Genes for tight adherence of *Actinobacillus actinomycetemcomitans*: from plaque to plague to pond scum

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The Gram-negative periodontal pathogen *Actinobacillus actinomycetemcomitans* forms an extremely tenacious biofilm on solid surfaces such as glass, plastic and hydroxyapatite. This characteristic is likely to be important for colonization of the oral cavity and initiation of a potentially devastating form of periodontal disease. Genetic analysis has revealed a cluster of *tad* genes responsible for tight adherence to surfaces. Evidence indicates that the *tad* genes are part of a locus encoding a novel secretion system for the assembly and release of long, bundled Flp pili. Remarkably similar *tad* loci appear in the genomes of a wide variety of Gram-negative and Gram-positive bacteria, including many significant pathogens, and in Archaea. We propose that the *tad* loci are important for microbial colonization in a variety of environmental niches.

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Actinobacillus actinomycetemcomitans is a fastidious, CO_2-requiring, Gram-negative human pathogen in the γ subdivision of the Proteobacteria. As a member of the family Pasteurellaceae, it is related to the well-studied genera *Haemophilus* and *Pasteurella*. Its species name is derived from the fact that the bacterium was first isolated from actinomycotic oral lesions along with *Actinomyces israelii*[1,2]. *A. actinomycetemcomitans* has been associated with a variety of human infections, most notably localized juvenile periodontitis (LJP), infective endocarditis and brain abscesses[2–7].

Localized juvenile periodontitis

Most studies of *A. actinomycetemcomitans* have focused on its role in LJP, an aggressive, highly destructive form of periodontal disease that occurs in infected individuals around the time of puberty. Left untreated, LJP results in rapid destruction of the gingival tissue and ultimately leads to the loss of alveolar bone and teeth at localized sites, specifically the incisors and the first molars[6,8]. LJP is relatively common among adolescents. In a nationwide survey of the USA, Loe and Brown[9] found the prevalence of LJP to be 0.53% among all adolescents. African-Americans were found to have a 15-fold greater incidence of disease than Caucasian Americans[9]. In Nigeria, prevalences of 0.75% among teenagers have been reported[10,11], and LJP was found in 3.7% of 15- and 16-year-old adolescents in Brazil[12]. Recently, Stabholz *et al.*[13] reported an astonishing 38% incidence of LJP in adolescents among the families of a closely knit community near Jerusalem. These studies indicate that LJP is a significant but largely unrecognized infectious disease of adolescents worldwide, although geographical location, socio-economic status and race are clearly important factors in the prevalence of this disease. LJP contributes to what US Surgeon General David Satcher calls the 'silent epidemic of oral diseases', which disproportionately burdens minorities and the poor[14].

Possible virulence factors

Although *A. actinomycetemcomitans* was first described in 1912 (Ref. 1), the genetic and molecular basis for its ability to cause disease is largely unknown. One important reason for this is that until recently, *A. actinomycetemcomitans* was refractory to genetic analysis. This was particularly true of fresh clinical isolates, which exhibit distinctive properties that can easily be lost upon subculture[15]. Additionally, there is currently no practical animal model for disease, although recent studies have established a new rat model for colonization and persistence[16]. Nevertheless, *A. actinomycetemcomitans*, like other pathogens, is likely to use a multitude of strategies to cause disease.

The bacterium has been shown to be capable of binding to and invading epithelial[17,18] and endothelial cells[19], which could be an important mechanism for evading host defenses and spreading beyond the initial site of infection[3]. *A. actinomycetemcomitans* has also been found to express several potential virulence factors, including cytolethal-distending toxin, catalase, IgA protease, OmpA-like protein, capsular polysaccharide biosynthetic enzymes, GroEL-like protein, several classes of lipopolysaccharide (LPS) and leukotoxin[20–30]. Of these, clearly the best studied is the 116-kDa leukotoxin (LtxA), which belongs to the RTX family of toxins typified by the *Escherichia coli* HlyA hemolysin[31–34]. In contrast to the other putative virulence factors, there is some evidence that LtxA expression can affect disease. Studies have indicated that the onset of disease occurs at an earlier age in individuals harboring a clonal variant thought to express high levels of LtxA as the result of a deletion upstream of the *ltxA* gene[35–37]. In addition, individuals

