

REVIEW ARTICLE

## ***Actinobacillus actinomycetemcomitans***

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### **Introduction**

The recent report of the American Surgeon General on oral health in the USA [1] has shone the spotlight on what is termed ‘the silent epidemic of oral diseases – which disproportionately burdens minorities and the poor’. This silent epidemic is caused primarily by certain members of the oral microbiota, which are responsible for two of our most prevalent diseases – dental caries and periodontitis. It is surprising to realise that in the USA, the richest country on our planet, dental caries is the single most common chronic childhood disease, being five times more common than asthma [1]. Severe periodontal disease in the USA affects 14% of adults aged 45–54 and 23% of those aged 65–74 years. These prevalence figures are alarming, particularly as in recent years evidence has begun to accrue to suggest that dental ‘infection’ may be a predisposing factor in systemic conditions such as coronary heart disease (CHD), diabetes and low birth weight [2, 3].

The periodontal diseases affect the gums (gingivae), the periodontal ligament and the alveolar bone. Inflammation of the gums affects all of us at some time in our lives, and is known as gingivitis. If, in addition to the inflammation in the gums, the periodontal ligament and alveolar bone that support our teeth are also affected, and begin to be destroyed, the disease is known as periodontitis. This term encompasses a number of clinically defined conditions [4]. The periodontal diseases are not classical exogenous infectious diseases, and there is good evidence that they are caused by certain members of the normal oral microbiota, principally gram-negative anaerobes [5].

The periodontal disease with the clearest association with an oral bacterium is a curious condition known as localised aggressive periodontitis (LAP), previously known as localised juvenile periodontitis [4]. This disease affects only certain teeth (incisors and premolars) and causes rapid loss of the alveolar bone of the jaw leading to tooth loss [6]. The major causative agent of LAP is *Actinobacillus actinomycetemcomitans* [7], which is the subject of this review. This organism has also been known to cause infective endocarditis [8].

In a US national survey the mean prevalence of LAP among adolescents of all racial origins was 0.53%, which makes it a reasonably common disease. However, adolescents of African-American descent were found to have a 15-fold higher incidence of disease than Caucasian Americans [9]. In Brazil, one study found that 3.7% of 15–16-year-old adolescents had LAP [10]. In Nigeria, a prevalence of 0.8% was found [11, 12]. Kilian and co-workers have established a relationship between LAP in individuals of African origin and a particular clone of *A. actinomycetemcomitans* (JP2) which has a 530-bp deletion from the promoter region of the leukotoxin gene operon – one of this organism’s many putative virulence factors. Furthermore, these workers found that in about half of the families from which the JP2 clone was isolated more than one family member had LAP [13]. This virulent clone is absent from Northern European Caucasians [14] who are colonised by a range of clonally distinct organisms. Thus, in most of the world, *A. actinomycetemcomitans* is presumably an opportunist pathogen. However, in adolescents of African origin *A. actinomycetemcomitans* (or at least a particular clone) appears to be an exogenous pathogen [13].

The possible role of genetic factors in LAP has been under investigation since the 1980s [15]. An early discovery was a defect in neutrophil chemotaxis to the bacterial chemoattractant formyl-methionine-leucine-phenylalanine (FMLP) [16]. Genetic analysis of 30

patients with LAP has recently shown that 29 had point mutations in the gene encoding the formyl peptide receptor [17] and these mutations have been found to be associated with almost complete loss of receptor signalling in response to FMLP [18]. Why this apparent major defect in neutrophils does not result in susceptibility to other bacteria, and only seems to increase susceptibility to *A. actinomycetemcomitans*, is unexplained.

This short review will introduce the reader to *A. actinomycetemcomitans*, an oral commensal which is also an opportunist pathogen with a distinct racial bias and a surprising range of potential virulence factors and virulence mechanisms. In addition, there is evidence that this bacterium may play some role in systemic diseases such as coronary artery disease.

