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Inactivation of DNA adenine methyltransferase alters virulence factors in *Actinobacillus actinomycetemcomitans*

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DNA adenine methyltransferase (DAM) plays critical roles in diverse biological pathways in gram-negative bacteria, and specifically in regulating the expression of virulence genes in several organisms. *Actinobacillus actinomycetemcomitans* plays an important role in the pathogenesis of juvenile and adult periodontal disease, yet little is known about its mechanisms of gene regulation. DAM is shown here to directly or indirectly affect well-known *A. actinomycetemcomitans* virulence factors. A mutant *A. actinomycetemcomitans* strain lacking the *dam* gene was created by homologous recombination and shows normal growth phenotypes when grown exponentially. This mutant strain has four sixfold increased levels of extracellular leukotoxin, altered cellular levels of leukotoxin, and significant changes in bacterial invasion of KB oral epithelial cells. These results provide a basis for further characterization of regulatory mechanisms that control *A. actinomycetemcomitans* virulence.

Key words: *Actinobacillus actinomycetemcomitans*; DNA adenine methyltransferase; virulence

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Actinobacillus actinomycetemcomitans plays an important role in the pathogenesis of juvenile and adult periodontal disease (9, 37). The link between periodontal disease and systemic diseases (3, 35) further demonstrates the significance of *A. actinomycetemcomitans* as a pathogen. The success of *A. actinomycetemcomitans* as a causative agent in disease can be attributed to its arsenal of virulence determinants. These include a variety of surface-associated ultra-structures such as fimbriae and vesicles, which are involved with epithelial cell adhesion, invasion and transcytosis to deeper tissue, as well as the production of toxins (e.g. leukotoxin) (9).

Long-term studies using an *in vitro* model have revealed the fundamental nature of *A. actinomycetemcomitans* adhesion and invasion (9, 36). Invasion is a dynamic, multi-step process that involves entry via a host-derived vacuole, escape from the vacuole, multiplication and microtubule-mediated intra- and intercellular spread (36). Genes associated with the adhesion and invasion mechanisms of *A. actinomycetemcomitans* are just now being described. Adhesion of *A. actinomycetemcomitans* to epithelial cells involves an autotransporter (Aae) (30) that is homologous to the autotransporters of *Haemophilus influenzae* and *Neisseria* species (34). To date, two genes have been implicated in *A. actinomycetemcomitans* invasion. One shares homology with the *apaH* gene, which encodes an RGD sequence; this peptide binds integrin, the receptor for invasin-mediated *Yersinia pseudotuberculosis* mammalian cell entry (33). The *apaH* gene is a homolog of *ialA*, *ygdP* and *invA*, genes which are associated with invasion by *Bartonella bacilliformis*, *Escherichia coli* K1 and *Rickettsia prowazekii*, respectively (4, 13, 26). Invasion by *A. actinomycetemcomitans* has also been linked to *spa*, a gene involved in protein export from cells (9, 36). Factors that regulate the expression of genes involved in either adhesion or invasion have not been identified.

A. actinomycetemcomitans leukotoxin, a member of the RTX family of pore-forming hemolysins, is a well-studied potential virulence factor. The leukotoxin promoter architecture is well understood

(7) and leukotoxin expression is regulated by various environmental signals (15, 17, 27). The *tad* gene cluster, which is required for tight non-specific adherence by *A. actinomycetemcomitans*, is involved in the release of leukotoxin from the cell surface (17) and a suitable animal model was developed to demonstrate the importance of this non-specific adhesion locus to *A. actinomycetemcomitans* pathogenesis (31). However, little is known about how *A. actinomycetemcomitans* virulence determinants are coordinately regulated.

DNA methylation is an epigenetic, post-replicative modification catalysed by a large and broadly dispersed class of enzymes, DNA methyltransferases (MTases). DNA methylation has many biological roles, including the regulation of gene expression in both prokaryotes and eukaryotes (6, 21, 29). The majority of prokaryotic MTases have a cognate restriction endonuclease, but a small set of MTases lack such a partner. DAM (DNA adenine methyltransferase) is one such orphan enzyme, and is prevalent among enteric bacteria, such as *E. coli*, *Salmonella* species, *Yersinia* species and *Vibrio cholerae* (2). DAM enzymes have also been identified in the oral bacteria *Porphyromonas gingivalis* (2) and *A. actinomycetemcomitans* (8). Patterns of DNA methylation are crucial to the regulation of important cellular processes (5, 20, 22, 28), including the expression of various virulence genes. Significantly, a *Salmonella typhimurium* DAM mutant displays numerous cellular defects (12). Such mutants are also unable to colonize deep tissue in a murine model and are avirulent (14).

