt;+&lt;/sup&gt; Sp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>24</td>
</tr>
<tr>
<td>pKM2</td>
<td>pPK1 containing chloramphenicol acetyltransferase, Cm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>10</td>
</tr>
<tr>
<td>pKM2/emaA</td>
<td>pKM2 containing ~500-bp upstream sequence of emaA (the putative promoter region)</td>
<td>This study</td>
</tr>
<tr>
<td>pKM2/ltxP</td>
<td>pKM2 containing ~500-bp ltx promoter, Cm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pKM2/ltxP/mlc</td>
<td>pKM2 containing ~500-bp ltx promoter + mlc sequence, Cm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pKM2/ltx/Pwaal</td>
<td>pKM2 containing ~500-bp ltx promoter + waal sequence, Cm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
</tbody>
</table>

<sup>*</sup> Ap, ampicillin; Cm, chloramphenicol; Sp, spectinomycin.

(24) The gene with the inserted transposon was identified based on the A. actinomycetemcomitans genomic database (strain HK1651; Oralgen, Los Alamos, NM) (http://www.oralgen.lanl.gov/). The sequence was performed at the University of Vermont Cancer Center DNA Analysis Facility.

The shuttle plasmid pKM2 was used for genetic complementation of the O-PS mutants (10) (Table 2). The 520-bp leukotoxin (ltx) promoter of A. actinomycetemcomitans (5) was used as the promoter for the expression of the mlc and waal genes in the complemented strains. The EmaA-overproducing strain was developed by transformation of the wild-type strain VT1169 with a plasmid containing the emaA sequence and 500 bp upstream of the start codon (pKM2/emaA).

Complementation of mlc. The complete mlc (pBl) sequence (GenBank accession no. DQ119107) was amplified using the following primers, 5′-CTCG AGATGAAAGTTATTG-3′ and 5′-GAATGTTAAATTTACGCGGTT-3′, which were engineered with 5′-XhoI and 5′-EcoRI restriction endonuclease sites, respectively (itales indicate restriction sites). The 550-bp fragment amplified from the chromosomal DNA of VT1169 was sequenced and found to be identical to that of the mlc<sub>G</sub> gene of HK1651 (http://www.oralgen.lanl.gov/). The PCR product was cloned into a pCR2.1-TOPO cloning vector (Invitrogen), transformed into TOP10 One Shot E. coli competent cells, and selected on LB plates containing 100 μg/ml ampicillin and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) and isopropyl-beta-D-thiogalactopyranoside (IPTG). The 5′-XhoI/mlc-EcoRI-3′ fragment was isolated and ligated with the complementary sites of pKM2/ltx, which was restricted with the same enzymes, followed by dephosphorylation with shrimp alkaline phosphatase (USB Corporation, Cleveland, OH). The ligation mixture was transformed into DH10B cells, and colonies were selected on LB agar containing 20 μg/ml chloramphenicol. The pKM2/ltx/mlc plasmid was isolated and transformed into the A. actinomycetemcomitans mlc<sub>G</sub> mutant by electroporation (42). Colonies were selected on TSBYE agar containing 1 μg/ml of chloramphenicol and 50 μg/ml of spectinomycin.

Development of a waal<sub>G</sub> mutant strain by insertion mutagenesis. A DNA fragment corresponding to base pairs 5 to 545 of the waal<sub>G</sub> gene was amplified from the wild-type strain VT1169 using the following primers: 5′-CTCGAGATGAAAGTTATTG-3′ and 5′-GAATGTTAAATTTACGCGGTT-3′, which were engineered with 5′-XhoI and 5′-EcoRI restriction endonuclease sites, respectively (itales indicate restriction sites). The 550-bp fragment amplified from the chromosomal DNA of VT1169 was sequenced and found to be identical to that of the mlc<sub>G</sub> gene of HK1651 (http://www.oralgen.lanl.gov/). The PCR product was cloned into a pCR2.1-TOPO cloning vector (Invitrogen), transformed into TOP10 One Shot E. coli competent cells, and selected on LB plates containing 100 μg/ml ampicillin and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) and isopropyl-beta-D-thiogalactopyranoside (IPTG). The 5′-XhoI/mlc-EcoRI-3′ fragment was isolated and ligated with the complementary sites of pKM2/ltx, which was restricted with the same enzymes, followed by dephosphorylation with shrimp alkaline phosphatase (USB Corporation, Cleveland, OH). The ligation mixture was transformed into DH10B cells, and colonies were selected on LB agar containing 20 μg/ml chloramphenicol. The pKM2/ltx/mlc plasmid was isolated and transformed into the A. actinomycetemcomitans mlc<sub>G</sub> mutant by electroporation (42). Colonies were selected on TSBYE agar containing 1 μg/ml of chloramphenicol and 50 μg/ml of spectinomycin.

