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Abstract

Aggregatibacter actinomycetemcomitans, a common inhabitant of the human upper aerodigestive tract, produces a repeat in toxin (RTX), leukotoxin (LtxA). The LtxA is transcribed as a 114-kDa inactive protoxin with activation being achieved by attachment of short chain fatty acyl groups to internal lysine residues. Methyl esters of LtxA that were isolated from A. actinomycetemcomitans strains JP2 and HK1651 and subjected to gas chromatography/mass spectrometry contained palmitoyl (C16:0, 27-29%) and palmitolyl (C16:1 cis Δ9, 43-44%) fatty acyl groups with smaller quantities of myristic (C14:0, 14%) and stearic (C18:0, 12-14%) fatty acids. Liquid chromatography/mass spectrometry of tryptic peptides from acylated and unacylated recombinant LtxA confirmed that Lys^{562} and Lys^{687} are the sites of acyl group attachment. During analysis of recombinant LtxA peptides, we observed peptide spectra that were not observed as part of the RTX acylation schemes of either Escherichia coliα-hemolysin or Bordetella pertussis cyclolysin. Mass calculations of these spectra suggested that LtxA was also modified by the addition of monohydroxylated forms of C14 and C16 acyl groups. Multiple reaction monitoring mass spectrometry identified hydroxymyristic and hydroxypalmitic acids in wild-type LtxA methyl esters. Single or tandem replacement of Lys^{562} and Lys^{687} with Arg blocks acylation, resulting in a >75% decrease in cytotoxicity when compared with wild-type toxin, suggesting that these post-translational modifications are playing a critical role in LtxA-mediated target cell cytotoxicity. © 2011 John Wiley & Sons A/S.

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Aggregatibacter actinomycetemcomitans leukotoxin is post-translationally modified by addition of either saturated or hydroxylated fatty acyl chains

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SUMMARY

***Aggregatibacter actinomycetemcomitans*, a common inhabitant of the human upper aerodigestive tract, produces a repeat in toxin (RTX), leukotoxin (LtxA). The LtxA is transcribed as a 114-kDa inactive protoxin with activation being achieved by attachment of short chain fatty acyl groups to internal lysine residues. Methyl esters of LtxA that were isolated from *A. actinomycetemcomitans* strains JP2 and HK1651 and subjected to gas chromatography/mass spectrometry contained palmitoyl (C16:0, 27–29%) and palmitolyl (C16:1 cis Δ9, 43–44%) fatty acyl groups with smaller quantities of myristic (C14:0, 14%) and stearic (C18:0, 12–14%) fatty acids. Liquid chromatography/mass spectrometry of tryptic peptides from acylated and unacylated recombinant LtxA confirmed that Lys^{562} and Lys^{687} are the sites of acyl group attachment. During analysis of recombinant LtxA peptides, we observed peptide spectra that were not observed as part of the RTX acylation schemes of either *Escherichia coli* α-hemolysin or *Bordetella pertussis* cyclolysin. Mass calculations of these spectra suggested that LtxA was also modified by the addition of monohydroxylated forms of C14 and C16 acyl groups. Multiple reaction monitoring mass spectrometry identified hydroxymyristic and hydroxypalmitic acids in wild-type LtxA methyl esters. Single or tandem replacement of Lys^{562} and Lys^{687} with Arg blocks acylation, resulting in a >75% decrease in cytotoxicity when compared with wild-type toxin, suggesting that these post-translational modifications are playing a critical role in LtxA-mediated target cell cytotoxicity.**

INTRODUCTION

Production and secretion of protein virulence factors can be observed in many bacterial pathogens. Leukotoxin (LtxA, 114 kDa) is produced by *Aggregatibacter actinomycetemcomitans*, a member of the family Pasteurellaceae and a common inhabitant of the upper aerodigestive tract of man and higher primates. As an RTX (Repeats in ToXin) toxin, LtxA shares with other members of the toxin family a

common operon organization and the functional domains of its structural toxin gene product. LtxA is synthesized as an inactive protoxin from the *ltxA*, which is part of a four-gene operon (*ltxC*, *ltxA*, *ltxB*, *ltxD*). The LtxB and LtxD, along with TdeA (Crosby & Kachlany, 2007), a TolC homolog (Balakrishnan *et al.*, 2001), form a type I secretion system that facilitates the secretion of the toxin from the bacterial cytoplasm directly into the extracellular environment.

Both gram-positive and gram-negative bacteria are capable of producing protein toxins; gram-positive organisms translate an active toxin protein whereas toxin proteins produced by gram-negative bacteria are biologically inactive protoxins that undergo a subsequent modification to exert an effect on the host. The most common post-translational modification is either C-terminal or N-terminal proteolytic cleavage that produces a catalytic fragment or facilitates oligomerization and subsequent membrane damage. The RTX toxins share a different mechanism of activation whereby acyl groups are covalently linked to internal lysine residues of RtxA through amide linkages (Hardie *et al.*, 1991; Issartel *et al.*, 1991; Hackett *et al.*, 1994).

Based upon the acylation studies of other RTX toxins such as *Escherichia coli* α-hemolysin (HlyA) and *Bordetella pertussis* CyaA the requirement for post-translational modification to achieve toxin maturation is absolute (Betsou *et al.*, 1993; Stanley *et al.*, 1994; Hackett *et al.*, 1995; Masin *et al.*, 2005); there is variability as to the number and length of the fatty acid chains found with the various RTX toxins. *In-vivo*-activated CyaA is modified by attachment of a palmitoyl group (C16:0) on Lys^{983} (Hackett *et al.*, 1994, 1995; Basar *et al.*, 1999). However, when CyaA is expressed recombinantly in *E. coli*, with CyaC, the recombinant CyaA is now acylated at two sites, Lys^{983} and an additional site, Lys^{860} (Hackett *et al.*, 1995). HlyA is modified at Lys^{564} and Lys^{690} with one of several C14:0, C15:0 and/or C17:0 carbon amide-linked side chains (Lim *et al.*, 2000). A combination of two HlyA acylation sites and three fatty acids suggests that *in vivo*, HlyA is not a single molecular species but a heterogeneous family of toxin molecules. The promiscuity observed with HlyA fatty acid modifications suggests that at least some wild-type RTX may exhibit structural heterogeneity; however, the significance of this observation as it relates to microbial pathogenesis of individual toxins is not currently understood.

In this series of studies we identified the fatty acyl sites of *A. actinomycetemcomitans*, LtxA as Lys^{562} and Lys^{687}. Both of these residues are within classic Gly-rich acylation motifs. Proteolytic fragments of LtxA were then analysed using reverse-phase liquid chromatography-tandem mass spectrometry (LC-MS/MS). Fatty acyl species were identified and quantifed using gas chromatography/mass spectrometry (GC/MS) with electrospray ionization and label-free LC-MS/MS. Four different sources of LtxA were compared; a recombinant LtxA that was expressed in tandem with LtxC, LtxA expressed in the absence of LtxC (LtxAΔC) and wild-type LtxA purified from *A. actinomycetemcomitans* strains JP2 and HK1651. These analyses identified not only the expected 14-carbon and 16-carbon fatty acyl moieties but also a novel set of monohydroxylated acyl derivatives.

METHODS

Bacterial strains, growth conditions and plasmids

Aggregatibacter actinomycetemcomitans strains, JP2 (Tsai *et al.*, 1984) and HK1651 (Haubek *et al.*, 1997) were grown for 24 h in 4 l AAGM medium (Fine *et al.*, 1999) with distilled H_2O at 37°C in an atmosphere of 5% CO.