We previously identified and characterized the *A. actinomycetemcomitans* DAM (M.AacDAM) of strain Y4 (8). In an effort to further investigate the role of DAM in controlling the expression of *A. actinomycetemcomitans* genes in general, and virulence-related genes in particular, we constructed and characterized *A. actinomycetemcomitans* SUNY 465, which lacks the *M.AacDAM* gene. We show here that this knockout strain shows altered leukotoxin secretion and oral epithelial cell invasion, both of which are critical *A. actinomycetemcomitans* virulence determinants.

Materials and methods

Bacterial strains and KB cells

The bacterial strains used in this study are listed in Table 1. *A. actinomycetemcomitans* strains were cultured in Trypticase soy broth supplemented with 0.6% yeast extract (TSBYE) at 37°C in a humid atmosphere containing 10% CO_2 in air or in TSBYE supplemented with 0.8% dextrose and 0.4% sodium bicarbonate (AAGM), where indicated. VT1169, a nalidixic acid–rifampin-resistant *A. actinomycetemcomitans* strain, was derived from SUNY 465 (25). Strains of *E. coli* were grown in Luria–Bertani (LB) medium at 37°C with aeration. For solid medium, the liquid medium was supplemented with 15 g/l agar. All cells were kept frozen at −70°C in 10% dimethyl sulfoxide. The KB cells, an oral epithelial cell line, were derived from a human epidermoid carcinoma. Cells were maintained in RPMI-1640 medium (Sigma Chemical Co., St Louis, MO) supplemented with 5% fetal bovine serum (Gibco, Grand Island, NY) and 50 μg/ml gentamicin (Sigma). KB cells were cultured in 75-cm^2 flasks in a humidified atmosphere of 5% CO_2 in air. Cultures were split by treatment with 0.02% EDTA (Sigma) followed by trypsin (Gibco) to detach cells. Antibiotic-free medium was used in adhesion or invasion assays.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics	Source
Strains		
A. actinomycetemcomitans		
SUNY 465	Clinical isolate, smooth phenotype, invasive	(24)
VT 1169	Derivative of SUNY 465, rif^r, nal^r	(24)
VT 1560	Derivative of VT1169; *dam*$^-$, rif^r, nal^r	This study
E. coli		
VT1461	Harbors pVT1461; sp^r	(24)
VT1541	Harbors pVT1541; *dam*$^-$, sp^r, k^{mr}	This study
DH5α*λpir*	Cloning host for mobilizable plasmids	Lab. stock
SM10*λpir*	Conjugation host for mobilizable plasmids	Lab. stock
Plasmids		
pVT1461	Mobilization vector; sp^r	(24)
pVT1541	Derivative of pVT1461; *dam*$^-$, sp^r, k^{mr}	This study

rif^r, rifampin resistant; nal^r, nalidixic acid resistant; sp^r, spectinomycin resistant; kn^r, kanamycin resistant.

Cloning of *A. actinomycetemcomitans* SUNY 465 *dam*

S. typhimurium dam was used to probe the University of Oklahoma *A. actinomycetemcomitans* strain HK 1651 genome database. A contig with sequence homology to *dam* was identified. Primers (aaaagcggacattgtggcac and ccaacaacaaagcgccccacgcag) flanking the *A. actinomycetemcomitans dam* homolog were used to amplify a 1.5-kilobase (kb) fragment of *A. actinomycetemcomitans* SUNY 465. The amplified fragment, sequenced at the University of Vermont Sequence Facility, contained a complete 855-nucleotide open reading frame for the *dam* gene of *A. actinomycetemcomitans* SUNY 465.

Construction of a *M.AacDAM* minus *A. actinomycetemcomitans* strain by allelic replacement

The 1.5-kb polymerase chain reaction (PCR) fragment with the *dam* flanking regions was cloned into pGEM-T Easy according to the manufacturer's instructions (Promega, Madison, WI). The resulting plasmid was digested with a unique restriction enzyme *Sgr*AI, which cuts only once within *dam*. The restricted fragment was ligated with a kanamycin cassette isolated from pUC4k. The ligation mixture was transformed into *E. coli* DH5α to isolate and amplify a plasmid that contained the disrupted *dam* fragment and the pGEM-T Easy vector backbone. The 3.3-kb inactivated *A. actinomycetemcomitans dam* fragment was restricted from pGEM-T Easy and ligated into the *Eco*RI site of the previously constructed mobilization vector, pVT1461 (24). The resulting construct, pVT1541 was transformed into *E. coli* SM10λpir. All constructs were confirmed by restriction mapping and sequence analysis.