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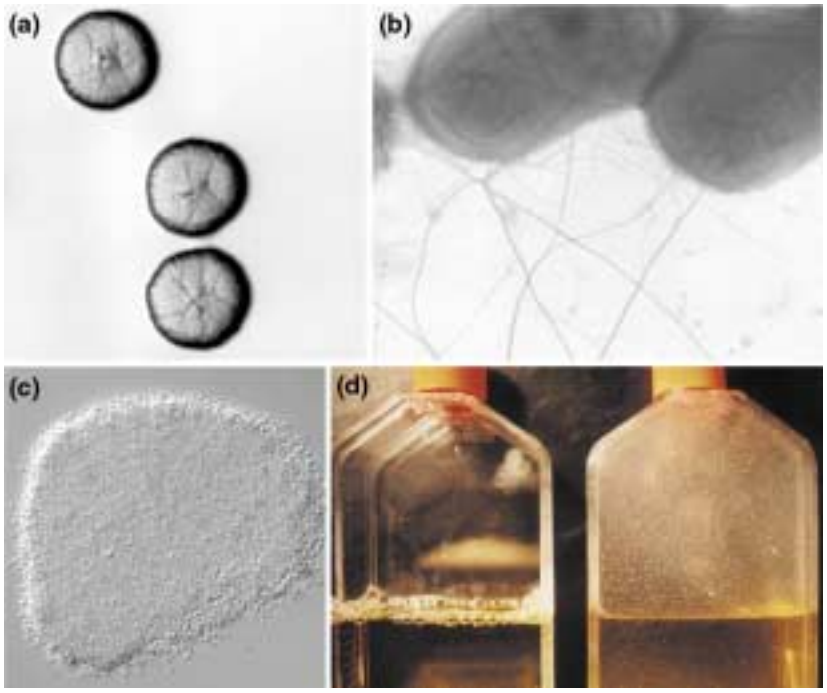

Fig. 1. Properties of the *Actinobacillus actinomycetemcomitans* clinical isolate, CU1000N (Ref. 44). (a) Colonies display a characteristic crinkled or 'rough' morphology. Star-like structures are often apparent in the centers. (b) Electron microscopic examination of negatively stained fibrils (bundles of pili) associated with the bacterial cells. (c) Bacteria adhere to each other to form microcolonies or autoaggregates that are difficult to disperse. (d) In liquid culture, the bacteria form a tenacious biofilm on solid surfaces, such as tissue culture plastic. Left, uninoculated tissue culture flask. Right, flask inoculated with cells and incubated for several days on its side. Even after vigorous shaking, the microcolonies remain attached to the surface.

infected with this variant are more likely to have disease than those with infected with wild-type bacteria[38]. LtxA is unusual among RTX toxins because it exhibits a high degree of target specificity, acting to kill only polymorphonuclear leukocytes, monocytes and T-cells in humans, apes and Old World monkeys[39,40]. LtxA was also previously thought to be the only RTX toxin to remain entirely associated with the bacterial cell[31,32,41,42]. This property was curious in light of the fact that the *A. actinomycetemcomitans ltxA* gene is coexpressed in an operon with two genes highly related to those typically used for type I secretion of all other RTX toxins[34,43]. Recent studies have revealed that LtxA can indeed be secreted in abundance by *A. actinomycetemcomitans* under certain conditions[33].

