Lipopolysaccharides mediate leukotoxin secretion in *Aggregatibacter actinomycetemcomitans*

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SUMMARY

We previously reported that lipopolysaccharide (LPS) -related sugars are associated with the glycosylation of the collagen adhesin EmaA, a virulence determinant of *Aggregatibacter actinomycetemcomitans*. In this study, the role of LPS in the secretion of other virulence factors was investigated. The secretion of the epithelial adhesin Aae, the immunoglobulin Fc receptor Omp34 and leukotoxin were examined in a mutant strain with inactivated TDP-4-keto-6-deoxy-D-glucose 3,5-epimerase (*rmlC*), which resulted in altered O-antigen polysaccharides (O-PS) of LPS. The secretion of Aae and Omp34 was not affected. However, the leukotoxin secretion, which is mediated by the TolC-dependent type I secretion system, was altered in the *rmlC* mutant. The amount of secreted leukotoxin in the bacterial growth medium was reduced nine-fold, with a concurrent four-fold increase of the membrane-bound toxin in the mutant compared with the wild-type strain. The altered leukotoxin secretion pattern was restored to the wild-type by complementation of the *rmlC* gene in *trans*. Examination of the *ltxA* mRNA levels indicated that the leukotoxin secretion was post-transcriptionally regulated in the modified O-PS containing strain. The mutant strain also showed increased resistance to vancomycin, an antibiotic dependent on TolC for internalization, indicating that TolC was affected. Overexpression of TolC in the *rmlC* mutant resulted in an increased TolC level in the outer membrane but did not restore the leukotoxin secretion profile to the wild-type phenotype. The data suggest that O-PS mediate leukotoxin secretion in *A. actinomycetemcomitans*.

INTRODUCTION

The gram-negative, non-motile, microaerophilic bacterium *Aggregatibacter actinomycetemcomitans* is an endogenous oropharyngeal colonizer of humans and primates. The bacterium can be recovered from the subgingival sulcus, tongue, buccal mucosa and saliva from 10 to 15% of healthy young individuals in the USA (Sirinian *et al.*, 2002) and Brazil (Cortelli *et al.*, 2008). *Aggregatibacter actinomycetemcomitans* is strongly implicated with localized aggressive periodontitis (Haubek *et al.*, 2008), which is featured with pubertal onset and results in rapid deterioration of the periodontium. Localized aggressive periodontitis affects approximately 0.5% of adolescents in the USA (Löe & Brown, 1991). In addition, *A. actinomycetemcomitans* causes disseminated infections,

including lung and brain abscesses (Stepanović *et al.*, 2005; Hagiwara *et al.*, 2009), and it is also the most common HACEK (*Haemophilus* spp., *Aggregatibacter actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella* spp.) microorganism associated with infective endocarditis (Paturel *et al.*, 2004; Tang *et al.*, 2008). Recent clinical studies (Gaetti-Jardim *et al.*, 2009), as well as *in vivo* mouse model experiments (Zhang *et al.*, 2010), suggest that *A. actinomycetemcomitans* accelerates the pathogenesis of atherosclerosis.

The outer membrane of gram-negative bacteria is an asymmetric bilayer, composed of phospholipids, proteins and lipopolysaccharides (LPS) (Raetz & Whitfield, 2002). Lipopolysaccharides are comprised of lipid A, core oligosaccharides and O-antigen polysaccharides (O-PS). Lipid A moieties are associated with the hydrophobic compartment of the bilayer. Core oligosaccharides are covalently attached to the lipid A moieties, which are bonded to the O-PS. The O-PS define the serotypes of this organism, and *A. actinomycetemcomitans* has been assigned seven different serotypes (Takada *et al.*, 2010). Lipopolysaccharides are the predominant molecules on the bacterial surface and are important for the viability and membrane stability of gram-negative bacteria (Raetz & Whitfield, 2002). It has also been suggested that LPS is involved in lateral diffusion of membrane proteins, including large autotransporters and porin protein OmpF (Jain *et al.*, 2006; Straatsma & Soares, 2009), protein secretion (Wandersman & Létoffé, 1993; Bulieris *et al.*, 2003; Bengoechea *et al.*, 2004), and biological activities of proteins (Stanley *et al.*, 1993; Iredell *et al.*, 1998).

Multiple virulence factors contribute to the colonization and pathogenesis of *A. actinomycetemcomitans.* These factors include adhesins: epithelial cell adhesins ApiA and Aae (Rose *et al.*, 2003; Yue *et al.*, 2007), and the extracellular matrix protein adhesin (EmaA) (Mintz, 2004; Tang *et al.*, 2008; Yu *et al.*, 2008). These adhesins are autotransporters secreted using the Type V secretion system (T5SS), which comprises an N-terminal passenger domain that typically mediates a virulence function and a C-terminal β-barrel domain for member insertion (Henderson *et al.*, 2004). In addition to these adhesins, other virulence factors are characterized including toxins: leukotoxin (Tsai *et al.*, 1979; Taichman *et al.*, 1980) and cytolethal extending toxin (Shenker *et al.*, 2006);

and the immunoglobulin Fc receptor: OmpA-like heat-modified protein Omp34(Omp29) (Mintz & Fives-Taylor, 1994; Komatsuzawa *et al.*, 1999).