Mobilization of pVT1541 from *E. coli* SM10λpir to the nalidixic acid–rifampin-resistant *A. actinomycetemcomitans* strain, VT1169, was accomplished by conjugation as previously described (24, 25). Kanamycin-resistant colonies were obtained and replica-plated onto TSBYE agar containing spectinomycin (50 μg/ml). Several clones that were kanamycin resistant and spectinomycin susceptible were selected as putative allelic replacement transconjugants.

Southern blot analysis

Confirmation of the disruption of *dam* in *A. actinomycetemcomitans* was obtained by Southern blot analysis. Chromosomal DNA was isolated using the Puregene

DNA Extraction Kit (Gentra Systems, Minneapolis, MN) and restricted with *Eco*RI; the fragments were separated by electrophoresis on a 0.8% agarose gel. The DNA fragments were transferred to Hybond nylon membranes (Amersham Life Sciences, Buckinghamshire, UK), which were treated according to the standard recombinant DNA methods. DNA was immobilized by ultraviolet light fixation. DNA probes were labeled and hybridized with the DNA according to the enhanced chemiluminescence (ECL™) direct nucleic acid labeling and detection system (Amersham Life Sciences). Fragments hybridizing with the probes were visualized using the ECL™ detection system and exposure to photographic film (XAR-5; Eastman Kodak, Rochester, NY).

Construction and analysis of VT1560 complemented with a functional *dam* (strain VT1560/*ltxP-dam*)

The leukotoxin promoter (*ltxP*) driven GFP reporter plasmid pVT1303(*ltxP-gfp*/pDMG4) (19) was used as a backbone to construct the *dam* complementation plasmid. The *dam* open reading frame was amplified by PCR using the 5′ *Xho*I-embedded primer (gatcatctcgagatgcctgaacccgctaaacc) and the 3′ *Sal*I embedded primer (gatcatgtcgactgaacgcagcataaatgatg). The amplified PCR product was digested with *Sal*I and *Xho*I. Meanwhile pVT1303 was digested with *Sal*I and *Xho*I to remove the *ltx* promoter and the *gfp* gene. The remaining fragment (pDMG4) of pVT1303 was purified and ligated with the treated PCR product; then the ligation mixture was transformed into *E. coli* JM109. The spectinomycin-resistant colonies were selected and the plasmid was purified and confirmed by restriction enzyme digestion. The resulting promoterless *dam* construct was digested with *Xho*I and then ligated with the *Xho*I-treated *ltx* promoter derived from pVT1303. The correct orientation of the *ltx* promoter upstream of *dam* was determined by restriction enzyme analysis. The corresponding plasmid *ltxP-dam*/pDMG4 was purified and used to transform *A. actinomycetemcomitans* VT1560 by electroporation. The spectinomycin-resistant transformants were selected and the presence of the correct plasmid was confirmed.

Confirmation of loss and restoration of M.AacDAM activity

Chromosomal DNA from wild-type strains, putative mutants and complemented strains was purified using a Puregene™ DNA extraction Kit (Gentra Systems). The DNA was restricted with the methylation-sensitive restriction endonucleases, *Dpn*I and *Dpn*II, in the requisite buffers at 37°C for 2 h and analysed on a 1% agarose gel stained with ethidium bromide.

Growth and viability studies

The two strains, VT1169 (parent strain) and VT1560 (M.AacDAM mutant), were grown concurrently, using standard TSBYE culture conditions (19, 24) and initial culture optical densities (OD at 600 nm) of 0.08. At various time intervals the ODs of the cultures were measured and aliquots were removed, plated onto TSBYE agar plates and colony-forming units (c.f.u.) were enumerated.

Protein secretion

Isolation and analysis of secreted *A. actinomycetemcomitans* proteins were performed as previously described (17). VT1169 (wild-type) and VT1560 (M.AacDAM mutant) were cultured overnight in AAGM broth at 37°C in air with 10% CO_2. Overnight cultures were diluted 1 : 100 in AAGM broth and at various time intervals 1.0 ml cell culture was removed. The amount of cells was normalized, based on OD_{600}, cells were pelleted, conditioned growth medium was harvested and secreted proteins were precipitated by the addition of 1.0 ml of −20°C 100% ethanol to 500 μl of the conditioned growth medium. Proteins were collected by centrifugation at 16,000 g at 4°C for 15 min, resuspended in sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer (300 mM Tris–HCl, pH 6.5, 12% SDS, 0.6% bromophenol blue and 60% glycerol), separated on 10% SDS–PAGE gels at 10 V/cm and stained with Coomassie brilliant blue (R250) for densitometry analysis using Scion imaging software (Scion Corporation, Frederick, MD).