Isolation of LPS. LPS was isolated using different procedures for different strains as described in the text. The LPS samples were stored at −20°C until further analysis.

Gas chromatography/mass spectrometry (GC/MS) of LPS. The glycosyl composition of the LPS extracted from the wild-type A. actinomycetemcomitans strain and the isogenic mutants was analyzed using a combination of GC/MS with the p-O-trimethylsilylated (TMS) glycans or with the p-O-trimethylsilylated (TMS) glycans, using acidic methanolysis at the Complex Carbohydrate Research Center, the University of Georgia. A total of 240 μg of each LPS sample was used for the analysis. Methyl glycosides were prepared by methanolysis of the samples in 1 M HCl in methanol at 80°C for 30 min, followed by re-N-acetylation with pyridine and acetic anhydride in methanol for detection of amino sugars. The samples were then derivatized using 2% (v/v) acetic anhydride in methanol for detection of amino sugars. The samples were then analyzed using a Hewlett-Packard 5890A gas chromatograph equipped with a HP-5 capillary column (30 m × 0.25 mm, 0.25 μm film).

Immunoreactivity of O-PS, determined by ELISA. The hot phenol-water-extracted LPS preparations were dissolved in deionized water and incubated in the wells of a 96-well microtiter plate overnight at 4°C. The wells were rinsed with water and blocked for 30 min in PBS with 0.05% Tween 20, 1 mM EDTA, and 0.25% bovine serum albumin (BSA) (19). The wells were incubated with purified rabbit anti-A. actinomycetemcomitans immunoglobulins (24) for 1 h at room temperature. The nonbinding immunoglobulins were removed, and the wells were washed with buffer and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Jackson Laboratory, Bar Harbor, ME) in PBS for 1 h. Immunoglobulin complexes were detected using 100 μl of tritiated-phosphate buffer (2.4 mM tritium acid monohydrate, 51.4 mM dibasic sodium phosphate, pH 5.0) containing 0.04% o-phenylenediamine and 0.012% hydrogen peroxide. The reaction was stopped by addition of 50 μl of 4 M H<sub>2</sub>SO<sub>4</sub>, and absorbance was measured at 490 nm.
Immunoblot analysis of LPS. A total of 0.5 µg of the isolated LPS sample from each strain was dissolved in loading buffer containing 10 mM HEPES, 2% SDS, 5% (vol/vol) 14.3 M β-mercaptoethanol, 10% (vol/vol) glycerol, and 0.05% (wt/vol) bromophenol blue, boiled for 5 min, and loaded into a 4 to 15% polyacrylamide Tris-HCl Ready Gel (Bio-Rad, Hercules, CA). The electrophoresis was performed at 60 V at 4°C for 120 min. Separated carbohydrate molecules were transferred to Optitran nitrocellulose membranes (Whatman Incorporated, Piscataway, NJ) at 70 V at 4°C for 90 min and probed with the purified rabbit anti-A. actinomycetemcomitans immunoglobulins mentioned above. Immune complexes were detected using HRP-conjugated goat anti-rabbit IgG and visualized using the chemiluminescent substrate (Pierce Biotechnology, Rockford, IL). Membranes were exposed to Kodak X-OMAT LS films (Carestream Health, Rochester, NY).