Production of recombinant *Ec*-LtxA has been described previously (Lally *et al.*, 1994; Korostoff *et al.*, 1998). Briefly, *E. coli*, strains AR120 and pSKF201 (pOTS-*Nco*I/pMG1) (Devare *et al.*, 1984; Shatzman & Rosenberg, 1987; Shatzman *et al.*, 2001) expression vectors were used for DNA manipulation and for expression of both wild-type and mutant LtxA. The plasmid cloning vehicle is derived from pKC30 (Rosenberg *et al.*, 1983) and uses the bacteriophage A promoter (λp_L) and is carried in the lysogenic host, AR120. In addition to providing a strong, tightly controlled promoter, the system also ensures that P_i directed transcription efficiently traverses the entire *ltxC*/*ltxA* gene construct by providing the phage A anti-termination function, *N*, and a site for *N* utilization (*Nut* site) within the P_i, transcription unit. The *E. coli* transitional regulatory efficient ribosome recognition and translation sites were also engineered into the P_L transcription unit (Shatzman *et al.*, 2001). The *E. coli* strain AR120 containing pSKF201/*ltx*CA was grown at 37°C in Luria–Bertani medium supplemented with 0.1 mg ampicillin ml^{-1}. After the cultures had

grown to an optical density at 500 nm of 0.4, nalidixic acid (Sigma, St. Louis, MO) was added to a final concentration of 60.00 μg ml^{-1} to induce expression of the *ltx* genes. Nalidixic acid induces the endogenous *E. coli* SOS response, resulting in cleavage of the *c*I repressor by the RecA protein and induction of expression. Induced cultures were grown for an additional 4 h, at which time the bacteria were pelleted, washed with cold phosphate-buffered saline, divided into aliquots and stored at −20°C before sonication.

Production and purification of LtxA

Wild-type LtxA and *Ec*-LtxA recombinant proteins were purified from ammonium sulfate precipitates (32.5%, 4°C, 1 h) from bacterial culture supernatants (Kachlany *et al.*, 2002) and sonic extracts, respectively. The precipitate was then recovered by centrifugation (15 146 **g**, 20 min), resuspended in 10 mM PO_4, pH 6.5 and dialysed overnight. After dialysis, the supernatant was filtered, diluted to 200 ml in 10 mM phosphate buffer (pH 6.5), and applied to a HiTRAP® SP column (GE Healthcare, Piscataway, NJ) that had been equilibrated in the same buffer. After sample application, the column was washed with 10 mM phosphate buffer, pH 6.5 until the optical density at 280 nm returned to background. Following sample application, the buffer was changed to 20% NaCl in 10 mM phosphate buffer, pH 6.5 and washed until the protein level was again at background level. LtxA was eluted by increasing NaCl to 60% in 10 mM phosphate buffer pH 6.5. Purity of LtxA was confirmed by cytotoxicity, Western blotting and sodium dodecyl sulfate–polyacrylamide gel electrophoresis and quantified by the Bio-Rad® Protein Assay (Bio-Rad Laboratories, Hercules, CA). A typical yield of toxin was 200 μg l^{-1} supernatant.

Analysis of LtxA acylation by LC-MS/MS

The location and identity of the acyl modifications were determined by LC-MS/MS on an LTQ-Orbitrap XL™ mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled with a NanoAcquity™ UPLC™ system (Waters, Milford, MA). Purified LtxA was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and gel bands containing the protein were excised using a scalpel and digested in-gel with trypsin (Speicher *et al.*, 2000). Tryptic peptides were separated by reversed phase on a nanocapillary column, 75 μm inner diameter × 25 cm PicoFrit (New Objective, Woburn, MA), packed with MAGIC C18 resin, 3-μm particle size (Michrom BioResources, Auburn, CA). Solvent A was 0.1% formic acid in Milli-Q water, and solvent B was 0.1% formic acid in acetonitrile. Peptides were eluted at 200 nl min^{-1} using an acetonitrile gradient consisting of 3–28% B over 42 min, 28–50% B over 25.5 min, 50–80% B over 5 min, 80% B for 4.5 min before returning to 3% B in 1 min. Duplicate LC-MS/MS analyses were performed on each tryptic sample. Data were acquired on the LTQ-Orbitrap XL™ with data-dependent acquisition and FT master scan preview enabled. Each survey scan on the Orbitrap (*m*/*z* 400–2000, at 60,000 resolution) was followed by six MS/MS scans with 30-s dynamic exclusion on the ion trap. Multiple charged ions with intensity values above 1000 counts were selected for MS/MS sequencing. Peptide sequences were interpreted from MS/MS spectra by searching against a custom database consisting of human and *A. actinomycetemcomitans* protein sequences using BioWorks®, version 3.3.1, SP1 (Thermo Fisher Scientific). All database searches allowed for up to two missed cleavages, a fixed modification of Cys with iodoacetamide, variable oxidation of methionine, and variable acylation of lysine with saturated acyl groups from 10 to 18 carbons. Outputs from BioWorks® searches were filtered using mass tolerance of 10 ppm and $\Delta Cn \geq 0.05$, and identified acylated peptides were manually verified. The composition of the identified acyl groups was also determined and confirmed using an elemental composition calculator (http://www.wsearch.com.au).

Label-free LC-MS/MS quantification

Quantification of LtxA tryptic peptides was performed from the area of the LC-MS peaks. LC-MS/MS RAW files were imported and processed using Elucidator® v3.1 (Rosetta Biosoftware®, Seattle, WA). Retention time alignment across the chromatographic runs, and feature (MS peak with uniquely defined retention time and *m*/*z* values) identification were performed using the PeakTeller® algorithm. The intensity value for each acylated LtxA peptide was obtained from the aligned dataset at the charge group level. Peaks with fewer than 2000 counts were manually examined using BioWorks® and were only considered to be present if they

were observed in both replicate runs. To correct for variation in sample load, the intensity values were normalized to a factor calculated for each sample based on the intensity values of eight non-acylated LtxA peptides (Fig. S1). Quantification of acylated peptides with C10:0–C14:0 acyl chains was compared based on the assumption that the minor difference of two to four CH_2 units would not significantly alter the ionization potential of the acylated peptides.

Identification of LtxA methyl esters

Samples for GC/MS analysis were prepared from JP2 and HK1651, as described above. The fatty acyl species associated with the LtxA toxin preparation were isolated in the chloroform phase using a chloroform/methanol extraction procedure (Bligh & Dyer, 1959). The lipids were dried under nitrogen and resuspended in methanol before analysis. Fatty acid profiles of each preparation were acquired using capillary gas chromatography (Clarus® 600 Gas Chromatograph/Mass Spectrometer; PerkinElmer®, Waltham, MA) with electron impact ionization mass spectrometry detection and compared with a standard of fatty acid methyl esters (C11:0 through to C20:0 including six monohydroxylated species) all of known identity, total content and common to bacterial extracts, with formulation of known identity and total content. Methyl esters of the samples were prepared initially at 30 mg protein per 1.0 ml, and 1 μl of extract was injected.

Identification and quantification of hydroxylated fatty acids in LtxA extracts using multiple reaction monitoring mass spectrometry

Fatty acyl species associated with the purified LtxA were isolated in the chloroform phase as described above and dried protein extracts were dissolved with 1.0 ml HPLC grade methanol. Samples were assayed by triple quadrupole mass spectrometry on an API 4000 QTrap® (Applied Biosystems® Inc., Foster City, CA) in negative ionization mode. The $[M^+CH_3COO]^-$ ion adducts of C14-OH and C16-OH were monitored by detection of their respective $[M\text{-}H]^-$ fragments using multiple reaction monitoring (MRM). The optimized detection, fragmentation parameters and chromatography were developed using an authentic standard of 3R-hydroxymyristic acid (Avanti Polar Lipids, Alabaster, AL). The sample was injected onto a Zorbax® (E.I. du Pont de Nemours & Co., Wilmington, DE) Eclipse XDB-C18, 2.1 × 50 mm, 1.8-μm column (E.I. du Pont de Nemours & Co.), with gradient elution of 10 mM NH_4OAC + 0.01%/v NH_4OH in water to 10 mM NH_4OAC + 0.01%/v NH_4OH in methanol from 50 : 50 to 100% over 10 min at a flow rate of 200 μl min^{-1} from a Dionex® 3000U HPLC system (Dionex Corporation, Chelmsford, MA). The detected compounds and C14:0-OH and C16:0-OH values were quantified from a linear curve of C14:0-OH.