### ***Actinobacillus actinomycetemcomitans* – the cell**

First described in 1912 and variously named over the intervening years, *A. actinomycetemcomitans* was recognised as a member of the normal human oral microbiota in the 1950s [19]. It is also found in the oral cavities of a number of primates and other mammals. The primary habitat has not been definitively identified but is most probably dental plaque in the gingival crevice [20], as it is not found in edentulous individuals [21, 22]. This organism is a gram-negative, non-spore-forming, non-motile, facultatively anaerobic coccobacillus that grows best in an aerobic environment enriched with CO<sub>2</sub> 5–10%. Growth is optimal at 37°C, over the pH range 7–8.5, and can be stimulated by a number of low molecular mass reagents including various steroid hormones [23]. The cells are straight or curved rods with rounded ends *c.* 1.0–1.5 µm by 0.4–0.5 µm. Freshly isolated bacteria produce small crinkled adherent (rough) colonies (1–2 mm diameter) on agar with a star-shaped (Greek *aktinos*, ray) structure in the centre. Certain strains and fresh isolates of *A. actinomycetemcomitans* in medium have the ability to adhere tightly to themselves or to substrata such as glass, plastic and hydroxyapatite [24–28]. Adhesion has been thought to be due to the presence of large bundled fibres on the bacterial surface [25, 26]. Analysis of the tight binding of *A. actinomycetemcomitans* has led to the discovery of a gene cluster, termed *tad* (tight adherence), containing seven novel genes [28–31]. Another gene, *impA*, has been proposed to contribute to this tight binding phenotype [32]. It is also established that *A. actinomycetemcomitans* can bind to extracellular matrix components such as collagen and fibronectin [33]. Serologically, six serotypes (a–f) of the O-polysaccharide component of lipopolysaccharide are recognised currently [34] and it is suggested that all have pathogenic potential [35].

### **Virulence factors of *Actinobacillus actinomycetemcomitans***

It must be emphasised that, although a large number of ‘virulence factors’ of *A. actinomycetemcomitans* have been identified, there have been no in-vivo studies with isogenic mutants to establish that any of these proteins are implicated in tissue pathology. The tissue pathology that this bacterium is responsible for includes inflammation of the gingivae (gums) and destruction of the periodontal ligament and alveolar bone, which link the teeth to the jawbone. The putative virulence factors of *A. actinomycetemcomitans* can be subdivided into those that: (i) modulate inflammation, (ii) induce tissue destruction and (iii) inhibit tissue repair. As expected, many of the individual putative virulence factors have overlapping actions.

#### *Immunomodulatory ‘virulence’ factors*

*A. actinomycetemcomitans* appears to employ multiple gene products to inactivate or evade immune defences. The most actively studied gene product of this organism is a leukotoxin and a member of the RTX (repeats in toxin) family [36, 37] whose cellular receptor is the β<sub>2</sub>-integrin, LFA-1 [38], thus accounting for its selective effect on leucocytes (although only those from primates). The leukotoxin operon consists, in transcription order, of four genes *ltxC*, *A*, *B* and *D*. The LtxA protein is the active toxin and this has to be acylated, by LtxC and an acyl carrier protein, to be biologically active. The other two proteins are responsible for transport and secretion of LtxA [39]. The RTX leukotoxins are secreted, and the only exception to this is the LtxA toxin of *A. actinomycetemcomitans* which had been thought to be entirely cell associated; either bound to cell surface-associated nucleic acids [40] or within membranous vesicles which bud from the bacterium’s surface [41, 42]. This means that the bacterium itself is toxic to target cells [43]. More detailed analysis has revealed that adherent (or rough) colonies of *A. actinomycetemcomitans* do not secrete leukotoxin but non-adherent (smooth colonies) do [44]. Bacteria with mutations in the *tad* operon were found to secrete the leukotoxin, suggesting that binding of this toxin to *A. actinomycetemcomitans* is in some manner dependent on proteins expressed by *tad* genes [44]. The response of target cells to the *A. actinomycetemcomitans* leukotoxin is to undergo apoptosis [45] by a mechanism involving mitochondrial perturbation [46].

Various other undefined and ill-defined proteins have been claimed to be either directly immunomodulatory or immune-modulating as a result of the ability to inhibit cell cycle progression. Indeed, injection of *A. actinomycetemcomitans* into mice has been claimed to induce immunosuppression [47] and sonicates of this organism suppressed the IgG response to sheep red blood cells in mice [48]. A 14-kDa immunosuppressive

protein able to inhibit lymphokine production was isolated from *A. actinomycetemcomitans* [49] and the N-terminal sequence identified it as thioredoxin, an intracellular protein which is a redox controlling/cell stress protein, known to have cytokine-like properties [50]. However, when the thioredoxin of *A. actinomycetemcomitans* was cloned and expressed, the recombinant protein, which was enzymically active, failed to inhibit lymphokine or cytokine synthesis [51]. This immunosuppressive protein presumably co-purifies with thioredoxin and still remains to be identified.