Isolation of total membrane proteins. The membrane protein fraction of A. actinomycetemcomitans was prepared as described previously (24, 46). Briefly, 200 ml late logarithmic phase cells were harvested and resuspended in 2.5 ml of 10 mM HEPES (pH 7.4) with 1 mM phenylmethylsulfonyl fluoride (PMSF; USB Corporation, Cleveland, OH) and 1% complete protease inhibitor cocktail (Roche Diagnostic Corporation, Atlanta, GA). Cells were lysed by three cycles of 9,000 hertz (22, 100 kPa) at 4°C using a French pressure minicell. Whole-cell lysates were centrifuged at 7,650 g to remove cell debris, followed by centrifugation at 100,000 × g for 40 min to pellet the membrane fraction. The protein concentration was estimated using absorbance at 280 nm (43).

Immunoblot analysis of EmaA. Equivalent amounts of membrane protein, determined by absorbance at 280 nm, were prepared in the loading buffer as described by the immunoblotting of LPS and loaded into a 4 to 15% polyacrylamide Tris-HCl gel. Electrophoresis was performed at 40 V and 4°C for 15 h. The separated proteins were transferred to an Optitran nitrocellulose membrane at 70 V and 4°C for 105 min and probed with an anti-EmaA monocular antibody (46). The immune complex was detected using HRP-conjugated goat anti-mouse IgG (Jackson Laboratory, Bar Harbor, ME) and visualized as described by the immunoblotting of LPS.

Lectin blot analysis. Equivalent amounts of membrane protein were prepared as described above and transferred to nitrocellulose membrane filters as described for the immunoblotting. The filter was blocked in PBS (pH 7.4) with 0.5% Tween 20 (PBS-T), and probed with biotinylated fucose-specific Lens culinaris agglutinin (LCA; Vector Laboratories, Burlingame, CA) for 1 h. The membrane was washed in PBS-T for 5 min with 6 changes and then incubated with HRP-conjugated avidin D (Vector Laboratories, Burlingame, CA) for 1 h. After being washed six times, the lectin-avidin complex was visualized as described by the immunoblotting of LPS.

Liquid chromatography/mass spectrometry (LC/MS) analysis. Equivalent amounts of membrane proteins from the parent and emaA mutant strains were dissolved in electrophoresis loading buffer as described above, boiled for 5 min, and loaded onto a 5 to 15% gradient polyacrylamide-SDS gels. Electrophoresis was performed at 40 V and 4°C for 15 h. The separated proteins were transferred to an Optitran nitrocellulose membrane at 70 V and 4°C for 10 min and probed with an anti-EmaA monoclonal antibody (46). The immune complex was detected using HRP-conjugated goat anti-mouse IgG (Jackson Laboratory, Bar Harbor, ME) and visualized using the chemiluminescent substrate (Pierce Biotechnology, Rockford, IL).

RESULTS

Characterization of A. actinomycetemcomitans serotype b LPS. Visualization of LPS isolated from the wild-type strain using the proteinase K method (15), by silver staining the polyacrylamide-SDS gels, revealed the presence of a dark brown stain located in the high-molecular-weight region of the gel (75,000 to 250,000) (Fig. 1B), which represents polymerized O-PS of serotype b strains (Fig. 1A). A similar LPS profile was demonstrated for the emaA mutant strain. These two profiles differed from the LPS profiles of both the rmlC and wz mutant strains, due to the absence or reduction of O-PS (Fig. 1B). However, the LPS isolated from the rmlC and wz mutant strains contained core oligosaccharides profiles similar to those of the wild-type or emaA mutant strains (Fig. 1B).

Hot phenol-water-extracted LPS (48) was used both in ELISA and in immunoblot assays, in which polyclonal antisera raised against the whole bacteria was used to determine the relative amount of O-PS associated with the LPS. Antibody binding in wells absorbed with as little as 10 ng of LPS isolated from the wild-type strain was observed. The signal increased with increasing amounts of LPS. In contrast, a weak signal in wells adsorbed with 1,000-fold increase (10 µg) of LPS isolated from the rmlC mutant was detected. A binding pattern similar to that of the wild-type strain was observed, using an LPS sample isolated from the mutant strain complemented with rmlC, driven by the ltx promoter in trans (Fig. 2A).