Mass spectrometric identification of secreted proteins

Proteins were separated on a 12% SDS–PAGE and visualized by silver-staining (Invitrogen, Carlsbad, CA) (32). The 120-kDa band was excised, destained, washed twice with 100 μl 50% 50 mM NH_4HCO_3/50% acetonitrile, dried in a vacuum centrifuge and 100 μl 12.5 ng/μl sequencing-grade modified trypsin (Promega, Madison, WI) was added. After 20–30 min on ice, excess trypsin was removed and 30 μl 50 mM NH_4HCO_3 was added. After a brief vortexing, tubes were covered with parafilm and incubated at 37°C overnight. NH_4HCO_3 (25 mM, 15 μl) was added and the mixture was incubated at room temperature for 10 min. The supernatant was transferred to a siliconized 1.5-ml tube. Proteins were eluted from gel pieces by three sequential treatments as follows. The addition of 50% acetonitrile/5% formic acid (50 μl), vortexing for 10 min, sonication in a water bath for 5 min (only for first organic extraction) and centrifugation. Combined supernatants were dried and the pellets were redissolved in 15 μl of 5% formic acid and concentrated/desalted in borosilicate nano-electrospray capillaries (Protana, Odense, Denmark) packed with POROS R2 resin (Perseptive Biosystems, Foster City, CA). Desalted peptides were eluted into gold/palladium-coated capillaries with 5 μl 5% formic acid in 50% H_2O/methanol solution. Nano-electrospray was performed on a pESCIEX QSTAR (Applied Biosystems, Foster City, CA) tandem mass spectrometer using AnalystQS data acquisition and analysis software. The obtained mass fingerprint data sets were analysed using ProFound (38).

Western blot analysis of leukotoxin production and secretion

Wild-type *A. actinomycetemcomitans* and the M.AacDAM mutant cultures grown to early exponential phase ($A_{495} \cong 0.4$) were centrifuged at 5000 g for 5 min. Cell pellets were resuspended and boiled in sample buffer prior to SDS–PAGE. Loading amount was normalized by c.f.u. calculated from the original cultures. A small-scale protein precipitation method described previously (17) was used to harvest proteins from culture supernatants. The precipitated protein pellet was air-dried and boiled in sample buffer prior to SDS–PAGE. The amount used for protein loading was normalized by cell number. Proteins were separated by SDS–PAGE in commercially available precast 4–12% gradient gels following the manufacturer's instructions (Cambrex, Baltimore, MD). Proteins were transferred from gels onto nitrocellulose membranes and blocked in 5% non-fat dry milk. Membranes were probed with anti-leukotoxin antibody (gift of Dr Edward Lally) followed by peroxidase-conjugated anti-rabbit immunoglobulins. Signals were detected by ECL Western blotting reagents (Amersham). The Western blot was exposed for only 5 seconds to display antibody-specific reactive bands.

Standard quantitative invasion assay

The gentamicin protection assay, standardized for *A. actinomycetemcomitans*, was performed as previously described (23). Approximately 10^5 KB cells were seeded onto 12-mm diameter glass coverslips in the wells of 24-well tissue culture plates and allowed to adhere overnight at 37°C. Bacterial cells were harvested in early exponential phase of growth and adjusted to obtain a multiplicity of infection of 1000 bacteria to one KB cell. Bacteria were centrifuged onto the semi-confluent monolayer and incubated at 37°C in 5% CO_2 for 2 h. After incubation, the monolayers were washed to remove unattached bacteria. Extracellular bacteria were killed by the addition to each well of 100 μg gentamicin per ml followed by incubation at 37°C in 5% CO_2 for 1 h. After removal of the gentamicin and washing, KB cells were lysed and the internalized bacteria were released by the addition of 0.5% Triton X-100 in phosphate-buffered saline. Appropriate dilutions in phosphate-buffered saline were plated on TSBYE agar plates to enumerate c.f.u. All the quantitative determinations were carried out in quadruplicate.

Results

Development of a *dam* insertional mutant strain of *A. actinomycetemcomitans* SUNY 465

To define the role of DAM in *A. actinomycetemcomitans* virulence, a DAM-deficient mutant was constructed. The wild-type copy of *dam* in *A. actinomycetemcomitans* was replaced with the kanamycin-resistant-gene-disrupted *dam* by allelic replacement mutagenesis via conjugation (25). Kanamycin-resistant colonies were counter-selected for sensitivity to spectinomycin to select for an allelic replacement mutant resulting from a double cross-over event. This counter-selection revealed that approximately 3% of the kanamycin-resistant transconjugates exhibited sensitivity to spectinomycin, suggesting that the *kan*-disrupted *dam* fragment had integrated into the chromosome, but that the *spc*-containing vector, pVT1461, had not. These putative allelic replacement mutants were confirmed by Southern blot analysis. This analysis revealed that a 4.5-kb DNA fragment bound to the *dam* probe in the wild-type, whereas a 5.6-kb fragment appeared in the putative mutant (Fig. 1A). This increase of 1.1 kb is consistent with the size of the inserted *kan* cassette. In fact, the *kan* probe recognized the 5.6-kb fragment (Fig. 1B), but did not react with any band in the wild-type. Together, these data confirmed that wild-type *dam* was replaced by the *kan* insertionally inactivated *dam* gene. This *A. actinomycetemcomitans dam*-deficient strain was designated VT1560.