The leukotoxin is a 114 kDa protein, which targets human polymorphonuclear leukocytes and monocytes (Tsai *et al.*, 1979; Taichman *et al.*, 1980). The biosynthesis, activation and secretion of leukotoxin is determined by the four-gene operon *ltxCABD* and *tolC* (or *tdeA*, toxin and drug export protein A) (Lally *et al.*, 1989a; Crosby & Kachlany, 2007). The *ltxCABD* operon structure and secretion pathway is homologous to haemolysin (HlyA) found in *Escherichia coli* (Fath & Kolter, 1993). The pro-toxin of LtxA is synthesized in the cytoplasm and is covalently attached (acylated) with short-chain fatty acyl groups to the internal lysine residues by LtxC (Fong *et al.*, 2011). Analogous to the haemolysin secretion, the leukotoxin is translocated across the inner membrane by an integral inner membrane ATPase, LtxB (HlyB in *E. coli*) and transits the periplasmic space through a channel formed by LtxD (HlyD in *E. coli*) and TolC (Koronakis *et al.*, 1993, 2004). In *E. coli*, TolC is a trimeric outer membrane protein, composed of a β-barrel domain and an α-helical domain (Koronakis *et al.*, 2000). The β-barrel domain integrates into the outer membrane to form a pore, whereas the α-helical domain forms a channel extending into the periplasm and interacts with HlyD (Koronakis *et al.*, 2004). In addition to the TolC-dependent type I secretion system (T1SS) (Fath & Kolter, 1993), leukotoxin is also found secreted with membrane vesicles in *A. actinomycetemcomitans* (Kato *et al.*, 2002).

The heat-modified outer membrane protein Omp34 is a homologue of the structural outer membrane protein OmpA found in *E. coli*, and one of the major outer membrane proteins of *A. actinomycetemcomitans* (Komatsuzawa *et al.*, 1999, 2002). This protein is secreted using the general secretion system, Type II secretion system (T2SS), which uses the Sec translocon for transport across the inner membrane (Henderson *et al.*, 2004). This group of proteins plays a pivotal role in maintaining membrane structure (Kleinschmidt, 2006). Omp34 also binds to the Fc portion of IgG, which may inhibit complement activation in the host (Mintz & Fives-Taylor, 1994).

In this study, the impact of LPS in the secretion of virulence determinants was investigated. A mutant strain of *A. actinomycetemcomitans* with an O-PS defect showed an elevated amount of membrane-

associated leukotoxin, whereas the secreted leukotoxin in the culture medium was diminished when compared with the wild-type strain. Further examination of the mRNA level of *ltxA* indicated the changes in leukotoxin secretion occurred post-transcriptionally. Additional evidence suggested that the impaired leukotoxin secretion in this mutant was associated with disruption of the function of the TolC-dependent T1SS, but not the relative amount of TolC in the outer membrane. In contrast, no change was associated with either Omp34 or Aae, which are secreted by Sec-dependent pathways.

METHODS

Bacterial strains

All strains used in this study are listed in Table 1. VT1169, the wild-type strain, was transformed with the shuttle plasmid, pKM2 containing the 520-bp leukotoxin promoter (*ltx*P) of *A. actinomycetemcomitans* (Tang & Mintz, 2010). This strain was used as the positive control and for the effect of the plasmid on gene expression. The *ltx*P was used as the promoter for the expression of *rmlC* and *tolC* in the strains used in this study (Table 1). The *rmlC* mutant, which was transformed with pKM2/*ltx*P, and the complemented strains were described previously

(Tang & Mintz, 2010). All *A. actinomycetemcomitans* strains were stored at −80°C and grown in 3% Trypticase Soy Broth and 0.6% Yeast Extract (TSBYE) with/without 1.5% agar (Becton Dickinson and Company, Franklin Lakes, NJ), and 1 μg ml^{-1} chloramphenicol (Sigma, St Louis, MO) in a 37°C incubator with 10% humidified carbon dioxide.

Analysis of LPS by gas chromatography/mass spectrometry (GC/MS)

The glycosyl composition of the LPS extracted from the wild-type *A. actinomycetemcomitans* strain and the isogenic mutants was analyzed using combined GC/MS, as described before (Tang & Mintz, 2010), at the Complex Carbohydrate Research Center, the University of Georgia.

Cell fractionation of *A. actinomycetemcomitans*

Membrane and cytoplasmic proteins of *A. actinomycetemcomitans* were prepared as described previously (Mintz, 2004). Briefly, 200 ml late-logarithmic phase cells were harvested and resuspended in 3.0 ml 10 mm HEPES (pH 7.4) with 1 mM phenylmethylsulphonyl fluoride (Roche, Mannheim, Germany) and complete protease inhibitor Cocktail (Roche). The cells were lyzed using a French press minicell,

Table 1 Strains and plasmids

Strains and plasmids	Genotypes/Remarks	Sources and references
Aggregatibacter actinomycetemcomitans		
VT1169	a Rif^r, Nal^r derivative of SUNY465, serotype b	Mintz, (2004)
VT1169/pKM2/*ltx*P	VT1169 transformed with plasmid pKM2/*ltx*P, which is referred as the 'wild-type'	This study
aae mutant (*aae*::Kan)	An isogenic *aae* (epithelial adhesin) mutant of SUNY465	Rose *et al.*, (2003)
rmlC (*rmlC*::pLOF/Sp)	Rhamnose mutant, transposon pLOF/Sp inserted into *rmlC*(TDP-4-keto-6-deoxy-D-glucose 3,5-epimerase)	Tang & Mintz, (2010)
rmlC/pKM2/*ltx*P	*rmlC* mutant transformed with pKM2/*ltx*P	This study
rmlC/*rmlC*$^+$	*rmlC* mutant complemented with plasmid pKM2/*ltx*P/*rmlC*	Tang & Mintz, (2010)
rmlC/*tolC*$^+$	*rmlC* mutant transformed with plasmid pKM2/*ltx*P/*tolC* to overexpress TolC	This study
Plasmids		
pLOF/Sp	Tn*10*-based transposon vector; Ap^r, Sp^r	Mintz, (2004)
pKM2	A plasmid containing chloramphenicol acetyltransferase, which can be transformed into serotype b *A. actinomycetemcomitans*, Cm^r	Gallant *et al.*, (2008)
pKM2/*ltx*P	pKM2 containing ∼500-bp *ltx* promoter, Cm^r	Tang & Mintz, (2010)
pKM2/*ltx*P/*rmlC*	pKM2 containing ∼500-bp *ltx* promoter + *rmlC* sequence, Cm^r	Tang & Mintz, (2010)
pKM2/*ltx*P/*tolC*	pKM2 containing ∼500-bp *ltx* promoter + *tolC* sequence, Cm^r	This study