Determination of leukotoxic activity

Leukotoxic activity of recombinant *ltxA* gene products was determined by bioassay as described previously (Lally *et al.*, 1994). Sonicates were prepared as described above and co-cultured with Jurkat cells in RPMI–1640 containing 10% fetal calf serum, 1% glutamine, 1% minimal essential medium vitamin solution (Life Technologies, Inc., Carlsbad, CA), 1% minimal essential medium non-essential amino acid solution (Life Technologies, Inc.), and 50 μg ml^{-1} gentamycin at 37°C under 5% CO_2. Before use, target cells were washed twice in RPMI-1640 to remove gentamycin and suspended in RPMI-1640 at 4×10^6 cells ml^{-1}. Then 50 μl of cells were added to sonicated bacterial supernatant (50 μl), and the suspensions were incubated at 37°C overnight. Negative controls consisted of target cells incubated in RPMI-1640 and sonicates that contained the plasmid without an insert. The cells were placed on ice, 100 μl trypan blue (0.4%) was added, and surviving cells were counted in a hemocytometer. At least four fields were counted in triplicate and averaged for each dilution assayed. Percent lysis was calculated by dividing the number of surviving cells by the number of cells in RPMI-1640 and subtracting from 100 (Brogan *et al.*, 1994).

Site-directed mutagenesis of *ltxA*

A series of acylation mutants was constructed by changing the appropriate lysine to arginine in the *ltx*A gene. The mutated *ltxA* gene was then expressed in tandem with *ltx*C by incorporating the mutated A gene cassette into an *ltx*C/*ltx*A expression system (Lally *et al.*, 1994). Ec-*ltx*A K562R was constructed using the mutation-specific primer CGG AAA ACA CGT CAG TCA GGTCGA TAT GAA TTT ATT ACT GAA TT using

the Sculptor™ *in vitro* mutagenesis system (Amersham Life Science, Arlington Hts, IL) according to the manufacturer's instructions. Ec-*ltx*A K687Rwas obtained by polymerase chain reaction using forward primer GGA TCC GGT TCA ACA ATA GTT AAT GCT and reverse mutation-specific primer GGT AAC CCT CCC TCG TTT AGT TTC TTG CTT and cloning the fragment into the *Bam*HI/*Bst*EII sites of *ltx*A. The double Ec-*ltx*A K562R/K687R mutant was obtained by replacing the *Bam*HI/*Sst*I fragment of Ec-*ltx*A K562R with the corresponding fragment of Ec-*ltx*A K687R.

The C gene was deleted via polymerase chain reaction using forward and reverse primers CAT CAA ATT AAA CCA CAC CTA TGG TGT ATG CAT and TAT GGT ACC TGC CAT ATT AAA TCT CCT TGT TAA TAA TTT AT to place a *Kpn*I site into our pOTS/pMG1 vector and using the *Bst*XI/*Kpn*I pOTS/pMG1 vector fragment to replace the C gene cassette in our *ltx*C/*ltx*A expression system.

RESULTS

Identification of fatty acyl modificationsto LtxA

LtxA is synthesized as a 114-kDa inactive protoxin. Analogous to other members of the RTX toxin family, LtxA is then post-translationally modified at lysine residues with the addition of fatty acyl groups. Previous studies of other RTX toxins have identified fatty acyl moieties of C14:0, C15:0, C16:0 and C17:0 associated with these toxins (Basar *et al.*, 1999; Lim *et al.*, 2000). Methyl esters of LtxA isolated from *A. actinomycetemcomitans* strains, JP2 and HK1651, prepared with the addition of sodium methoxide, were subjected to GC/MS analysis using capillary chromatography with electron impact ionization. A bacterial fatty acid methyl ester standard (Fig. 1, BAME) was used for retention and spectral comparison. Lipids associated with the two LtxA strains (JP2 and HK1651) identified by GC/MS were found to include fatty acyl groups corresponding to C14:0 (Fig. 1 – A and A′; elution time 32.4 min, 14%), C16:0 (Fig. 1 – B and B′; elution time 36.9 min, 27–29%), C16:1 (Fig. 1 – C and C′; elution time 37.5 min, 44–43%), C18:0 (Fig. 1 – D and D′; elution time 41.0 min, 12–14%) and C20:0 (Fig. 1 – E and E′; elution time 44.9 min) carbons. In the analysis of the data, Peaks B and B' appeared to contain a peak and a shoulder, suggesting that it was a mixture of a C16:0 fatty acid and a heretofore uncharacterized acyl group. Acyl groups with C15:0 and C17:0 were not detected in either JP2 or HK1651 preparations.

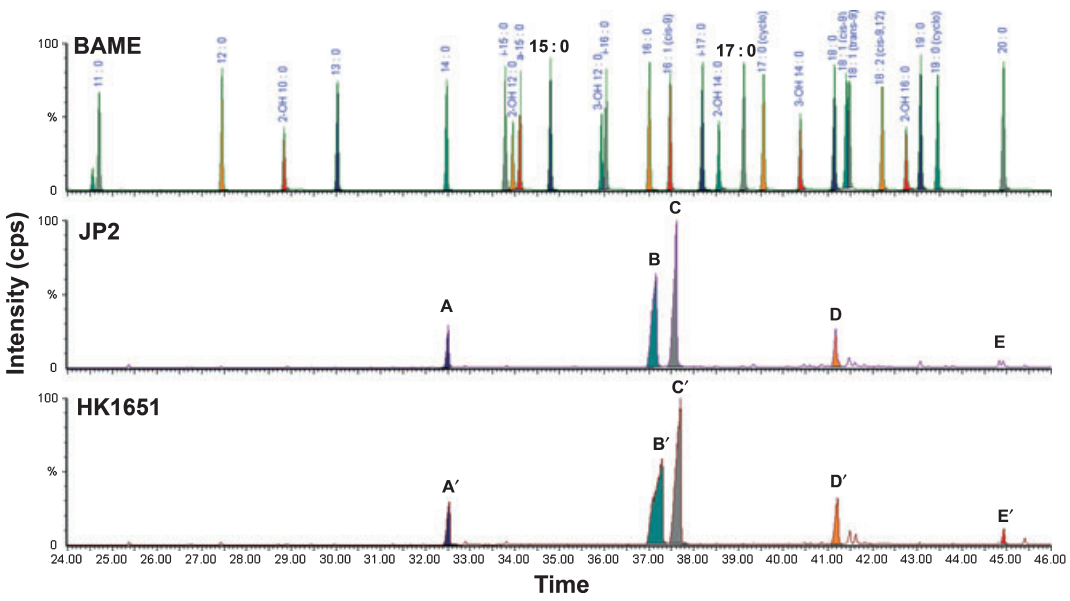

Figure 1 Chromatogram comparison of leukotoxin A (LtxA) isolated from *Aggregatibacter actinomycetemcomitans*, strains JP2 and HK1651 at 20× concentration to a bacterial fatty acid methyl ester standard (BAME). Methyl esters of the standards and samples were prepared by the addition of sodium methoxide and incubation. BAMEs were extracted into hexane and 1 μl was injected. Gas chromatography/mass spectrometry data were acquired using a capillary column (DB-225MS) with electrospray ionization. Both samples consist of C14:0, C16:0, C16:1, C18:0 and C20:0 fatty acids.