Superantigens are generally bacterial proteins that activate T cells bearing specific V $\beta$  T-cell receptors. One consequence of such activation is that the T cells apoptose and thus superantigens can be considered as immunosuppressants. *A. actinomycetemcomitans* has been reported to produce an as yet unidentified superantigen which rapidly causes T-cell apoptosis [52, 53].

*A. actinomycetemcomitans* has been reported to produce a number of, as yet unidentified, proteins with cell cycle-inhibitory activity causing arrest in the G<sub>2</sub> phase of the cell cycle. These proteins range in molecular mass from the 8-kDa protein termed gapstatin [54, 55] to 60 kDa [56] and all the way up to 80 kDa [57]. A proportion of the sera from patients with LAP contained antibodies that were able to inhibit the cell cycle-inhibiting activity of gapstatin [54]. One cell cycle-modulatory protein with immunosuppressive function that has recently been identified as being produced by *A. actinomycetemcomitans* is cytolethal distending toxin (CDT) [58, 59]. This toxin is the product of a three gene operon (*cdtA*, *cdtB*, *cdtC*) which is found in a range of bacteria including *Escherichia coli*, *Shigella* spp., *Campylobacter* spp., *Helicobacter* spp. and *Haemophilus ducreyi* [58]. The mechanism of action of this toxin is believed to be due to the nuclease activity of CdtB [60]. In some, as yet unexplained manner, CdtA and CdtC are believed to facilitate the entry of CdtB into host cells. The CdtB is believed to enter into the nucleus and degrade chromosomal DNA, thus inducing cell cycle arrest *via* specific checkpoint kinases [59]. There is significant sequence variation in the *cdt* genes between bacteria, and phylogenetic analysis reveals that the *cdt* genes of *A. actinomycetemcomitans* [61, 62] most closely resemble those of *H. ducreyi*, the bacterium responsible for the sexually transmissible disease, chancroid [59]. The CDTs of all bacteria, other than *A. actinomycetemcomitans*, require all three CDT proteins for cell cycle inhibition. However, Shenker and colleagues have reported that purified or recombinant *A. actinomycetemcomitans* CdtB is sufficient to block human lymphocyte cell cycle progression [63–65]. We have found that the combination of CdtB and CdtC from *A. actinomycetemcomitans* will induce cell cycle arrest of HEp-2 cells [66]. The combination of all three toxin proteins will also stimulate human

monocytes to produce certain cytokines. Most unusually, the cytokine network produced by these toxin proteins includes the early response cytokine, IL-1 $\beta$ , but not its usual partner cytokine, tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) [66]. We have also found that CdtC binds to cell membranes and acts as a receptor for CdtB allowing the latter to enter into cells [67]. Thus, at the moment it appears that the CDT proteins of *A. actinomycetemcomitans* have some differences in their interaction with host cells compared with homologous proteins from other bacteria. The possible role of CDT in the virulence of *A. actinomycetemcomitans* has yet to be determined and the finding that inactivation of the CDT operon in *H. ducreyi* had no effect on virulence in a human skin model [68] is suggestive.

Another potentially immunosuppressive virulence factor of *A. actinomycetemcomitans* is an Fc-binding protein [69]. This was identified as a heat-modifiable outer-membrane protein [70]. The gene encoding this protein was cloned and encodes a protein of 35 kDa, which was termed Omp34 [71]. The Omp34 protein has 54% identity with OmpA of *E. coli*, a protein implicated in the virulence of this organism [72]. *A. actinomycetemcomitans* produces a 65-kDa macromolecule able to bind to the IL-10 receptor [73]. IL-10 is a major macrophage-de-activating cytokine and the biological activity of this molecule may either mimic or antagonise IL-10, thus modulating monocyte/macrophage function.

In addition to being able to inhibit lymphocyte and monocyte function, *A. actinomycetemcomitans* has been reported to produce a low molecular mass inhibitor of neutrophil chemotaxis to FMLP [74].

While many immunosuppressive factors have been described, there are fewer reports of immunostimulatory factors from *A. actinomycetemcomitans*. Direct culture of *A. actinomycetemcomitans* with human gingival epithelial cells induces the production of IL-1 $\beta$ , IL-8 and ICAM-1 but not IL-6 [75, 76]. In contrast, human gingival fibroblasts cultured in the presence of *A. actinomycetemcomitans* expressed mRNA for IL-6 and IL-8 but not IL-1 $\beta$  [76]. We know very little about the cytokine-inducing components of *A. actinomycetemcomitans*. The activity of lipopolysaccharide will be discussed in the next section. A 2-kDa peptide, which induced human gingival fibroblasts to synthesise IL-6 without also inducing IL-1 $\beta$  and TNF- $\alpha$ , is secreted by *A. actinomycetemcomitans* [77] and may explain the results found with the intact organism [76]. A 37-kDa secreted immunogenic glycoprotein has also been reported to stimulate murine macrophages to produce IL-1 $\beta$ , IL-6 and TNF- $\alpha$  [78]. *A. actinomycetemcomitans* has also been reported to secrete mitogenic proteins [79], including a 13-kDa B-cell mitogen [80].