The potential that other LPS biosynthetic enzymes are involved in EmaA modification was determined by characterizing a waaL mutant strain. The putative A. actinomycetemcomitans waaL gene is predicted to encode the lipid A-core O-antigen ligase. The waaL gene of A. actinomycetemcomitans was identified based on the homology of the translated protein sequence with Haemophilus influenzae (42% amino acid identity) (GenBank accession no. ZP 0020155). A single open reading frame was identified in the A. actinomycetemcomitans genome (gene ID AA01434) (http://www.oralgen.lanl.gov), which is homologous to known O-antigen ligases. The waaL gene is located outside of the 21-gene serotype b O-PS operon, which includes rmlC and wz.

Insertional inactivation of the waaL gene resulted in a strain that synthesized a reduced level of LPS. The reduction corresponded to a decrease in the binding of anti-A. actinomycetemcomitans antibodies, determined by ELISA (Fig. 2B), compared with that of the wild-type strain. However, the amount of immunoreactivity was greater than that found with the LPS preparation of the rmlC mutant strain (Fig. 2A). The amount of antibody binding to the LPS preparation of the waaL complemented strain was similar to the wild-type strain. Collectively, the reduction in the level of O-PS isolated from this mutant and the high protein homology with other O-antigen ligases suggest a similar role of the WaaL protein in A. actinomycetemcomitans LPS biosynthesis.

The difference in antibody binding between the O-PS mutants and the wild-type LPS preparations was also visualized by immunoblotting. A larger amount of immunoreactive material was present in the LPS samples isolated from the wild-type and the complemented strains than in those isolated from the mutant strains. The presence of a small amount of O-PS staining in the rmlC mutant may be attributed to some non-O-PS poly-
mers that also reacted with the antibodies or to another rmlC-like gene that partially complemented the mutation. The data clearly demonstrate a gradient in the amount of sugars associated with the LPS isolated from the waaL, wzt, and waaL mutant strains (Fig. 3).

The quantification of the carbohydrates associated with the LPS isolated from different mutants and the wild-type strain was determined using GC/MS. Carbohydrate analysis revealed that the LPS sample from the three mutants contained less carbohydrate on a mass basis than the wild-type LPS (Table 3). This difference can be attributable to the absence or reduction in the saccharides associated with the serotype b O-PS of A. actinomycetemcomitans, L-Rha, D-Fuc, and GalNAc. Rha and GalNAc were not detected, and Fuc was greatly diminished in the rmlC mutant compared with that in the wild-type strain. Different from the rmlC mutant, Rha and GalNAc were present in the wzt mutant but at reduced levels compared to those in the wild-type LPS sample. The level of Fuc in the wzt mutant was comparable to the level found in the rmlC mutant. Similar to the other two mutants, the waaL mutant LPS showed a reduction in the amounts of both Rha and Fuc in comparison with those in the wild-type strain. In contrast to the LPS isolated from the rmlC and wzt mutants, an increase in the amount of GlcNAc was observed in the waaL mutant LPS.

Characterization of EmaA in the wild-type and O-PS mutant strains. The EmaA monomer of serotype b strains is identified as a 202-kDa molecule, based on the predicted

<table>
<thead>
<tr>
<th>Glycosyl residue</th>
<th>Mass (µg) of LPS in a</th>
<th>Wild type (3-LOF/Sp)</th>
<th>wzt (Plof/Sp)</th>
<th>waaL::pKM221</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetradecanoic acid</td>
<td>9.1</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ribose (Rib)</td>
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<tr>
<td>Rhamnose (Rha)</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Mannose (Man)</td>
<td>2.6</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Galactose (Gal)</td>
<td>2.6</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glucose (Glc)</td>
<td>3.1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N-acetyl galactosamine (GalNAc)</td>
<td>3.0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N-acetyl glucosamine (Gal)</td>
<td>22.6</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Heptose (Hep)</td>
<td>11.7</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3-Deoxy-2-manno-2-octulosonic acid</td>
<td>12.2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sum</td>
<td>96</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Total loading amt</td>
<td>400</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