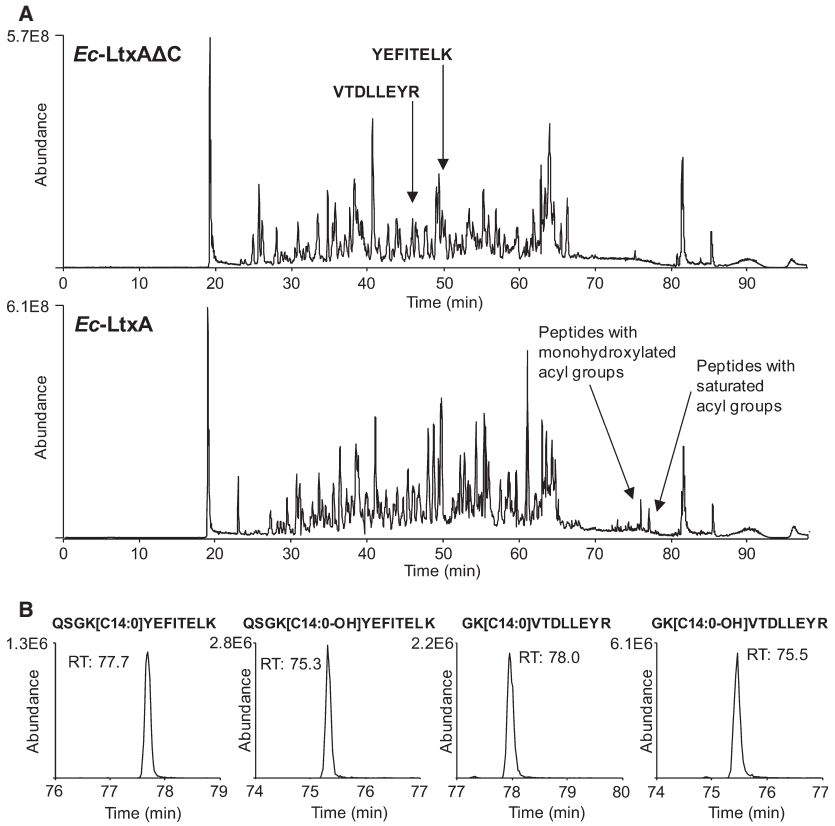

Figure 2 Reverse phase liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis of recombinant leukotoxin A (LtxA) proteins. (A) Total ion chromatograms of *Ec*-LtxAΔC and *Ec*-LtxA tryptic digests. The tryptic peptides VTDLLEYR and YEFITELK were observed predominately in *Ec*-LtxAΔC because of the unacylated preceding Lys residues. Acylated peptides were detected in *Ec*-LtxA, and the positions of the major acylated forms of QSGK^{562}YEFITELK and GK^{687}VTDLLEYR are shown. (B) Extracted ion chromatograms of doubly charged ions corresponding to the major acylated peptides from *Ec*-LtxA. These acylated peptides were not detected in any significant amount in *Ec*-LtxAΔC.

LtxA Lys^{562} and Lys^{687} are post-translationally modified

Acylation sites of LtxA were determined by LC-MS/MS analysis of recombinant toxin peptides following trypsin proteolysis. LtxA contains 16 GlyLys motifs and a sequence alignment analysis with HlyA had predicted that Lys^{562} and Lys^{687} are potential acylation sites (Stanley *et al.*, 1994; Ludwig *et al.*, 1996). Acylated peptides containing Lys^{562} and Lys^{687} were identified in trypsin digests of *Ec*-LtxA, but not in *Ec*-LtxAΔC (Figs 2 and 3A). Lys^{562} was located within the acylated peptide, QSGK^{562}YEFITELK of *Ec*-LtxA, indicating that acylation of this residue had rendered it insensitive to trypsin digestion. QSGK^{562}YEFITELK was not detected in *Ec*-LtxAΔC peptide digests as the K^{562} site was cleaved to yield QSGK^{562}, which is too small to be detected by our LC-MS/MS method and YEFITELK, which was detectable.

Three different saturated acyl modifications were found to be associated with Lys^{562}. Acylated QSGK^{562}YEFITELK peptides were detected at retention times of 72.0, 75.1 and 77.7 min with MH$^+$ (observed mass of the protonated peptide) of 1596.90, 1624.93 and 1652.96, respectively (Table 1). The increase in mass by 154.1, 182.2 and 210.2 Da indicates the addition of an acyl group C10:0, C12:0 and C14:0, respectively. This is confirmed by MS/MS fragmentation of these peptides, which showed that the mass increase occurred on the Lys^{562} residue. For example, the MS/MS spectrum of the peptide that eluted at 77.7 min contained b- and y-ions that correspond to the sequence of acylated QSGK^{562}YEFITELK, and the mass difference between y8 and y9 ions (210.2 Da greater than

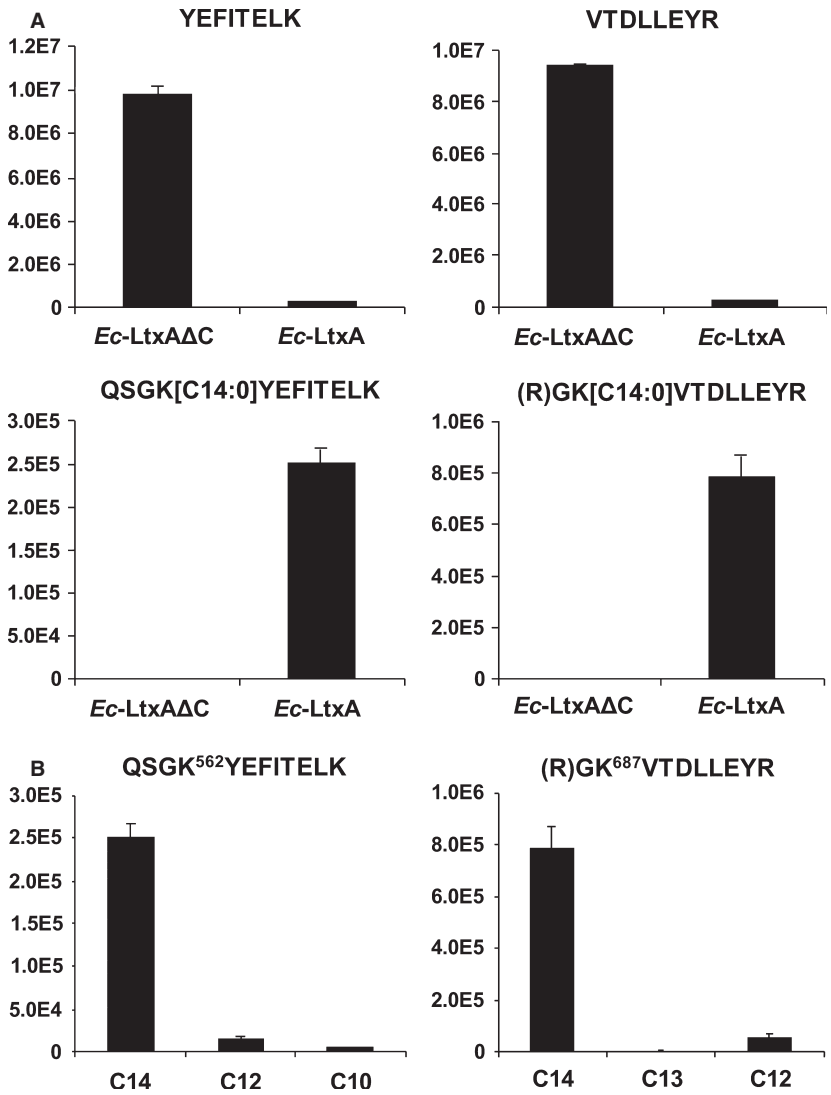

Figure 3 Label-free quantification of acylated peptides from recombinant leukotoxin A (LtxA) proteins. (A) Bar charts showing the quantification of YEFITELK and VTDLLEYR representing unacylated K^{562} and K^{687} sites and the C14:0 acylated peptides from *Ec*-LtxAΔC and *Ec*-LtxA. (B) Quantification of the various acylation forms of K^{562} and K^{687}. Quantification was determined from peak areas of all charge states of the peptide. For K^{687}, the areas were determined from the sum of GK^{687}VTDLLEYR and RGK^{687}VTDLLEYR peaks. Error bars, standard deviation from replicate run.

the expected lysine residue mass) indicated the attachment of a C14-saturated acyl group to the ε-amino group of Lys^{562} (Fig. 4A). In like manner, analysis of peptides with retention times of 72.0 and 75.1 min indicated mass additions of 154.1 and 182.2 Da to the Lys^{562} residue confirming that these peptides contained C10:0 and C12:0 saturated acyl groups attached at Lys^{562} (Table 1; Fig. S2).