Ap, ampicillin; Cm, chloramphenicol; Nal, nalidixic acid; Rif, rifampicin; Sp, spectinomycin

and intact cells and debris were removed. The cytoplasmic and membrane fractions were separated by ultra-centrifugation (100,000 **g** for 40 min at 4°C). Inner and outer membrane proteins were separated by detergent solubilization using 1% *N*-lauroylsarcosine sodium salt (L-5125, Sigma) in HEPES, at room temperature for 30 min, without agitation (Filip *et al.*, 1973; Nikaido, 1997), followed by centrifugation at 15,600 **g** for 30 min at 4°C. The proteins that are insoluble in lauroylsarcosine are defined as outer membrane proteins, and the detergent-soluble proteins are defined as inner membrane proteins.

Leukotoxin isolation from bacterial culture medium

Two hundred milliliters of *A. actinomycetemcomitans* were grown to the late-logarithmic growth phase, and the cells were centrifuged at 7650 **g** for 30 min. The resulting supernatant was filtered through a low protein binding polyethersulfone membrane (0.22 μm) (Corning, Lowell, MA) to remove any remaining bacteria. Then, 15 ml of filtered spent medium was concentrated 150-fold using Amicon Ultra centrifugation filter devices with a 50,000 molecular weight cutoff (Millipore, Billerica, MA), by centrifugation at 5000 **g** for 30 min at 4°C, to a final volume of 100 μl.

Immunoblot analysis for the detection of Aae, Omp34 and leukotoxin (LtxA)

Protein concentrations were determined using the bicinchoninic acid protein assay (Pierce Thermo Scientific, Rockford, IL). Protein samples were diluted in 10 mM HEPES with 2% sodium dodecyl sulfate (SDS) before addition to the assay. Equivalent concentration of protein was dissolved in electrophoresis loading buffer containing 10 mM HEPES, 2% SDS, 5% (v/v) β-mercaptoethanol, 2% (v/v) glycerol and 0.05% (w/v) bromophenol blue, at 100°C for 5 min, and loaded into 4–15% gradient polyacrylamide, Tris–glycine Procast Gels (Bio-Rad, Hercules, CA). The separated proteins were transferred to 0.45-μm Westtran polyvinylidene fluoride (PVDF) membrane (Whatman Inc., Piscataway, NJ), probed with anti-Aae polyclonal antibody (Rose *et al.*, 2003), anti-Omp34 monoclonal antibody (Komatsuzawa *et al.*, 1999), and anti-leukotoxin polyclonal antibody (Lally et al., 1989b; Gallant *et al.*, 2008). The immune complexes were detected with horseradish peroxi-

dase-conjugated goat anti-rabbit IgG (Jackson Laboratory, Bar Harbor, ME) for the polyclonal antibody, or goat anti-mouse IgG (Sigma) for the monoclonal antibody. Signal was detected using a chemiluminescent substrate (Pierce Biotechnology, Rockford, IL) and visualized by exposure to Kodak X-OMAT LS film (Carestream Health, Rochester, NY).

Protein levels were quantified based on integrated signal densities. Immunoblots were performed four times using samples prepared from four independent experiments. Densities were determined using IMAGEJ 1.43u (http://rsb.info.nih.gov/ij/). The paired *t*-test was used to compare two groups of data, and one-way analysis of variance was used to compare three or more than three groups of data (GRAPHPAD, Version 3.00, La Jolla, CA). The level of the proteins in the wild-type strain was arbitrarily set as 1.0. A value of $P < 0.05$ was considered significant.

Isolation of total RNA from *A. actinomycetemcomitans*

The *A. actinomycetemcomitans* wild-type and mutant strains were recovered directly from −80°C. After a 2-day growth on TSBYE plates, one colony of the wild-type strain (VT1169) and the *rmlC* mutant were transferred into 8 ml freshly prepared TSBYE broth, with or without spectinomycin, and grown overnight. One milliliter of the bacterial suspension was transferred into 9 ml fresh TSBYE without antibiotics and grown for an additional 4 h (one and a half doublings) to reach mid-logarithmic growth phase (optical density at 495 nm 0.25–0.3). A total of 5×10^8 bacteria in 1 ml TSBYE were mixed with 2 ml RNAprotect Bacteria Reagent (Qiagen Inc., Valencia, CA) to stabilize the RNA. The cells were centrifuged at 5000 **g** for 10 min and resuspended in 100 μl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) containing 1 mg ml^{-1} lysozyme Type VI (MP Biomedicals, Aurora, OH). The cells were incubated for 5 min at room temperature with agitation. Total RNA was purified from the lysate using an RNeasy Mini kit (Qiagen Inc.).

Quantitative, real-time, reverse transcription polymerase chain reaction analysis of leukotoxin expression

The purified total RNA was pretreated with deoxyribonuclease I (DNase), amplification grade (Invitrogen,

Carlsbad, CA), to remove contaminating DNA. A total of 200 ng DNase-treated RNA from each sample was reverse transcribed into complementary DNA (cDNA) in a 20-μl reaction using the SuperScript III First-Strand Synthesis system (Invitrogen). Random hexamer primers, provided by the manufacturer, were used in the reverse transcription reaction. The quantitative real-time polymerase chain reaction (PCR) was performed using an ABI Prism 7900HT Sequence Detection System in the Vermont Cancer Center at the University of Vermont. The 16S ribosomal RNA gene (16S rRNA) was chosen as the endogenous housekeeping gene. The primers and probes used in this study are listed in Table 2. The probes were labeled with the 5′ reporter dye 6-FAM and the 3′ quencher dye BHQ-1. Three cDNA samples, derived from three individual RNA samples, were prepared for each strain. Each cDNA sample was run in duplicate for each real-time PCR. The data were analyzed using paired Student's *t*-test, and $P < 0.05$ was set as a significant difference. The relative expression level was calculated based on the threshold cycle (C_T):

$$\text{Expression level} = 2^{-\Delta\Delta C_T},$$

$$\Delta\Delta C_T = C_{T(\text{target})} - C_{T(\text{16s rRNA})} - C_{T(\text{calibrator})}$$

The wild-type strain VT1169 was chosen as the calibrator.