% Carbohydrate* 41.5 19.0 13.6 23.9

* The total percentage of carbohydrate of each LPS sample (400 µg per sample).

a ND, not detectable; +, detected; +, the most predominant glycosyl residues.
In wild-type phenotype after the complementation of the mutants EmaA in the O-PS mutant strains can be reverted to the strains. The aggregation state and molecular weight change of material found associated with the stacking gel in the mutant correlated with a decrease in the amount of immunoreactive wild-type strain. The increase in the amount of the monomer the three O-PS mutant strains was greater than that in the wild-type EmaA monomer. In addition to the change in the mobility, which corresponded to a lower molecular mass than that of the wild-type EmaA monomer. The LC/MS results indicated that EmaA was the most abundant protein present in the EmaA-overproducing strain, except for the absence of EmaA. However, some variation in the concentration of the individual proteins was observed. The electrophoretic mobility change of the EmaA monomer in the O-PS mutants, the lectin blot, and the LC/MS data support the hypothesis that EmaA contains a sugar associated with serotype b O-PS.

Assessment of collagen binding activity of the O-PS mutant using an in vitro tissue model. Equivalent amounts of bacteria were incubated with trypsin-treated rabbit mitral valves to assess the role of the modification of EmaA in collagen binding activity (Fig. 6). The competitive index (CI) between the rmlC mutant and the wild type was 0.33 (paired t test; \( P = 0.008 \)), which was equivalent to that of the emaA mutant strain (CI = 0.27). A CI value of 1 indicates no difference in competitiveness between the mutant and wild-type strains. These data suggest that the rmlC mutant strain, as well as the emaA mutant strain, colonized the heart valve approximately 3-fold less effectively than the wild-type strain. These data suggest that the modification of the adhesin is important for the interaction with collagen.

**DISCUSSION**

The *A. actinomycetemcomitans* serotype b O-PS is a trisaccharide-repeating unit composed of \( \alpha \)-Fuc, \( \alpha \)-Rha, and \( \alpha \)-GalNAc residues (2, 29, 33, 50). Rha and Fuc were the main sugars
identified in the analysis of the LPS isolated from the serotype b strain used in this study. The isolated LPS gave a typical silver stain profile for serotype b A. actinomycetemcomitans following electrophoresis (29, 50). The O-PS of serotype b appeared as a broad smear in the high-molecular-weight region of the polyacrylamide-SDS gel, and the sugars and fatty acids that are typical of the LPS core oligosaccharides and lipid A were observed in the lower-molecular-weight region of the gel (29, 50). The smear is most likely due to different numbers of repeating saccharide units present in the O-PS. O-PS is the immunodominant material when probed with anti-A. actinomycetemcomitans antibodies, which is similar to the observation obtained with serum samples from patients with periodontal disease (29, 50).

Rha and Fuc, the predominant sugars of the serotype b O-PS, are synthesized following a shared biosynthetic pathway involving D-glucose-1-phosphate and dTTP (53).

FIG. 5. Fucose-specific Lens culinaris agglutinin (LCA) blots of membrane proteins from EmaA-producing and emaA mutant strains. Equivalent amounts of membrane proteins from the EmaA-overproducing strain VT1169 (pKM2/emaA) and the emaA mutant (emaA) were prepared, loaded into the 4 to 15% polyacrylamide Tris-HCl gel, and transferred to nitrocellulose membranes. (A) The same protein-transferred membrane was probed with anti-EmaA monoclonal antibody (panel 1); biotinylated LCA, with different exposure times of the film (panels 2 and 3); and avidin alone (nonlectin control) (panel 4). Antibody binding was detected using goat anti-mouse antibodies, and lectin binding was detected using avidin. The solid arrow at ~200 kDa corresponds to the EmaA monomer. The dashed arrow corresponds to EmaA aggregates associated with the stacking gel. (B) Colloidal blue stain of membrane proteins. Equivalent amounts of membrane proteins from the EmaA-overproducing strain VT1169 (pKM2/emaA) and the emaA mutant (emaA) were separated in a 5 to 15% polyacrylamide-SDS gel with a 3% stacking gel. Following electrophoresis, the gel was stained with colloidal blue. The region of the gel corresponding to the EmaA aggregates, shown with the square bracket ([), and a similar region of the gel in the emaA mutant ([) were excised and analyzed using LC/MS (Table 4).