A second LtxA acylation site (K^{687}) is contained within the peptide sequence $K^{684}RGK^{687}$VTDLLEYR (Table 1). The LC-MS/MS analysis of *Ec*-LtxA trypsin digests indicated that K^{687} was identified within two forms of the same post-translationally modified peptide, GK^{687}VTDLLEYR and RGK^{687}VTDLLEYR because of the close proximity of Lys/Arg residues. Because peptides GK^{687}VTDLLEYR and RGK^{687}VTDLLEYR contain the same acylation site, the data were combined and represented as (R)GK^{687}VTDLLEYR.

Similar to acylated Lys^{562} peptides, multiple forms of acylated Lys^{687} peptides were observed. One of the acylated versions, GK^{687}VTDLLEYR and

Table 1 LtxA peptide fragments with saturated acyl groups

Sequence	R.T. (min)	Obs MH+	Unmodified MH+	Obs mass addition (Da)	Predicted composition	Acyl mass (Da)	Expected MH+	ppm
K562 peptide fragments								
Non-acylateci peptides								
YEFITELK	50.0	1042.5479	1042.5455	0.0024	None	0.0000	1042.5455	2.3
Peptides with saturated acyl groups								
QSGKYEFITELK	72.0	1596.8965	1442.7526	154.1439	C10H18O	154.1358	1590.8884	5.1
QSGKYEFITELK	75.1	1624.9281	1442.7526	182.1755	C12H22O	182.1671	1624.9187	5.2
QSGKYEFITELK	77.7	1652.9576	1442.7526	210.2050	C14H26O	210.1984	1652.9510	4.0
K687 peptide fragments								
Non-acylated peptides								
VTDLLEYR	45.9	1008.5384	1008.536	0.0024	None	0.0000	1008.5360	2.4
Peptides with saturated acyl groups								
GKVTDLLEYR	75.2	1375.8270	1193.6525	182.1745	C12H22O	182.1671	1375.8196	5.4
GKVTDLLEYR	78.0	1403.8567	1193.6525	210.2042	C14H26O	210.1984	1403.8509	4.1
RGKVTDLLEYR	71.9	1531.9275	1349.7536	182.1739	C12H22O	182.1671	1531.9207	4.4
RGKVTDLLEYR	75.2	1559.9591	1349.7536	210.2055	C14H26O	210.1984	1559.9520	4.6

R.T., retention time; Obs MH^+, observed mass of protonated peptide; unmodified MH^+, theoretical MH+ of the peptide without any acyl modification; Obs mass addition, difference between Obs MH^+ and unmodified MH^+; Acyl mass, theoretical mass of the acyl modification as shown in 'Predicated composition' column; ppm, mass error between Observed MH^+ and Expected MH^+ in parts per million.

RGK^{687}VTDLLEYR peptides had retention time of 78.0 and 75.2 min respectively, and both are 210.2 Da larger than the unmodified peptides (Table 1). The MS/MS spectrum of acylated GK^{687}VTDLLEYR (Fig. 4B) contained a b2 ion with a mass of 396.3 Da, which is consistent with the combined masses of Gly and Lys plus a saturated C14:0 acyl group. Further evidence that a C14:0 saturated acyl group is associated with Lys^{687} is provided by the MS/MS spectrum of acylated RGK^{687}VTDLLEYR that showed y8 and y9 ions with a mass difference of 210.2 Da larger than the Lys residue mass (Fig. 4C). Additional (R)GK^{687}VTDLLEYR peptides were detected at elution times 75.2 and 71.9 min, which upon analysis contained a C12:0 acyl chain attached to Lys^{687} (Table 1; Fig. S2). Interestingly, a C13:0 acylated GK^{687}VTDLLEYR peptide was also detected at retention time of 76.6 min (Fig. S2). Even though multiple saturated acyl groups were detected for both Lys^{562} and Lys^{687} peptides, C14:0 is the dominant acylation for both sites (Fig. 3B).

Identification of fatty acyl modifications to LtxA

During LC-MS/MS analysis of *Ec*-LtxA peptides, we observed additional QSGK^{562}YEFITELK and (R)GK^{687}VTDLLEYR peptide spectra that were neither part of our predicted peptide maps nor did they follow traditional saturated fatty acid acylation schemes described for other RTX toxins. Mass additions of 154.1, 182.2 and 210.2 Da to QSGK^{562}YEFITELK or (R)GK^{687}VTDLLEYR were consistent with C10:0, C12:0 and C14:0 modifications; however additional increases in mass of 170.1, 198.2 and 226.2 Da to these peptides were also observed (Table 2). Initially, the observed mass increases were believed to be the result of an increase in length of saturated carbon chains as similar modifications have been described for *E. coli* α-hemolysin (Lim *et al.*, 2000). However, the addition of the masses of saturated C11:0, C13:0 and C15:0 carbon chains to the peptides each led to a difference of 1.987 Da between the expected and observed masses, which is beyond the acceptable mass error of the Orbitrap® mass spectrometer. Furthermore, although these peptides exhibited greater masses than those with saturated hydrocarbon chains, their shorter retention times (Fig. 2 and Table 2) provided an initial indication that they are less hydrophobic than the saturated modifications identified above. An elemental composition calculator was employed to determine the molecular composition that best fitted the observed mass. These calculations suggested that LtxA is modified by the addition of $C_{10}H_{18}O_2$, $C_{12}H_{22}O_2$ and $C_{14}H_{26}O_2$, which are monohydroxylated forms of the saturated C10:0, C12:0 and C14:0 acylations. These peptides have a mass error of <5 ppm (Table 2). Similar to the saturated acyl modifications, C14:0-OH