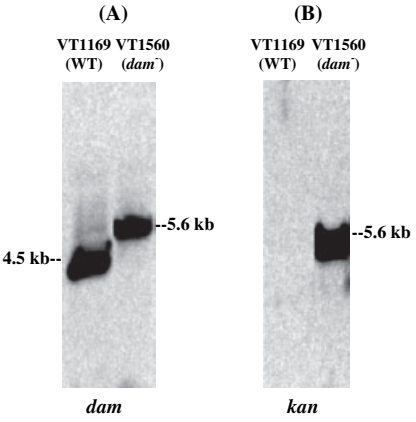

Fig. 1. Confirmation of *dam* disruption by Southern blot analysis. Wild-type VT1169 and the *dam* mutant, VT1560, probed with (A) *dam* or (B) *kan*.

Loss of methyltransferase activity in the *dam* insertional mutant

Our previous studies (8) suggest that that M.AacDAM is necessary and sufficient for DNA methylation in *E. coli*. To ascertain the loss of M.AacDAM activity in the *A. actinomycetemcomitans dam* mutant, restriction digests were carried out with genomic DNA using *Dpn*I and *Dpn*II, both of which are methylation-sensitive restriction enzymes; *Dpn*I cuts only methylated GATC, whereas *Dpn*II cuts only non-methylated GATC. *Dpn*I degraded VT1169 (Fig. 2; lane 1, left panel) genomic DNA, because it appeared as a smear, but did not degrade VT1560 DNA (Fig. 2; lane 2, left panel), as shown by the high-molecular-weight uncut DNA. Similarly, treatment with *Dpn*II (right panel) only results in the digestion of VT1560 (lane 2), not VT1169 (lane 1).

Construction and analysis of VT1560 complemented with a functional *dam* (strain VT1560/*ltxP-dam*)

To verify the functionality of the *dam* gene and the polarity of the *dam* mutation, we introduced the full-length *dam* gene into strain VT1560, which constitutes an insertional genomic mutation in the *dam* gene. The leukotoxin promoter was used to drive the expression of the *dam* gene in the complementation experiment. The results of complementation were assayed via restriction digests using *Dpn*I and *Dpn*II to demonstrate restoration of DAM activity (Fig. 2). While DNA from strain VT1560 was sensitive to digestion by *Dpn*II because of the lack of adenine methylation, genomic DNA from VT1560/*ltx-dam* remained intact (Fig. 2; left panel). This was paralleled by the substantial digestion of VT1560/*ltxP-dam* DNA by *Dpn*I, whereas no digestion was apparent with the VT1560 DNA (Fig. 2; lanes 2 and 3, right panel), confirming that VT1560/*ltxP-dam* was complemented with a functional *dam*. This is consistent with previous data of heterologous expression of M.AacDAM in *dam*-negative *E. coli* (8), clearly demonstrating that M.AacDAM is necessary and sufficient for DAM activity in *A. actinomycetemcomitans*. It also indicates that the *dam* mutant is non-polar.

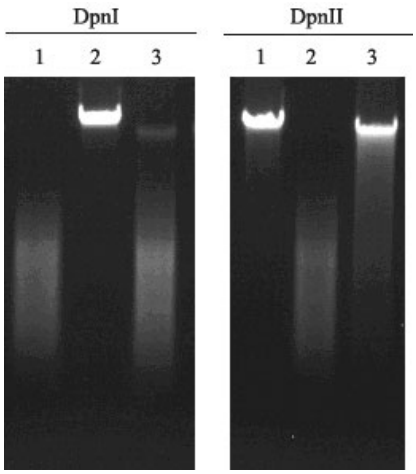

Fig. 2. Confirmation of *dam* disruption and complementation by agarose gel analysis of DNA restricted with methylation-sensitive enzymes. Genomic DNA isolated from VT1169 (wild-type, lane 1), VT1560 (*dam* knockout, lane 2), and VT1560/*ltxP-dam* (VT1560, complemented with functional *dam*, lane 3) were treated with *Dpn*I (left panel) or *Dpn*II (right panel) and analysed on a 1% agarose gel stained with ethidium bromide. The digestion by *Dpn*I of VT169 but not of VT1560 confirms the lack of DAM methylation (left panel) as does the lack of *Dpn*II digestion of VT1169 and the digestion of VT1560 (right panel). Similarly, the lack of *Dpn*I digestion of VT169 and VT1560/*ltxP-dam* genomic DNA (left panel) and digestion by *Dpn*II (right panel) confirms that VT1560/*ltxP-dam* was complemented with a functional *dam*.