FIG. 6. Assessment of collagen binding activities using trypsin-treated rabbit heart valves. Rabbit heart valves were surgically removed and treated with trypsin to remove the endothelia. Equal CFU numbers of the wild-type (WT) and the rmlC (rhamnose epimerase mutant) bacteria were added to the treated valves and incubated. The competitive index (CI) was calculated as the ratio of the numbers of mutant to wild-type CFU in the cardiac valve samples divided by the ratio of the numbers of mutant to wild-type CFU in the inoculum (CI, 0.33; Paired t test; P = 0.008), which was similar to that of the emaA mutant (CI, 0.27).

TABLE 4. Protein composition of the EmaA-overproducing strain and the emaA mutant

<table>
<thead>
<tr>
<th>Protein</th>
<th>VT1169 (pKM2/emaA) protein abundance</th>
<th>emaA mutant abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular matrix adhesin A (EmaA)</td>
<td>Not detected</td>
<td></td>
</tr>
<tr>
<td>NAD(P) transhydrogenase subunit alpha (PntA)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Protein-export membrane protein (SecD)</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Leukotoxin A (LtxA)</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Fumarate reductase flavoprotein subunit A</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Conserved hypothetical protein</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Signal peptide peptidase (SppA)</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Acriflavine resistance protein (AcrB)</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Glycerol-3-phosphatase transporter (GlpT)</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Conserved hypothetical protein</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

The proteins are listed based on their relative amount, as detected by LC/MS. Only the 10 most abundant proteins in the EmaA-producing strain are listed.

The numbers represent the relative abundance of the corresponding proteins of the EmaA-overproducing strain found in the emaA mutant strain, as determined by LC/MS (Fig. 5B).
cose, the intermediate is converted to dTDP-l-rhamnose via an epimerase (RmlC) and a reductase (RmlD) or to dTDP-d-fucose using a reductase (Fcd) (53). The integral nature of RmlC in Rha synthesis was demonstrated by the absence of Rha in the LPS isolated from the rmlC mutant, as analyzed by GC/MS. d-GalNAc was also not detected in the LPS isolated from this mutant. Both fcd and the genes associated with the synthesis of d-GalNAc are downstream of rmlC (53). Restoration of O-PS following complementation of the mutant with the full-length rmlC gene in trans indicates that disruption of the gene does not have a polar effect on the transcription of the downstream genes in the operon. There was, however, a decrease in the molar percentage of Fuc associated with the LPS isolated from the rmlC mutant. The presence of fucose may represent undecaprenyl phosphate (Und-PP)-linked fucose that copurifies with the modified LPS. The absence of Rha and GalNAc, the loss of high-molecular-weight staining material in the silver-stained SDS-PAGE gels, and the apparent decreased immunoreactivity in the ELISA and LPS immunoblots with the rmlC mutant LPS are consistent with those of a strain of A. actinomycetemcomitans lacking O-PS.

Wzt and WaaL are two enzymes that function in the latter stages of the LPS biosynthetic pathway. Wzt is an ATP binding cassette (ABC) transporter that exports saccharide polymers from the cytoplasm to the periplasmic space (36). The carbohydrate analysis and the antibody reactivity studies collectively indicate a substantial decrease in the amount of O-PS sugars associated with the LPS in the wzt mutant. The presence of O-PS sugar residues associated with the mutant LPS suggests that the mutant bacterium may be able to express either an active truncated protein or another enzyme, albeit with a lower affinity, for transport of the oligosaccharides across the cytoplasmic membrane. Mutation of wzt in a serotype f strain of A. actinomycetemcomitans has also been shown to cause defects in O-PS synthesis (19).