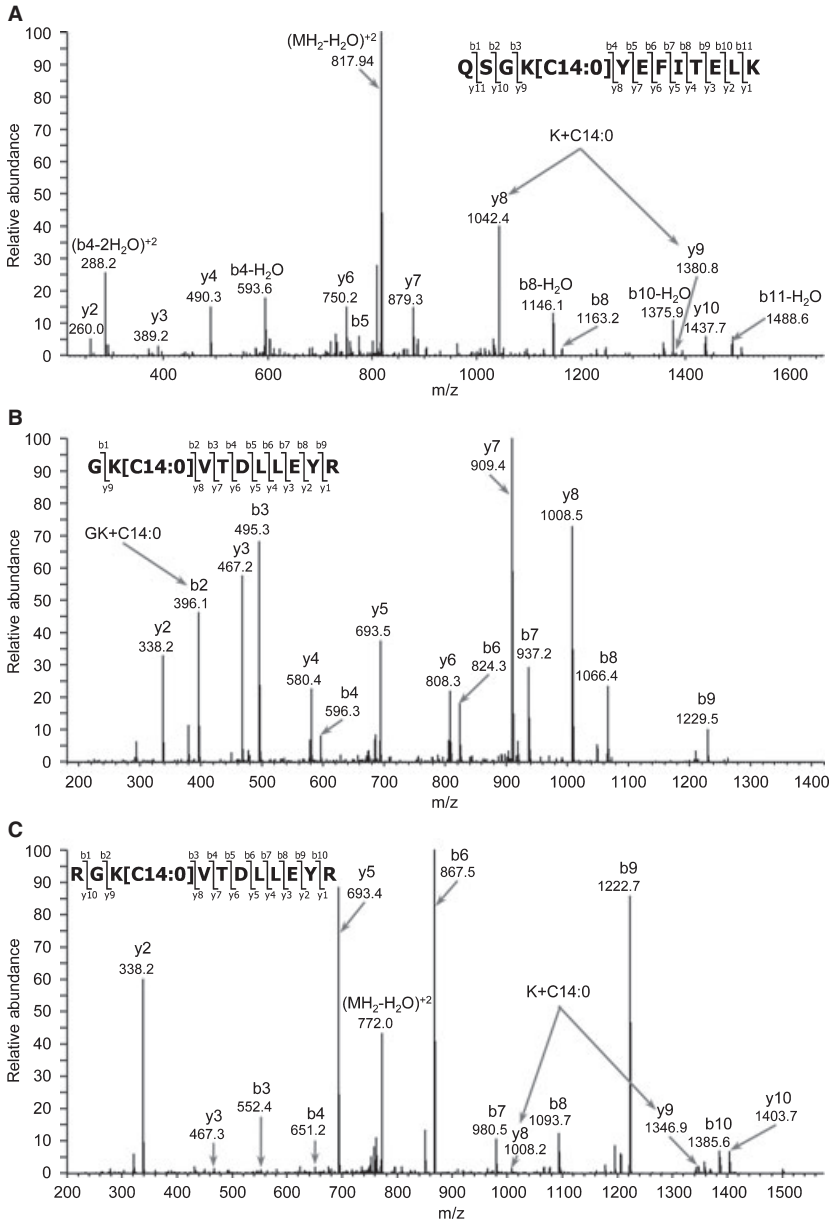

Figure 4 Tandem mass spectrometry (MS/MS) spectra of representative acylated peptides. (A) Acylated QSGK^{562}YEFITELK with precursor *m*/*z* 826.9824 and a charge state of +2. The mass difference between y8 and y9 ions indicates that K^{562} contains a C14:0 acyl group. (B) Acylated GK^{687}VTDLLEYR with precursor *m*/*z* 702.4320 and a charge state of +2. The mass of b2 ion corresponds to GK plus a C14:0 acyl group. (C) Acylated RGK^{687}VTDLLEYR with precursor *m*/*z* 780.4832 and a charge state of +2. The C14:0 acyl group is localized to K^{687}.

is the dominant form accounting for approximately 93 and 96% of Lys^{562} and Lys^{687} monohydroxylated acylation, respectively.

In addition to the monohydroxylated forms of the saturated acyl groups, trace amounts of monounsaturated and monohydroxylated acyl modifications were also identified by LC-MS/MS analysis of the *Ec*-LtxA sample (Table 2). Assuming that peptides of both groups have similar LC-MS ionizing efficiency, the amount of monounsaturated monohydroxylated acyl modification is <0.2% of the amount of saturated monohydroxylated modification. Even though the level of monounsaturated monohydroxylated acyl modification is low, the detection of this group further demonstrates the heterogeneity of LtxA acylation.

Table 2 LtxA peptide fragments with monohydroxylated acyl groups

Sequence	R.T. (min)	Obs MH+	Unmodified MH+	Obs mass addition (Da)	Predicted composition	Acyl mass (Da)	Expected MH+	ppm
K562 peptide fragments								
Peptides with monounsaturated and monohydroxylated acyl group								
QSGKYEFITELK	73.9	1666.9401	1442.7526	224.1875	C14H24O2	224.1776	1666.9302	5.9
Peptides with monohydroxylated acyl group								
QSGKYEFITELK	67.2	1612.8894	1442.7526	170.1368	C10H18O2	170.1307	1612.3833	3.8
QSGKYEFITELK	71.8	1940.9214	1442.7526	198.1688	C12H22O2	198.1620	1640.9146	4.2
QSGKYEFITELK	75.3	1668.9443	1442.7526	229.1917	C14H26O2	226.1933	1668.9459	−0.9
K687 peptide fragments								
Peptides with monounsaturated and monohydraxyloted acyl group								
GKVTDLLEYR	76.4	1445.8674	1193.6525	252.2149	C16H28O2	252.2089	1445.8614	4.1
RGKVTDLLEYR	70.1	1573.9351	1349.7536	224.1815	C14H24O2	224.1776	1573.9312	2.5
Peptides with monohydroxylated acyl group								
GKVTDLLEYR	66.7	1363.7878	1193.6525	170.1353	C10H18O2	170.1307	1363.7832	3.4
GKVTDLLEYR	71.7	1391.8187	1193.6525	198.1662	C12H22O2	198.1620	1391.8145	3.0
GKVTDLLEYR	75.4	1419.8494	1193.6525	226.1969	C14H26O2	226.1933	1419.8458	2.5
RGKVTDLLEYR	68.0	1547.9117	1349.7536	198.1581	C12H22O2	198.1620	1547.9156	−2.5
RGKVTDLLEYR	72.5	1575.952	1349.7536	226.1984	C14H26O2	226.1933	1575.9469	3.2

R.T., retention time; Obs MH^+, observed mass of protonated peptide; unmodified MH^+, theoretical MH^+ of the peptide without any acyl modification; Obs mass addition, difference between Obs MH^+ and unmodified MH^+; Acyl mass, theoretical mass of the acyl modification as shown in 'Predicted composition' column; ppm, mass error between Obs MH^+ and Expected MH^+ in parts per million.

Wild-type LtxA has similar type of acyl modifications to Ec-LtxA

Our results using the recombinant toxin suggested a heterogeneous distribution of acyl moieties; to confirm that this was also the case *in vivo,* toxin was isolated from overnight culture supernatants of two strains of *A. actinomycetemcomitans*, strains JP2 and HK1651. Both LtxA preparations contained the acylated QSGK^{562}YEFITELK and (R)GK^{687}VTDLLEYR peptides upon trypsinolysis and LC-MS/MS analysis, confirming that both Lys^{562} and Lys^{687} are post-transitionally modified by *A. actinomycetemcomitans in vivo*. No C13:0 acylation was detected in LtxA from JP2 and HK1651, but all other forms of acylation were detected. Peptides are modified by the addition of saturated (C10:0, C12:0, C14:0:0), monohydroxylated (C10:0-OH, C12:0-OH, C14:0-OH) or monounsaturated/monohydroxylated (C14:1-OH, C16:1-OH) acyl groups.

Wild-type LtxA also contains hydroxymyristic and hydroxypalmitic acid modifications

The identification of recombinant toxin molecules with monohydroxylated acyl group modifications provided an initial indication that one or several peaks we had observed in our initial GC/MS analysis of LtxA lipids (Fig. 1) might also represent the addition of monohydroxylated fatty acids to native leukotoxin. We used MRM, which is the most sensitive means available, to selectively detect species of hydroxymyristic (14:0-OH) and hydroxypalmitic (C16:0-OH) acids. A 14:0-OH standard was synthesized and subjected to LC-MS/MS analysis to determine the optimal LC and MRM transitions for measurement (data not shown). The detection of C14:0-OH was optimum when monitoring the MRM transition for $[M^+CH_3COO]^- \rightarrow [M^-H]^-$ (303.2/243.2 atomic mass unit [amu]) in negative ionization mode (Fig. 5). The MRM method was also expanded to monitor the $[M^+CH_3COO]^- \rightarrow [M^-H]^-$ transition for C16:0-OH (331.2/271.2 amu). Analysis of LtxA lipid extracts from *A. actinomycetemcomitans*, strains JP2 and HK1651, dissolved and injected under the same conditions of LC-MRM optimized with the standard showed that hydroxylated myristic and palmitic acids were detected in both samples (Fig. 5).