Bacterial capsules normally play a major role in bacterial virulence because of their role in immune

evasion. Little is known about the role of *A. actinomycetemcomitans* capsular polysaccharides in the interactions of this organism with the host. The finding that the various serotypes of *A. actinomycetemcomitans* contain the unusual sugar, 6-deoxy-D-talose, and the identification of a unique gene encoding the protein involved in the synthesis of this sugar [81, 82] suggests that the capsule of this bacterium may have unusual properties.

#### 'Virulence' factors inducing tissue destruction

LAP is a very aggressive disease resulting in rapid destruction of the periodontal ligament and alveolar bone. The promotion of bone resorption requires interactions between the bone-forming mesenchymal cell, the osteoblast, and the myeloid multinucleate bone-resorbing cell, the osteoclast [83]. The main signals transferred between these cells are cytokines – particularly members of the TNF family, including TNF- $\alpha$  and the newly discovered cell surface cytokine, RANKL (receptor-activator of NF- $\kappa$ B ligand), on the osteoblast. This cell-bound cytokine is the ligand of RANK on the osteoclast plasma membrane. Binding of RANKL to RANK on osteoclast precursor cells stimulates the production of mature osteoclasts. Osteoblasts also produce a soluble protein resembling RANK, called osteoprotegerin, which acts as a RANK antagonist or decoy receptor [84].

With all bacterial diseases involving inflammation and tissue destruction, LPS is always considered to be a key virulence factor. This needs to be re-examined with oral bacteria in light of reports that LPS from certain oral organisms is an antagonist of human toll-like receptor (TLR)4, one of the major cellular LPS receptors [85]. *A. actinomycetemcomitans* LPS is reported to stimulate bone resorption *in vitro* [86, 87] and *in vivo* [88]. However, the authors of this review have established that the LPS from *A. actinomycetemcomitans* is a significantly less active cytokine inducer than the proteins secreted by the bacterium [89]. Among these secreted proteins is a potent bone-degrading molecule that was identified as the cell stress protein, chaperonin 60 [90]. This molecular chaperone, which is normally intracellular, appears to be secreted by this bacterium [90, 91] and stimulates bone resorption by acting as an osteoclast 'growth factor' [92]. A serotype-specific capsular polysaccharide antigen of *A. actinomycetemcomitans* has been reported to stimulate osteoclast formation [93] and induce apoptosis of an osteoblast-like cell line [94]. A role for antigen-driven T cells in alveolar bone resorption induced by *A. actinomycetemcomitans* has been postulated. Gingival injection of the outer-membrane protein (Omp34) [71] in rats after transfer of antigen-specific Th1 lymphocytes, but not Th2 cells, resulted in alveolar bone resorption. Such bone destruction was blocked by antagonism of CD80, the co-stimulatory signal for T cells [95]. T cells express

the osteoclast-activating cytokine RANK described above. In studies with NOD/SCID mice, which can accept human tissues and cells without inducing graft rejection, it was found that mice repopulated with peripheral T cells from periodontitis patients undergo alveolar bone destruction when orally challenged with *A. actinomycetemcomitans*. Bone destruction was inhibited by administration of the decoy receptor, osteoprotegerin, demonstrating that the antigen-specific induction of RANK by human T cells was the probable cause of the bone resorption [96].