Antimicrobial susceptibility assay

Three compounds were chosen to determine the antimicrobial susceptibility of this O-PS mutant: bile salt, erythromycin and vancomycin hydrochloride. Bile salt (S719191, Fisher, Pittsburgh, PA), which contains

∼50% glycocholic sodium salt and ∼45% taurocholic sodium salt, was incorporated into TSBYE agar, initially with 10 mg ml^{-1}, and decreased by 0.5 mg ml^{-1} intervals to a final concentration of 5 mg ml^{-1}. Erythromycin (E6376, Sigma) was initiated with 10 μg ml^{-1}, and decreased by 1.25 μg ml^{-1} to a final concentration of 2.5 μg ml^{-1}. Vancomycin from *Streptomyces orientalis* (V2002, Sigma) was started with 350 μg ml^{-1} and decreased by 12.5 μg ml^{-1} to a final concentration of 150 μg ml^{-1}. Then, 1.0×10^3 to 2.5×10^3 mid-logarithmic growth phase cells were evenly spread on each plate and grown for 7 days in 10% humidified CO_2 at 37°C. The minimal inhibitory concentration (MIC) of each compound was defined as the lowest concentration of antibiotic on plates containing less than five colony-forming units (Wandersman & Létoffé, 1993). The assay was performed in triplicate.

Cloning of *tolC*

The *tolC* (*tdeA*) gene was amplified and engineered with *Xho*I at the 5 end and *Eco*RI at the 3′ end of the gene: sense primer (5′-G*CTCGAG*ATGTTCACAA TAAAAA-3′) and antisense primer (5′-*GAATT C*TTATTTTTTTACGGAATAAT-3′). The *tolC* gene of VT1169 was found to be 99.9% similar to the sequence of the HK1651 strain (http://www.oralgen. lanl.gov/; Gene ID: AA02077) and the IDH781 strain (Crosby & Kachlany, 2007). The gene was amplified using high-fidelity polymerase (Roche), cloned into the Topo vector (Invitrogen), and treated with restriction enzymes *Xho*I and *Eco*RI (New England Biolabs, Ipswich, MA). The fragment 5′-*Xho*I-*tolC*-*Eco*RI-3′ was gel-purified (Qiagen Inc.), ligated (Invitrogen) with the vector pKM2/*ltxP* treated with the same enzymes, and dephosphorylated using shrimp alka-

Table 2 Primers and probes for quantitative real-time, reverse transcriptional polymerase chain reaction

Targets	Primers and probes	Sequence (5′–3′)	Amplicons (bp)
LktA	Forward primer	5′-GGACCTGTCGCAGGGTTAATT-3′	98
	Reverse primer	5′-AGCATTCTCGCACGATCAAA-3′	
	Dual-labeled probe	5′-FAM-TCAGCTTGGCAATCAGCCCTTTGTC-BHQ-3′	
16S rRNA[1]	Forward primer	5′-GAACCTTACCTACTCTTGACATCC-3′	122
	Reverse primer	5′-GGACTTAACCCAACATTTCACAAC-3′	
	Dual-labeled probe	5′-FAM-CTGACGACAGCCATGCAGCACCTG-BHQ1-3′	

[1]Endogenous control.
Reporter dye: 6-FAM; Quencher dye: BHQ-1.

line phosphatase (USB, Cleveland, OH). The ligation mix was transformed into Top10 cells, and colonies were selected on Lysogeny Broth (LB)-Luria (0.5% sodium chloride) agar plates with 20 μg ml^{-1} chloramphenicol. The new construct pKM2/*ltxP*/*tolC* was transformed into the *A. actinomycetemcomitans rmlC* mutant. The *rmlC*/*tolC$^+$* strain was selected on the TSBYE plate containing 1 μg ml^{-1} chloramphenicol and 50 μg ml^{-1} spectinomycin.

Liquid chromatography/mass spectrometry (LC/MS) -based, label-free, relative quantification of TolC

Equivalent amounts of outer membrane fractions from the wild-type, the *rmlC* mutant and the TolC overexpression strain (*rmlC*/*tolC$^+$*) were dissolved in electrophoresis loading buffer, boiled for 5 min and loaded into a 5–15% gradient SDS–polyacrylamide gel, with a 3% stacking gel. Electrophoresis was performed at 60 V, 4°C for 24 h. The gel was fixed, stained with colloidal blue (Invitrogen), and destained in deionized water. The bands containing TolC were excised, washed, reduced and alkylated. The hydrated gel slices were treated with trypsin, and analyzed by ion trap LC/MS. The trypsin-generated peptide fingerprints were scanned by the mass spectrometer, with a mass-to-charge ratio between 400 and 1600 Daltons. The generated peptide spectrum was compared with the TolC protein sequence of an *A. actinomycetemcomitans* serotype b strain (HK1651) found in the oral pathogen database (http://www.oralgen.lanl.gov). The relative amount of TolC protein in each sample was quantified based on the total spectral counts (Zybailov *et al.*, 2005), which were matched with the TolC protein sequence in the database. The proteomic relative quantification was duplicated. The LC/MS was performed at the Vermont Genetics Network proteomics facility located at the University of Vermont.