Tight, non-specific adherence

By far the most striking property of fresh clinical isolates of *A. actinomycetemcomitans* is their ability to form extremely tenacious biofilms on a variety of solid abiotic surfaces, such as glass, plastic and hydroxyapatite[44–47]. This remarkable ability is believed to allow *A. actinomycetemcomitans* to colonize the tooth surface and initiate infection in the presence of salivary flow. When *A. actinomycetemcomitans* is first isolated from the oral cavity and cultured on solid medium, the bacterium forms a crinkled ('rough') colony with a characteristic star-like structure in the center (Fig. 1a)[15,48,49]. In broth cultures, the bacterial cells adhere tightly to the walls of the culture vessel, where they form a dense community of microcolonies (Fig. 1d)[5,15,44,46–48]. The adherent colonies are completely resistant to vigorous shaking or vortex mixing, and they are generally harvested by physically scraping them from the surface with a spatula[45]. The liquid broth remains clear as the biofilm develops, although older broth cultures will eventually become turbid from the growth of isogenic non-adherent variants[15,44,46–48]. These variants form large, smooth colonies that lack the ability to adhere in broth cultures. Clinical isolates from the oral cavity are always rough and adherent, and inability to adhere appears to be a general property of the spontaneous smooth variants. Recently, smooth variants were isolated from nine different clinical isolates, and all were non-adherent (D.H. Fine *et al*., unpublished). Reversion of non-adherent variants to a rough, adherent form has not been confirmed. Nevertheless, it is still an open question whether the spontaneous loss of adherence is indicative of a mechanism of phase variation important to pathogenesis or an artifact of laboratory subculturing.

Because non-adherent variants of *A. actinomycetemcomitans* can be so readily enriched during growth in liquid culture, most studies of *A. actinomycetemcomitans* have used smooth, non-adherent laboratory variants. We wished to investigate the genetic and molecular basis of tight adherence, a property likely to be crucial for initiation of pathogenesis. We therefore re-examined the stability of this phenotype in a series of independent clinical isolates[15]. All isolates tested gave rise to smooth, non-adherent variants. In broth, non-adherent variants eventually overgrew the culture, as observed previously. However, we found that the tight adherence property of purified, clinical *A. actinomycetemcomitans* strains could be maintained indefinitely on solid medium by picking single, rough colonies for subculture in broth or on plates. This finding was crucial for genetic studies on tight adherence.

Adherence, autoaggregation and fibrils

Tight adherence has been associated with two other important characteristics of *A. actinomycetemcomitans*: autoaggregation and long, bundled fibrils. If adherent bacteria are removed from the surface and dispersed in broth by gentle sonication, they will rapidly reform large clumps, or 'autoaggregates', which quickly settle to the bottom of the tube (Fig. 1c)[5]. Furthermore, electron microscopic studies of rough, adherent strains of *A. actinomycetemcomitans* have revealed that the bacterial cells are attached to a dense matrix of long, thick fibrils (Fig. 1b)[3,44,47,48]. Each fibril consists of a parallel array of individual pili of approximately 5–7 nm in diameter (Fig. 2)[48,50], and the fibrils become interlocked by sharing individual pili. Fibrils are often several microns long and up to 100 nm or more thick[50].