The O-antigen ligase (WaaL) of Enterobacteriaceae has been shown to be responsible for attachment of polysaccharides to the lipid A core (49). The enzyme ligates an undecaprenyl phosphate (Und-PP)-linked fucose that copurifies with the modified LPS. The absence of Rha and GalNAc, the loss of high-molecular-weight staining material in the silver-stained SDS-PAGE gels, and the apparent decreased immunoreactivity in the ELISA and LPS immunoblots with the rmlC mutant LPS are consistent with those of a strain of A. actinomycetemcomitans lacking O-PS.

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Glycosylation of Gram-negative bacterial adhesins has been identified. These include fimbriae (pili), found in E. coli (28, 47), Neisseria meningitidis (34), and Pseudomonas aeruginosa (35), as well as the nonfimbrial adhesin HMW1 found in Haemophilus influenzae. N. meningitidis pilin glycosylation proceeds through a homologue of O-antigen ligase (PgL), which is not associated with LPS assembly in this bacterium (34). The pilin glycosylation of P. aeruginosa requires an oligosaccharyltransferase (PilO), which is also independent from the LPS biosynthetic pathway, although the conjugated oligosaccharide is identical to the O-antigen-repeating unit of this organism (16, 35). Different from the above-mentioned O-linked pilin glycosylation pathways, the N-linked glycosylation of the nonfimbrial adhesin HMW1 found in H. influenzae uses an independent glycosylation machinery, requiring HMW1C and phosphoglucomutase (11, 12). The glycosylation of HMW1 occurs in the cytoplasm (11) instead of in the periplasmic space associated with the fimbrial adhesins (34, 35).

Secreted glycosylated proteins have been found in the periodontal pathogen Porphyromonas gingivalis (31). The extracellular cysteine proteinases Arg-gingipains (RgpsA and RgpsB) contain sugar moieties similar to those of the anionic polysaccharides (APS) of the cell surface associated with this organism (31). APS is identified as an essential surface structure different from either the LPS or the capsule polysaccharide (31). Recently, the O-antigen ligase (WaaL) of P. gingivalis has been shown to ligate the O antigen to the lipid A core and assemble the APS sugar repeat units (38). However, the role of WaaL in the modification of the gingipains is unknown.

In this study, we present both genetic and biochemical evidence that A. actinomycetemcomitans strain VT1169 synthesizes a trimeric autotransporter adhesin that contains carbohydrate. These data also suggest that the enzymes involved in the modification of EmaA overlap with the enzymes of the LPS biosynthetic machinery. The genetic studies imply that saccharide assembly for EmaA is mediated by the O-antigen ligase (WaaL), which is also required for ligation of the O-polysaccharide to the lipid A core oligosaccharide of LPS (Fig. 7). It is possible that the loss of the intact LPS molecules in the waaL mutant has destabilizing effects on another protein responsible for EmaA glycosylation. At this time, we cannot exclude this possibility. However, the data presented here support the least complicated hypothesis that EmaA modification is dependent on the LPS biosynthetic pathway. This dependency makes EmaA unique among the Gram-negative glycosylated proteins, which are independent of LPS biosynthetic enzymes.

EmaA is one of two reported glycosylated proteins associated with A. actinomycetemcomitans. The fimbriae of this organism are also suggested to be glycosylated (17); however, the
mechanism of fimbrillin glycosylation is unknown. In this study, we present the first evidence for the posttranslational modification of a trimeric autotransporter protein adhesin. The evidence presented in this study suggests that the nonfimbrial collagen adhesin EmaA of \textit{A. actinomycetemcomitans} is posttranslationally modified by enzymes associated with the LPS biosynthetic pathway. Furthermore, this modification may be important for full biological activity of this adhesin. Biochemical experiments are under way to confirm the nature and the site(s) of glycosylation of EmaA.

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8. Reference deleted.