RESULTS

Characterization of sugar residuals of the rhamnose mutant strain (*rmlC*)

The impact of O-PS on protein secretion was investigated in a strain with a mutation in the gene coding for the TDP-4-keto-6-deoxy-D-glucose 3,5-epimerase (*rmlC*). Carbohydrate analysis of isolated LPS from the *rmlC* mutant strain revealed differences in the sugar composition of the O-PS when compared with the wild-type strain (Table 3). The serotype b O-PS of *A. actinomycetemcomitans* consist of trisaccharide repeating units, and each unit is composed of L-rhamnose (L-Rha), D-fucose (D-Fuc) and D-*N*-acetyl galactosamine (D-GalNAc) (Perry *et al.*, 1996). Neither Rha nor GalNAc was detected, and the mole percentage of Fuc was reduced 75% when compared with the wild-type LPS. The changes in O-PS sugars could be restored to wild-type levels when the mutant strain was transformed with the wild-type *rmlC* gene *in trans* (Tang & Mintz, 2010).

Secretion of Omp34, Aae and leukotoxin in the rmlC mutant strain

Three proteins representing different secretion pathways in *A. actinomycetemcomitans* (Omp34, Aae and Ltx), were examined for the impact of the O-PS defect on secretion. As demonstrated in Fig. 1, no difference in the relative immunostaining intensity of Omp34 in the outer membrane was observed in the *rmlC* mutant strain compared with the wild-type strain. A similar result was observed for the epithelial cell adhesin Aae (Fig. 1). However, the relative amount of leukotoxin in the different bacterial cellular compartments (cytoplasm, membrane and culture medium) was altered compared with the wild-type strain (Fig. 2A,B). The leukotoxin detected in the membrane fraction of the *rmlC* mutant was about

Table 3 Glycosyl composition analysis

Glycosyl residues	Mole percentage (%)[1]	
	Wild-type	*rmlC* mutant
Ribose(Rib)	8.1	8.7
Rhamnose (Rha)	**20.5**	**n.d.**
Fucose (Fuc)	**15.7**	**3.9**
Galactose (Gal)	4.2	4.8
Glucose (Glc)	21.9[2]	36.2[2]
N Acetyl galactosamine (GalNAc)	**2.2**	**n.d.**
N Acetyl glucosamine (GlcNAc)	12.8	16.4
Heptose (Hep)	14.7	30.0
Total amount	100	100

[1]The values are mole percentage of total carbohydrate.
[2]The most predominant glycosyl residues.
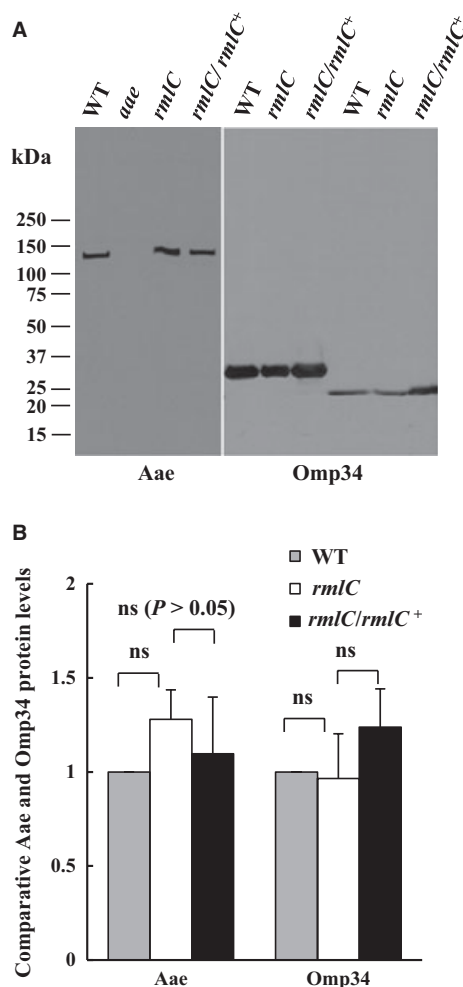Rha, Fuc and GalNAc form the backbone of trisaccharide repeating units of serotype b O-PS.