Effect of M.AacDAM disruption on *A. actinomycetemcomitans* growth and viability

DAM is known to alter multiple cellular functions and is essential for the viability

of certain pathogens (16). Thus, its disruption in *A. actinomycetemcomitans* could modify cell growth and/or viability. These characteristics were analysed by the concurrent growth of VT1169 (parent strain) and VT1560 (DAM mutant). Plots of both ODs and c.f.u revealed a 2–3 h longer lag phase for the mutant than for the parent strain. However, when the DAM mutant began growing exponentially, its rate of growth was identical to that of VT1169 (data not shown). These data suggest that the disruption of *dam* in *A. actinomycetemcomitans* has no apparent profound effects on viability.

M.AacDAM disruption results in increased leukotoxin secretion

Protein secretion by wild-type VT1169 and the M.AacDAM mutant was monitored by SDS–PAGE over a period of 49 h (Fig. 3A). The SDS–PAGE profiles of proteins secreted by cells grown in air with and without 10% CO_2 were identical at all time-points. Thus, only profiles from cells grown with CO_2 are shown. After the first 6 h of culture no secreted proteins were detected. At 12 h (mid exponential growth phase) one very prominent protein with an apparent molecular weight of ~120 kDa was associated with both the wild-type and the M.AacDAM mutant. The protein, subsequently determined to be leukotoxin (see below), was generated in greater amounts by the M.AacDAM mutant than by the wild-type. Several bands representing smaller proteins were also evident at this time. By 24 h the leukotoxin band generated by the M.AacDAM mutant was still more intense than the one generated by the wild-type. In addition, the bands representing the smaller proteins were more evident. By 49 h neither the wild-type nor the M.AacDAM mutant generated leukotoxin bands, but both strains were associated with increased numbers of more intensely stained bands representing the smaller proteins, which implicated degradation as previously reported (17). Quantitative analysis of the leukotoxin band intensities of the 24-h culture supernatants by densitometry showed a four-fold increase in leukotoxin secretion by the mutant compared with that of the wild-type. The overexpression of leukotoxin was confirmed by Western blot analysis (Fig. 3B), which showed that intracellular levels of leukotoxin are also increased two-fold in the M.AacDAM mutant. Therefore, while the production and perhaps degradation of intracellular leukotoxin is altered, protein secretion is also altered in the absence of DNA methylation.