Acylation of K^{562} and K^{687} is critical to LtxA-mediated cytotoxicity

We hypothesized that preventing acylation at Lys^{562} and/or Lys^{687} might result in loss of LtxA function.

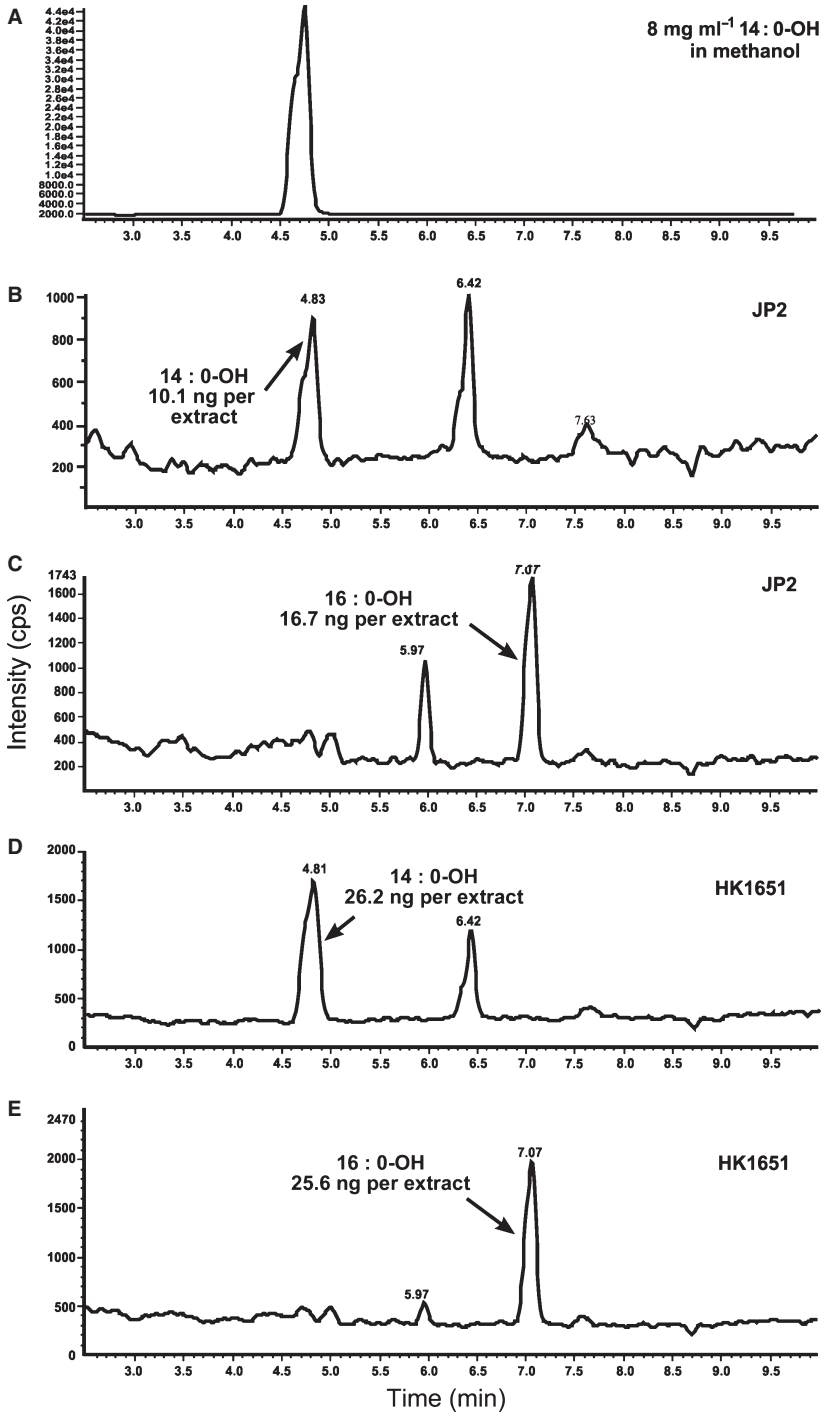

Figure 5 Identification of hydroxylated myristic and palmitic acids in leukotoxin A (LtxA) extracts from *Aggregatibacter actinomycetemcomitans*, strains JP2 and HK1651. Extracted ion chromatograms showing the multiple reaction monitoring (MRM) of $[M^+CH_3COO]^- \rightarrow [M^-H]^-$ for C14:0-OH (303.2/243.2 amu transition) and C16:0-OH (331.2/271.2 amu transition). The C14:0-OH standard was injected onto a reversed phase, Zorbax® Eclipse XDB-C18, 2.1 × 50 mm, 1.8 μm column to optimize the liquid chromatography and MRM conditions for measurement. The MRM method was subsequently expanded to monitor C16:0-OH. Dried samples of JP2 and HK1651 LtxA were then reconstituted in 1.0 ml HPLC-grade methanol and injected along with a calibration curve for C14:0-OH ranging from 0.05 to 12.6 μg ml^{-1}. Both C14:0-OH and C16:0-OH values were then calculated from the $y = mx$ curve of the C14:0-OH standard.

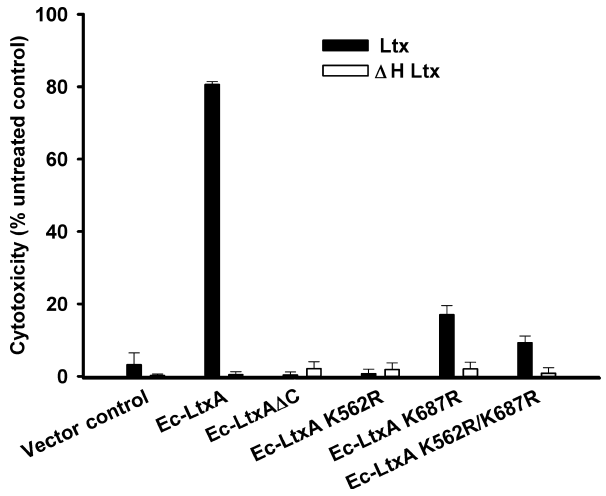

Figure 6 Acylation mutants of the *ltxA* gene were constructed by replacing K^{562} and K^{687} with R. The single mutations (K562R and K687R) and a double (K562R/K687R) mutant were ligated into the pOTS/pMG1 and expressed in tandem with *ltxC* in *Escherichia coli*. Ec-LtxAΔC was a leukotoxin A (LtxA) mutant where LtxA was expressed in pOTS/pMG1 in the absence of LtxC. Aliquots of each recombinant wild-type toxin were heat-inactivated (ΔH Ltx; 60°C) and serve as negative controls. Sonicates from each mutant and wild-type *ltxA* were assessed for cytotoxicity by a trypan blue exclusion assay using Jurkat target cells (Brogan *et al*., 1994).

To determine if Lys^{562} and Lys^{687} were critical to LtxA-mediated killing of target cells, acylation mutants were constructed in which Lys residues were replaced with Arg, which is not acylated. Both mutant and wild-type *ltx*A were then expressed in *E. coli*, sonicated, and tested for cytotoxicity by trypan blue exclusion assay (Brogan *et al*., 1994) using Jurkat target cells (Fig. 6). A bacterial sonicate containing wild-type *ltx*CA was then titrated to kill 80% of target cells. The cytotoxicity activity observed in this preparation could be completely inhibited by pre-heating the protein (60°C, 45 min) before assay. Cytotoxicity was not observed in sonicates containing LtxA that was expressed in the absence of *ltx*C (*Ec*-LtxAΔC). Mutation of Lys^{562} to Arg^{562} (Ec-LtxA K562R), Lys^{687} to Arg^{687} (*Ec*-LtxA K687R), or both residues (Ec-LtxA K562R/K687R) to Arg resulted in a loss of the ability to kill target cells.