Figure 1 Analysis of outer membrane proteins. (A) Outer membrane proteins were separated by SDS–PAGE, transferred to the PVDF membrane, and probed with specific antibodies for the indicated proteins. Proteins were detected from the wild-type (WT), *aae* mutant (*aae*), *rmlC* mutant (*rmlC*), and the *rmlC* complemented strain (*rmlC/rmlC*+). Omp34 migrates at a molecular mass of 34 kDa when the protein is heated at 100°C; and migrates at 29 kDa when the protein is denatured at room temperature (Komatsuzawa *et al.*, 1999). (B) Quantification of Aae and Omp34 proteins. The integrated signal intensity of each band shown in (A), which represents the relative protein level, was quantified using the IMAGEJ program and analyzed using one-way analysis of variance. The signal intensity of the wild-type strain was arbitrarily set as 1.0. (ns, non-significant).
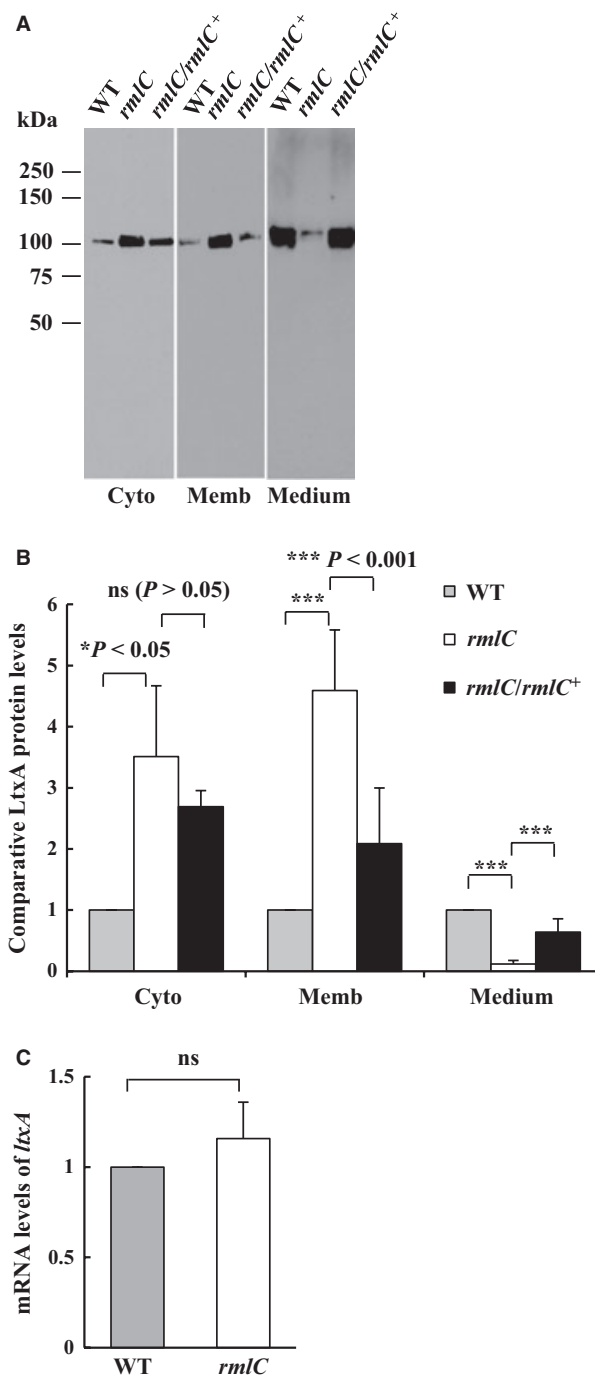
Figure 2 Analysis of leukotoxin in the cytoplasm, membrane and growth medium. (A) Immunoblot analysis. Equivalent amounts of proteins from the cytoplasm (Cyto), membrane (Memb) and culture medium (Medium) of the wild-type (WT), the *rmlC* mutant (*rmlC*) and the complemented strain (*rmlC/rmlC*+) were separated, transferred to PVDF, and probed with anti-LtxA antibody. (B) Quantification of leukotoxin (LtxA). The integrated intensity of each band in (A), which represents the relative protein level, was quantified using the IMAGEJ program and analyzed using one-way analysis of variance. The signal intensity of the wild-type in each fraction was arbitrarily set as 1.0. (C) Quantitative, real-time, reverse transcriptional PCR analysis of *ltxA* mRNA levels. The relative mRNA levels of *ltxA* were calculated based on the threshold cycle (C_T): relative expression level = $2^{-\Delta\Delta C_T}$ with $\Delta\Delta C_T = C_{T(ltxA)} - C_{T(16SrRNA)} - C_{T(calibrator)}$. The wild-type strain VT1169 was chosen as the calibrator. (ns, non-significant; $^*P < 0.05$, $^{***}P < 0.001$).

four-fold higher than the wild-type strain (Fig. 2A,B). Concurrent with the increase of leukotoxin in the cytoplasm and membrane, the amount of leukotoxin detected in the culture medium of the *rmlC* mutant decreased to 12% of the wild-type strain (Fig. 2A,B). The altered leukotoxin secretion pattern in the mutant was restored to the wild-type by complementation of the *rmlC* mutant (Fig. 2A,B). The change in the relative protein level of leukotoxin was not associated with transcriptional changes of the leukotoxin structural gene (*ltxA*). The transcription of *ltxA* was found to be similar in the *rmlC* mutant, when compared with the wild-type strain, as determined by quantitative real-time PCR (Fig. 2C). Taken together, these results suggest that the TolC-dependent T1SS appears to be impacted in the *rmlC* mutant, and the O-antigen polysaccharide is necessary for leukotoxin secretion in *A. actinomycetemcomitans*.

Susceptibility to antimicrobial compounds

The TolC-mediated influx/efflux system actively transports vancomycin and the internalization of vancomycin mediated by TolC is required for its antibiotic activity (Wandersman & Létoffé, 1993). To examine if the TolC-mediated influx/efflux system was affected in the *rmlC* mutant, vancomycin-induced bacterial cell death was examined. The *rmlC* mutant strain showed a significantly ($P < 0.05$) higher resistance to vancomycin (MIC 262.5 ± 12.5 µg ml^{-1}) when compared with the wild-type strain (MIC 212.5 ± 12.5 µg ml^{-1}). In contrast, the *rmlC* mutant showed similar susceptibility to bile salt (MIC 8.5 ± 0.5 mg ml^{-1}) and erythromycin (5.0 ± 1.25 µg ml^{-1}) when compared with the wild-type strain. The increased vancomycin resistance in the *rmlC* mutant of *A. actinomycetemcomitans* suggests that the influx/efflux function of TolC is altered in the mutant.

Quantification of outer membrane-associated TolC

The reduction in leukotoxin secretion and the increase in resistance to vancomycin in the *rmlC* mutant indicated that the transportation of leukotoxin via the channel-forming protein TolC may be impaired in the O-PS mutant. To address the question of whether the impact on secretion is the result of altered biological activities or the decreased

amount of TolC, the relative amount of TolC in the membrane of the mutant and wild-type strains was quantified using an LC/MS-based, label-free, proteomic approach, because of the lack of anti-*A. actinomycetemcomitans* TolC antibodies. Outer membrane proteins from these two strains were separated by SDS–polyacrylamide gel electrophoresis and the bands corresponding to TolC (~51 kDa) were excised and analyzed by mass spectroscopy (Fig. 3 A). The amount of TolC in the outer membrane of the mutant and wild-type strain was equivalent (Fig. 3 B). A two-fold increase in the amount of TolC in the outer membrane was observed in the *rmlC* mutant transformed with *tolC* on a replicating plasmid (Fig. 3 B). The overexpression of TolC in this background did not rescue the leukotoxin secretion phenotype of the *rmlC* mutant strain (Fig. 4A,B). The data indicate that the decrease in leukotoxin secretion in the *rmlC* mutant strain was not dependent on the amount of TolC in the membrane.