### Virulence mechanisms of *A. actinomycetemcomitans*

#### Adhesion

Bacterial adhesion is required for colonisation and is a key virulence mechanism [97]. The tight auto-adhesion of *A. actinomycetemcomitans* has been described [24–28] and is due to the expression of long, bundled fibrils [98, 99] composed of a 6.5-kDa subunit protein, Flp-1 (fimbrial low-mol. wt protein) [100], which has been reported to be glycosylated [101]. Transposon mutagenesis with a novel transposon, IS903 $\phi$ kan, identified a cluster of novel genes – *tadABCDEFG* where *tad* stands for 'tight adherence' – involved in bacterial adhesion [28–31]. IS903 $\phi$ kan insertion mutants of *flp-1* also lack tight adhesion [30]. The most satisfactory explanation is that *tadABCDEFG* genes, which are arranged like an operon, are linked to the *flp-1* gene. Indeed, the *flp-1* gene is found upstream of the *tad* operon in a low G+C region (similar G+C ratio to the *tad* operon) along with two other genes (*rcpA* and *rcpB*) expressed specifically in rough adherent bacteria but not in smooth mutants [102]. The relationship between the *tad* operon and *flp-1* is that the former is a secretion system for the latter [31]. The TadA protein has significant homology to NTP hydrolase proteins involved in type II and type IV secretion systems and similar *tad* loci are found in the genomes of gram-negative, gram-positive bacteria and in Archaea [29]. The low G+C content of the *tad* locus suggests that it may have been horizontally transferred [31]. The role of the *tadA* locus in the pathogenesis of LAP is not known but a *tadD* isogenic mutant of *Pasteurella multocida*, a bacterium showing many similarities to *A. actinomycetemcomitans*, was less virulent in a mouse model of infection [103].

#### Invasion

It is established that many bacteria can invade host cells, at least *in vitro*, and invasiveness is a prerequisite for the pathology of certain bacterial pathogens [97]. Bacterial invasion is a complex process dependent on both the bacterium and the host cell [104]. Most bacteria utilise the actin microfilaments for uptake and intracellular movement but there is increasing evidence that microtubules are also important [104]. Studies of

invasion by *A. actinomycetemcomitans* have been limited in respect of the bacterial strains and human cells used but have revealed that 25% of *A. actinomycetemcomitans* isolates are invasive [105, 106]. The identity of the host cell surface receptor that binds to *A. actinomycetemcomitans* and initiates invasion is not firmly established. It has been suggested that the transferrin and integrin receptors are involved [107]. Alternatively, uptake, at least into vascular endothelial cells, has been proposed to utilise the platelet-activating factor (PAF) receptor which binds phosphorylcholine [108]. Binding and invasion of *Haemophilus influenzae* also utilises the PAF receptor [109]. It is not known if the Flp-1 protein is involved in adhesion to host cells. The uptake of *A. actinomycetemcomitans* into the HEp-2 cell line is blocked by monodansylcadaverine, implicating a receptor-mediated endocytotic uptake pathway and is also blocked by cytochalasin D, implying a role for the actin cytoskeleton in uptake [110]. This is similar to reports with many other bacteria [97, 104]. However, unusual aspects of the behaviour of *A. actinomycetemcomitans* are its rapid exit from cells after invasion, its ability to move from one cell to another and its capacity to divide rapidly within host cells [111]. The process of intracellular movement and the cell spreading could be inhibited by agents that interfered with microtubule dynamics, suggesting that this bacterium interacts closely with the microtubules of the host cell [112].

The relevance of these in-vitro studies has been brought into focus by the report that buccal epithelial cells of 23 of 24 individuals harboured oral bacteria including *A. actinomycetemcomitans* [113]. This shows that invasion is not an artefact of the tissue culture process but raises the very important question of the role of bacterial invasion in health and disease. Is invasion an evasion process or does it represent a closer relationship between the host and its commensal bacteria than has hitherto been believed?

### Global control of virulence

Our understanding of the mechanisms involved in the global regulation of virulence genes is becoming clearer and quorum sensing is now identified as a key mechanism for such control [97]. It is now established that *A. actinomycetemcomitans* expresses a homologue of the LuxS protein of *Vibrio harveyi* and produces autoinducer II (AI-2). This AI-2 induced *A. actinomycetemcomitans* to produce both leukotoxin expression and a periplasmic iron transport protein [114]. Of interest is the fact that this AI-2 protein can interact with other bacteria and induce specific gene transcription [114].

### Conclusions

*A. actinomycetemcomitans* is a bacterium with an array

of diverse potential virulence characteristics, including multiple immune evasion mechanisms and novel mechanisms for binding to host matrices and invading host cells, any one of which may play a crucial role in the local tissue pathology of LAP. Our understanding of this organism still lags behind that of enteric pathogens, largely because methods for genetic manipulation have only just become available and the genome sequence, while almost complete, still awaits annotation. With the availability of such methodology and genome information we should begin to see rapid advances in understanding how *A. actinomycetemcomitans* produces such profound, but local, pathology and shed light on its ability to induce systemic pathology such as the recent report of glomerulonephritis caused by this bacterium [115].

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