DISCUSSION

Members of the RTX toxin family share an intracellular activation process whereby carbon amide-linked side chains are attached to the ε-amino group of internal lysine residues of the structural toxin gene product, RTXA. Acylation of eukaryotic and yeast proteins most commonly involves the attachment of the fatty acyl groups to the N-terminal α-amine of the protein in a reaction that involves covalent attachment of the fatty acid to the acyl coA transferase. The maturation of an RTX toxin such as LtxA is also an acylation process, but one that is both different and unique. The RTX acylation reaction involves the attachment of acyl groups to the ε-amino groups of internal lysine residues in a manner that does not involve coA as the fatty acid donor but rather by binding of the fatty acid to acylACP (Hardie *et al*., 1991; Issartel *et al*., 1991) in an ordered Bi-Bi reaction that involves the formation of a non-covalent ternary acylACP–HlyC–proHlyA complex (Stanley *et al*., 1999). AcylACP contains phosphopantetheine, which forms the prosthetic arm to which individual acyl chains are attached. Based upon *in vitro* acylation studies with *E. coli* HlyA the reaction is a sequential and ordered Bi-Bi reaction that involves the formation of a non-covalent ternary acylACP–HlyC–proHlyA complex. Whereas the bonding in has been described as 'tight' it is non-covalent perhaps allowing for some heterogeneity of different acyl groups.

The RTX lysines to which the fatty acyl groups are attached are highly conserved among the various toxin family members, leading Stanley *et al.* (1994) to predict that LtxA Lys^{562} and Lys^{687}would be modified in biologically active LtxA. Balashova *et al.* (2009) used MALDI-TOF to identify peptides containing unaltered Lys^{562} and Lys^{687} residues in an *ltx*C$^-$ *ltx*A mutant that were not observed in *A. actinomycetemcomitans*, strain JP2 LtxA, suggesting that these peptides are modified in wild-type LtxA. Our LC-MS/MS analysis identified a number of saturated acyl groups on the Lys^{562} and Lys^{687} residues. For each group of acylated peptides, the hydrophobicity of the peptide is expected to increase with increasing length of the acyl group. The observation that peptides with longer acyl groups had a later elution time on a reversed phase gradient further supports the LC-MS/MS acylated peptide identifications. Even though multiple saturated acyl groups of different lengths were identified for both Lys residues, the majority of the acylation as detected by LC-MS/MS, appeared to be C14:0. LC-MS/MS could not detect any C16:0 or higher acylation in contrast to GC/MS with electron impact ionization, which detected modifications of up to C20:0, The failure to

recover peptides with larger acyl groups is most likely the result of the strong interaction between these highly hydrophobic acylated peptides and the reversed phase resin used in the LC-MS/MS system resulting in poor elution and recovery.

Modification by addition of hydroxymyristic and hydroxypalmitic acyl derivatives has not been previously described and suggests that they may impart the unique biological properties of this toxin. Studies of either HlyA (Lin *et al.*, 1992) or CyaA (Hackett *et al.*, 1994) have found saturated acyl chains were being added to these RTX toxins. The addition of hydroxylated acyl chains may be simply an extension of the promiscuity brought about by ternary complex formation. The binding of acylACP to HlyC has been described as 'tight' but it is non-covalent (Stanley *et al.*, 1999), perhaps allowing for the acyl group heterogeneity we observe. Furthermore, studies with HlyC indicate that it does not preferentially select an acylACP carrying a specific fatty acid (Issartel *et al.*, 1991). It would therefore appear that LtxA acylation varies with the composition of the acylACP that is available. Variations noted in the hemolytic activity of secreted HlyA (Stanley *et al.*, 1993) and in the acylation profile of recombinant CyaA expressed in *E. coli* (Hackett *et al.*, 1994, 1995; Basar *et al.*, 1999). On the other hand hydroxylated acyl groups, specifically 3-hydroxypalmitic acid methyl ester, have been shown to be very potent autoregulator compounds controlling virulence in the phytopathogenic bacterium, *Ralstonia solanacearum* (Flavier *et al.*, 1997). Furthermore, 3-hydroxy fatty acids (16 or 18 carbons) with two conjugated double bonds found in Fijian green macroalgae (*Tydemania expeditionis*) have demonstrated moderate inhibitory activity against a panel of tumor cell lines (Jiang *et al.*, 2008).

We did not observe the attachment of either C15:0 or C17:0 acyl chains to either recombinant or wild-type LtxA. These additions had been reported with the transcription of native HlyA (Lim *et al.*, 2000). Odd-numbered carbon fatty acids are not part of the *E. coli* membrane composition (Neidhardt, 1996) nor do they appear to have a significant role in the endogenous fatty acid metabolism of the organism (Cronan & Rock, 2009). Although fatty acids with an odd number of carbons are found in the membranes of other gram-negative bacteria (O'Leary, 1962) and in other taxonomic groups, their occurrence in *A. actinomycetemcomitans* has not been reported. Recombinant expression of *hly*C in tandem with *ltx*A should prove useful in determining if the *E. coli* acylase can direct theC15:0 or C17:0 modifications that have been described. Based upon the current report it is also possible that HylA, like LtxA, contains hydroxymyristic and hydroxypalmitic acid additions. Although the mass differences between C15:0 and C14:0-OH or C17:0 and C16:0-OH are small (1.987 Da) the measurement of observed/expected charge differences of the HlyA peptides should indicate whether the HylA peptides contain saturated or hydroxylated acyl fatty acids.

In the current study, we have characterized the post-translational modifications that enable LtxA to lyse human immune cells. The exact role that these modifications play in LtxA-mediated cytotoxicity is a subject for future studies. Acylation has been shown to affect cell binding as well as cell lysis in other RTX toxin systems (Masin *et al.*, 2005) and several different models have been offered to explain the role of RTX toxins and their acyl groups in the lytic event. The simplest of these is the barrel stave pore whereby LtxA monomers oligomerize to form a protein-lined annulus of toxin 'staves' that produce little membrane disturbance beyond a pushing aside of the lipids by the protein (Baumann & Mueller, 1974; Yang *et al.*, 2001). As an alternative to the formation of transmembrane protein-lined pores, it has been suggested that RTX toxins may associate with the lipid head groups, and pore formation may occur through the bending of the lipid bilayer, yielding a pore lined with both protein and lipid (Martin *et al.*, 2004). A common feature of either model would be the necessity for a structural reordering of LtxA as it moves from an extracellular environment that is polar to the membrane interior, which is hydrophobic. In either case, fatty acyl modifications of LtxA would not simply provide a hydrophobic membrane anchor but would also provide a mechanism for bringing hydrophobic N-terminal LtxA domains into the proper juxtaposition with the membrane hydrocarbon of the target cell membrane to initiate damage (Charollais & van der Goot, 2009).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Determination of normalization factors to correct the variations in sample loading for quantification of leukotoxin A (LtxA) acylated peptides. Seven different sample preparations of LtxA (A to G), including those reported in this study, were digested with trypsin and analyzed by reverse phase liquid chromatography–tandem mass spectrometry (LC-MS/MS) in duplicate. The peak area (*y*-axis) of eight non-acylated LtxA tryptic peptides is shown. A normalization factor was determined for each sample from the sum of the eight peptide peak areas. Examples of the corrected sample loading using the normalization factor for the peptide HLSNSVGSTGNLTK and IGELAGITR are shown. Error bars, standard deviation from replicate LC-MS/MS analyses.

Figure S2. Tandem mass spectrometry (MS/MS) spectra of peptides with C10:0 and C12:0 on K^{562}, and C12:0 and C13:0 on K^{687}.

Figure S3. Tandem mass spectrometry (MS/MS) spectrum of the C14:0-OH synthetic standard. C14:0-OH was best detected by monitoring the $[M^+CH_3COO]^- \rightarrow [M^-H]^-$ transition (303.2/243.2 amu) in negative ionization mode.

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