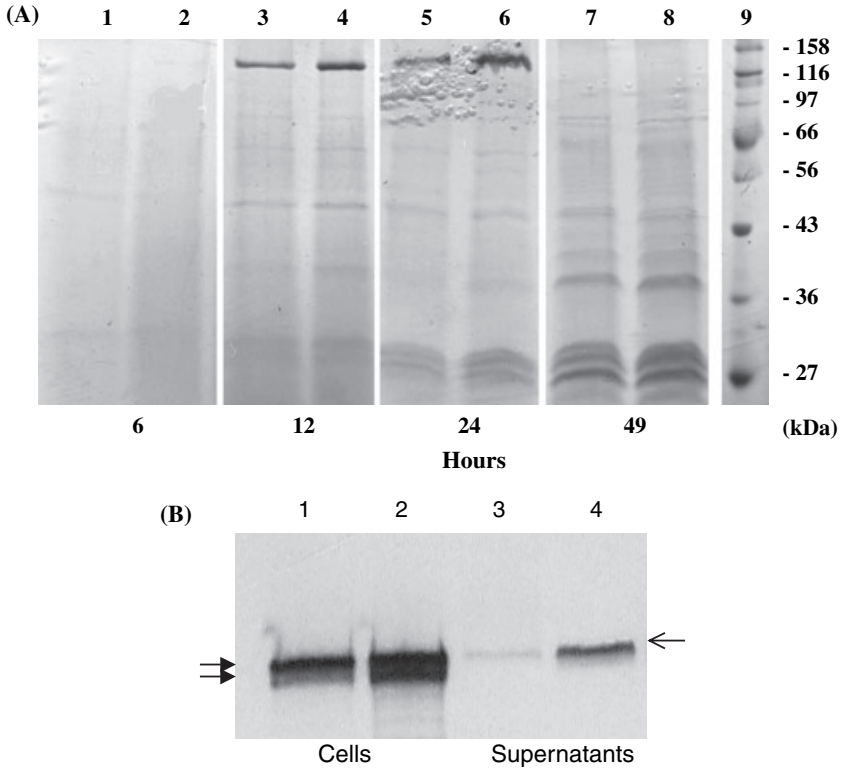

Fig. 3. (A) SDS–PAGE of protein secretion by *A. actinomycetemcomitans*. Wild-type VT1169 and the *dam* mutant were cultured for 6, 12, 24 and 49 h; culture supernatants were harvested and analysed for protein secretion profiles. VT1169 (lanes 1, 3, 5 and 7); VT1560 (lanes 2, 4, 6 and 8); molecular weight standards (in kDa): 158, 116, 97, 66, 56, 43, 36, and 27 are indicated in lane 9. Note that the ~120-kDa band, i.e. leukotoxin, is more intense in the lanes associated with the mutant than in those associated with wild-type. (B) Western blot analysis of leukotoxin production and secretion. Wild-type VT1169 and the *dam* mutant were cultured to the exponential growth phase, cell pellets and proteins precipitated from culture supernatants were subjected to Western blot analyses using anti-leukotoxin antibody. Cell pellets (lanes 1 and 2), culture supernatants (lanes 3 and 4); VT1169 (lanes 1 and 3), VT1560 (lanes 2 and 4).

Mass spectrometric identification of leukotoxin

The 120-kDa band was excised, digested in the gel with trypsin, and analysed by mass spectrometry as described above. Twenty-three mono-isotopic peptide masses were recorded from the mass spectrum and queried in ProFound by searching all taxa for matches of a single 80–150 kDa protein, assuming a maximum of two miscuts by trypsin. Eleven peptides matched 13 peptides within the 0.2 Da tolerance, covering 15% of the ~120 kDa protein (Table 2). Based on the matching peptides, the protein was identified unequivocally as leukotoxin of *A. actinomycetemcomitans* (Gi:79293).

Invasion of epithelial cells by the M.AacDAM mutant is reduced

Invasion is calculated as the ratio of c.f.u. recovered from VT1690 to c.f.u. recovered from VT1560 expressed as a percentage with the CFU VT1690 set at 100 (23). Assays were carried out using the standard gentamicin protection assay. The invasion efficiency of the M.AacDAM mutant, VT1560, was determined to be ~50% lower than that of VT1169, the wild-type (Fig. 4). These data show that disruption of *dam* can have a significant impact on the ability of *A. actinomycetemcomitans* to enter epithelial cells.

Discussion

DNA methylation occurs in diverse organisms and regulates various biological functions, including DNA replication, transcription, repair, and transposition.

Table 2. Monoisotopic peptide masses

Peptide sequence	Residues		Mass	
	From	To	Computed	Measured
IGELAGITR	482	490	928.53	928.60
KLLGLTER	97	104	928.57	928.60
AITQYLIAQR	284	293	1157.67	11758.60
TISPQELAGLIK	977	988	1268.74	1268.80
YEFITELKVK	563	572	1268.70	1268.80
FELQRGKVDKSL	944	955	1342.75	1342.80
QQAAQFANSVADR	11	23	1404.68	1404.60
DLSGTQVLQETVSK	667	680	1503.78	1503.60
ARQNGKNVTDVQLAK	166	180	1640.90	1640.80
SLNNKVEEIIGKDGER	944	959	1799.94	1800.00
VERDLSGTQVLQETVSK	664	680	1887.99	1887.90
GSTTLTFLNPLLTAGKEER	537	555	2047.10	2047.00
LIANLGAKDDYVFVGSGSTIVNAGDGYDVVDYSK	610	643	3521.72	3521.70

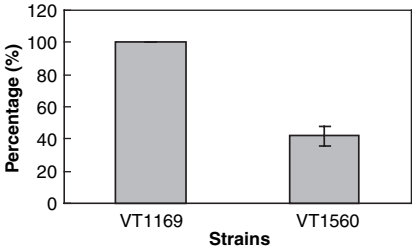

Fig. 4. Invasion of KB oral epithelial cells by the *dam* mutant and wild-type bacteria. Invasion is the ratio of c.f.u. recovered from VT1690 to c.f.u. recovered from VT1560 expressed as a percentage with the c.f.u. for VT1690 set at 100. Assays were carried out using the standard gentamicin protection assay. Experiments were performed in quadruplicate. Error bars represent the standard deviation of three experiments.

The DNA N^6-adenine methyltransferases (DAMs), found in many bacteria, are key regulators of bacterial viability and host virulence (14, 29). As a periodontal pathogen, *A. actinomycetemcomitans* expresses an array of virulence factors, including host cell adhesion and invasion, and leukotoxin production, both of which are involved in *A. actinomycetemcomitans* persistence and pathogenesis (9). However, mechanisms by which *A. actinomycetemcomitans* regulates the expression of genes associated with virulence are not well understood. The differential expression of many genes is attributed to changing environmental cues. The LuxS system is fairly well characterized in this regard (11). LuxS is important for iron uptake through the secretion of iron acquisition proteins; it also up-regulates the expression of leukotoxin (10). Although the regulation of leukotoxin expression and secretion has been extensively investigated, many aspects remain poorly understood (15, 18, 27). Here we provide initial insights into the relationship between a process known to regulate genes in various bacteria (DNA methylation), and two well-known virulence factors in *A. actinomycetemcomitans*.