DISCUSSION

Lipopolysaccharide, the predominant molecule on the bacterial surface, is composed of a hydrophobic domain (lipid A), non-repeating core oligosaccharides and distal O-PS. Although lipid A is critical for the onset of immune responses to gram-negative bacteria via activation of Toll-like receptor 4 (Raetz & Whitfield, 2002), O-PS are reported to be involved in protein secretion, including the α-haemolysin of *E. coli*, the proteases of *Erwinia chrysanthemi* (Wandersman & Létoffé, 1993) and virulence factors of *Yersinia* spp. (Bengoechea *et al.*, 2004). However, the role of O-PS in the secretion of virulent determinants in *A. actinomycetemcomitans* has not been investigated. This study suggests the composition or the structure of the O-PS impacts selective protein secretion in *A. actinomycetemcomitans*.

The dTDP-4-keto-6-deoxy-ᴅ-glucose 3,5-epimerase (*rmlC*) converts the precursor dTDP-4-keto-6-deoxy-ᴅ-glucose to dTDP-4-keto-ʟ-rhamnose, which is further reduced to dTDP-ʟ-rhamnose. The dTDP-4-keto-6-deoxy-ᴅ-glucose is also the substrate for dTDP-4-keto-6-deoxy-ᴅ-glucose reductase (*fcd*), which is metabolized to dTDP-ᴅ-fucose (Yoshida *et al.*, 1999). The absence of Rha in the LPS isolated from the *rmlC* mutant confirmed the inactivation of dTDP-4-keto-6-deoxy-ᴅ-glucose 3,5-epimerase. The
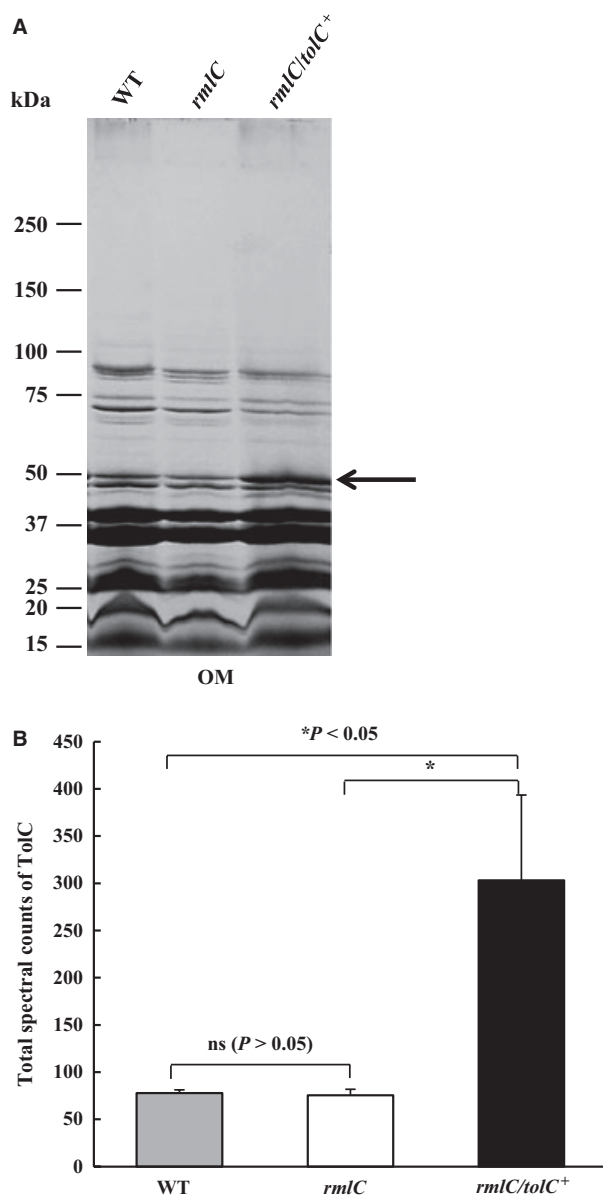
A

OM

B

Figure 3 Liquid chromatography/mass spectrometry (LC/MS)-based, label-free quantification of TolC. (A) Colloidal blue-stained SDS–polyacrylamide gel and LC/MS analysis. Equivalent amounts of outer membrane (OM) proteins of the wild-type (WT), the *rmlC* mutant (*rmlC*), and the *rmlC* mutant with overexpression of TolC (*rmlC/tolC*+) were separated by SDS–polyacrylamide gels, stained with colloidal blue, and the bands corresponding to TolC (shown by arrow) were excised, reduced, alkylated, digested and analyzed using ion trap LC/MS. (B) Relative quantification of TolC proteins using total spectral counts. The amount of TolC protein in each sample was quantified based on the total number of peptides generated by LC/MS and matched with the TolC protein sequence. (ns, non-significant; *$P < 0.05$).
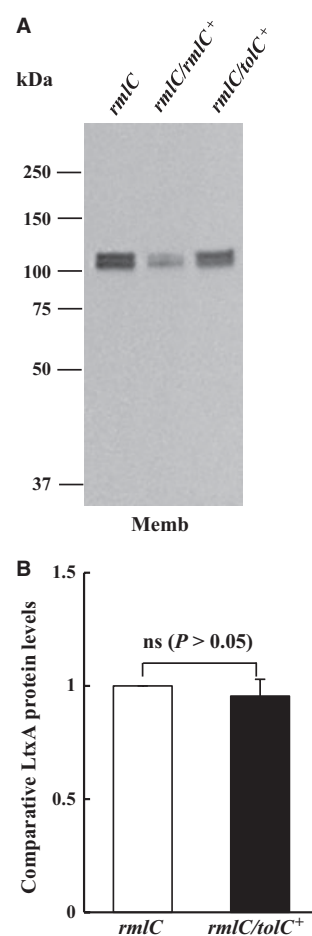
A