We isolated and sequenced the gene coding for *A. actinomycetemcomitans* DNA adenine methyltransferase from the *A. actinomycetemcomitans* invasion prototype SUNY 465. An allelic replacement M.AacDAM mutant was constructed for *A. actinomycetemcomitans* SUNY 465 using our previously developed directed mutagenesis scheme (24, 25). The knockout strain (VT1560) lacks any detectable DAM activity (Fig. 2), confirming our earlier results that M.AacDAM is a functional DAM (8); complementation with a functional *dam* gene (Fig. 2) confirms that the original knockout has not disrupted other parts of the *A. actinomycetemcomitans* genome. Interestingly, the viability of *A. actinomycetemcomitans* was differentially affected in a growth phase-dependent manner. The *dam* mutant exhibited normal growth phenotype when grown exponentially. However, the lag phase for the *dam* mutant was 2–3 h longer than for wild-type cells, in which half the cells were not viable. This finding differs from the observations made for other DAM-deficient bacteria. DAM is not essential for viability in *E. coli* and *Salmonella*, however it is required for growth and viability of both *V. cholerae* and *Y. pseudotuberculosis* (16). These results indicate that DAM from different bacteria may impact various cellular processes. While the colony morphology of M.AacDAM mutants was identical to that of wild-type, electron micrographs revealed that the surfaces of M.AacDAM mutant cells demonstrated a deficiency in the expression of peritrichous fimbriae and fewer surface-associated vesicles (unpublished data). These observations suggest that the membrane components are altered, a change that could be related to altered invasion and protein secretion.

The well-studied *A. actinomycetemcomitans* virulence factor, leukotoxin, was secreted in four sixfold greater amounts by the M.AacDAM mutant, whereas cellular leukotoxin was increased by only two-fold. These increases represent altered steady-state amounts of leukotoxin that involve the rate of expression/secretion and the rate of degradation, suggesting that M.AacDAM may regulate leukotoxin expression, secretion and degradation. Although both cellular leukotoxin levels and secretion are increased, the majority of the proteome is maintained. [(Two-dimensional PAGE-based proteomics involving mass spectrometric identification of proteins was used to identify additional proteins that are altered more than two-fold in the DAM knockout (Oza, J and Reich NO, in preparation).] This further supports the role of DNA methylation in leukotoxin biogenesis. Mutagenesis of *Salmonella dam* resulted in the accumulation of numerous extracellular proteins, partly as a result of increases in the release of membrane vesicles. We are currently determining if the changes in leukotoxin expression and secretion involve direct changes in DNA methylation of the leukotoxin promoter, or indirect mechanisms involving protein secretion or stability alterations.

The efficiency of invasion of KB oral epithelial cells by the M.AacDAM mutant was decreased, although the underlying mechanism remains obscure. Certainly DAM-mediated methylation may regulate genes required for invasion and adhesion, thereby affecting the invasion process. DAM methylation in *S. typhimurium* appears to be involved in bacterial invasion, because DAM mutants of *S. typhimurium* are defective in invasion of the intestinal epithelium and macrophages (12). The reduced ability to invade epithelial cells may involve the reduced secretion of InvJ and SipC, two invasion proteins encoded by the *Salmonella* pathogenicity island 1 (SPI-1) (12). In *E. coli*, DNA methylation modulates the expression of virulence-related type I fimbriae that are required for adherence to host cells, while the loss of DNA methylation has no apparent effect on the integrity or stability of the *E. coli* envelope; the role of DNA methylation in *E. coli* invasion is not known.

An outer membrane protein found in *A. actinomycetemcomitans* Y4, Omp100, is implicated in the adhesion to, and invasion of oral epithelial cells (1). The invasion deficiency is therefore proposed to be an adhesion defect rather than a change in the ability to invade. We are

currently investigating the profile of altered putative adhesins and invasins expressed by the *dam* mutant, using proteomic methods. Loss of DNA methylation function in the M.AacDAM mutant strain has altered *A. actinomycetemcomitans* leukotoxin production, secretion and bacterial invasion, suggesting that the *A. actinomycetemcomitans* DAM is an important regulator of *A. actinomycetemcomitans* virulence. Characterizing how virulence genes are regulated in *A. actinomycetemcomitans* and the roles of DNA methylation in this process, are important next steps for understanding the pathogenesis of *A. actinomycetemcomitans*.

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