Memb

B

Figure 4 Detection of leukotoxin in the *rmlC* mutant strain overexpressing TolC. (A) Immunoblot analysis. Equivalent amounts of outer membrane (OM) protein of the *rmlC* mutant (*rmlC*), the *rmlC* complemented strain (*rmlC/rmlC*+), and the TolC overexpression strain (*rmlC/tolC*+) were separated, transferred to PVDF, and probed with anti-LtxA antibody. (B) Quantification of leukotoxin (LtxA). The integrated intensity of each band in (A), which represents the relative protein level, was quantified using the IMAGEJ program. The membrane-bound leukotoxin found in the *rmlC/tolC*+ strain remained the same as the *rmlC* mutant. The signal intensity of the *rmlC* mutant was arbitrarily set as 1.0. (ns, non-significant).

D-GalNAc moiety of the O-PS is linked to the O-3 position of L-Rha (Perry *et al.*, 1996). Therefore, the absence of GalNAc in the LPS of the *rmlC* mutant might be attributed to the inability of this sugar to covalently bond to the O-PS backbone in the absence of Rha. The sugar components of the core oligosaccharides were found to be similar to the wild-type LPS. Together, the data indicated that the composition and structure of the O-PS in the *rmlC* mutant were altered, as compared with the wild-type O-PS.

The change in the O-PS had little effect on the amount of selected outer membrane proteins, including Omp34, Aae or TolC. We did, however, observe an increase in the amount of leukotoxin associated with the membrane and a concurrent decrease in the amount of toxin in the bacterial growth medium. The reversion to a wild-type secretion profile by complementation of the *rmlC* mutant strain with a plasmid expressing dTDP-4-keto-6-deoxy-D-glucose 3,5-epimerase supports the thesis that leukotoxin secretion is indirectly dependent on this enzyme.

The change in the leukotoxin secretion profile was not attributed to alteration in *ltxA* transcription, which suggested a post-transcriptional mechanism to describe this defect. In *E. coli*, the secretion of α-haemolysin is reduced in an *rfaH* mutant, a strain producing LPS with the core oligosaccharides lacking hexose (Wandersman & Létoffé, 1993). Although the mutations in these two bacteria targeted different moieties of LPS, the impact of the LPS defect on toxin secretion appeared to be similar. In *E. coli*, however, the change in α-haemolysin secretion is attributed to the reduction in the amount of TolC present in the outer membrane (Wandersman & Létoffé, 1993). This is in contrast to our observation that the amount of membrane-associated TolC in the *rmlC* mutant did not differ from the wild-type strain (Fig. 4). Furthermore, overexpression of TolC in the membrane of the *rmlC* mutant did not revert to the wild-type leukotoxin secretion profile (Fig. 4). However, different membrane isolation and solubilization methods were employed in this study, as compared with Wandersman & Létoffé (1993), which may have resulted in different interpretations. In addition, the difference in the membrane morphology of these bacteria, convoluted in *A. actinomycetemcomitans* (Gallant *et al.*, 2008) vs. smooth in *E. coli*, may also be a contributing factor. Nonetheless, our data indicate that the leukotoxin secretion defect in this LPS mutant of *A. actinomycetemcomitans* was not dependent on the amount of TolC in the membrane.

Multidrug influx/efflux machineries are associated with TolC (Wandersman & Létoffé, 1993; Koronakis *et al.*, 2004; Crosby & Kachlany, 2007). In the *E. coli* system, the reduction in hemolysin secretion was associated with an increase in the resistance to vancomycin (Wandersman & Létoffé, 1993), a glycopeptide that inhibits peptidoglycan biosynthesis of gram-positive bacteria (Reynolds, 1989). Vancomy-

cin, because of its large molecular size (1486 Da), cannot passively diffuse across the outer membrane of gram-negative bacteria; instead, it is actively transported via the influx/efflux system mediated by TolC (Wandersman & Létoffé, 1993). The increase in vancomycin resistance of the *rmlC* mutant of *A. actinomycetemcomitans* suggests a defect in the influx/efflux activity of TolC, because the amount of TolC in the membrane is unaffected by the mutation.

TolC or TdeA in *A. actinomycetemcomitans* facilitates the influx/efflux of antimicrobials (Crosby & Kachlany, 2007). The MIC of two selected agents used in this study, bile salt and erythromycin, were equally effective in inhibiting the growth of the wild-type and the *rmlC* mutant strains. Bile salt and erythromycin are the most sensitive antimicrobials in differentiating *tolC* mutations in *A. actinomycetemcomitans* (Crosby & Kachlany, 2007) and *Vibrio cholera* (Bina & Mekalanos, 2001) from the wild-type strains. The apparent activity of TolC for these antibiotics may be attributed to the smaller size of these antimicrobials, as compared with vancomycin. The crystal structure of TolC suggests that the protein acts as a gating mechanism, which has an 'open' or 'closed' state (Eswaran *et al.*, 2003; Koronakis *et al.*, 2004). The change in O-PS may influence the structure of the protein to limit the pore size of the 'open' state and allow only for the passage of low molecular weight compounds and exclude molecules such as leukotoxin and vancomycin. However, we cannot exclude the possibility that other membrane protein(s) may be altered in the *rmlC* mutant, which lead to changes in the microenvironment of the membrane resulting in a structural perturbation of the transport system.

In summary, this study suggests that the O-PS components of LPS are necessary for secretion of leukotoxin in *A. actinomycetemcomitans*. Intact O-PS appear to be important for the maintenance of an environment for the membrane integration of a competent TolC-dependent leukotoxin secretion apparatus.

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