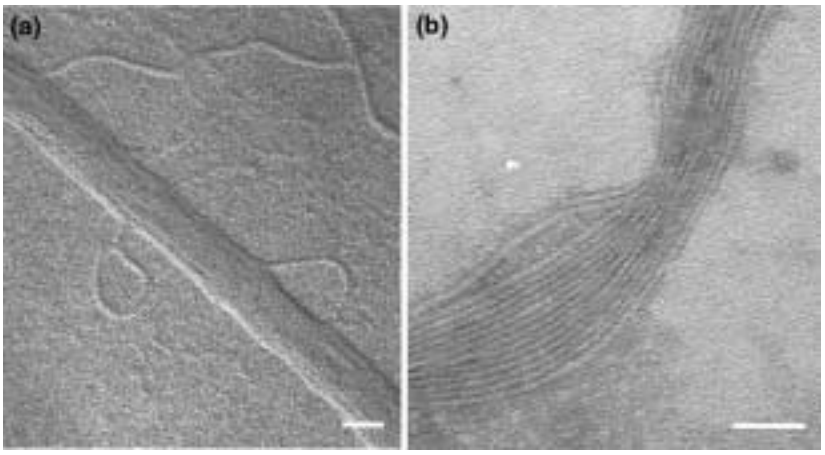

Fig. 2. High-resolution electron micrographs of *Actinobacillus actinomycetemcomitans* fibrils. (a) Individual pili are visible as they become separated from the thick fibril. The sample was prepared by unidirectional metal shadowing (at 45° angle) with platinum and carbon. (b) A fibril that is composed of approximately 14 individual pili is clearly discernable after negative staining with 1% uranyl acetate. Scale bars = 50 nm.

The major pilin subunit (Flp1) is a 6.5-kDa polypeptide that was identified from purified fibrils[51]. Amino acid sequence analysis led to the identification of the *flp-1* gene, which predicts that the Flp1 precursor has a signal sequence that is likely to be processed during fibril assembly[50,51]. Inoue *et al*. have recently reported that Flp1 extracted from purified fibrils is post-translationally modified, probably by glycosylation[52].

By contrast, the spontaneous smooth, non-adherent variants of *A. actinomycetemcomitans* that arise in culture are incapable of autoaggregation, and they do not have long, bundled fibrils[5,15,44,48]. This correlation indicated that the fibrils are responsible for both tight non-specific adherence and autoaggregation. Electron microscopy of adherent *A. actinomycetemcomitans* has provided support for this idea. *A. actinomycetemcomitans* cells growing on hydroxyapatite were observed by scanning electron microscopy to be connected by a matrix of fibril-like structures attached to the hydroxyapatite surface[45]. With transmission electron microscopy, single long fibrils can be seen to be associated with several bacterial cells[44,50]. Remarkably, high resolution electron microscopy failed to show the fibrils or pili projecting from bacterial cells, nor do bacteria appear to be attached to the tips of fibrils or pili[50]. Perhaps the pili are assembled and released from the cells. The pili might then associate to form a matrix of thick, bundled fibrils, which bind along their lengths to the bacterial cell surface to form a dense aggregate of cells. Regardless of the manner in which fibrils associate with the bacteria, the apparent correlation of adherence to surfaces, fibril formation and autoaggregation suggested that it should be possible to isolate well-defined mutations of a single gene of *A. actinomycetemcomitans* that affects all three properties.

High-frequency transposon mutagenesis in *A. actinomycetemcomitans*

One of the major difficulties faced by workers in the field has been the dearth of useful tools for the genetic analysis of *A. actinomycetemcomitans*. To obtain well-defined, single-gene knockouts defective in adherence, it was first necessary to develop an efficient transposon-mutagenesis system for *A. actinomycetemcomitans.* Transposon mutagenesis had been reported in *A. actinomycetemcomitans*, but the mutation frequencies were low[53,54]. The primary limiting factor is the low efficiency with which suicide vectors containing a transposon could be introduced into

Box 1. Is Flp1 sufficient for biofilm formation?

Bacterial biofilms are ubiquitous in natural environments[a]. A biofilm is defined as a community of microorganisms closely associated with each other and attached to surfaces. The community can be held together by a macromolecular matrix of protein and/or polysaccharide[a, b]. Are Flp1 fibrils solely responsible for biofilm production by *A. actinomycetemcomitans*? Current data suggest that another, non-proteinaceous component plays a role in adherence and biofilm formation. Electron microscopic examination of ruthenium red-stained *A. actinomycetemcomitans* cells by Holt *et al.*[c] revealed densely staining material surrounding and associated with bacterial cells. It was proposed that exopolysaccharide forms a capsule or matrix around the cells. Work by others has supported a possible role for exopolysaccharide in biofilm formation. *A. actinomycetemcomitans* adherence to abiotic surfaces could be neither prevented nor disrupted by treatment with trypsin[d]. Proteinase K and pronase failed to disrupt *A. actinomycetemcomitans* biofilms or Flp1 fibrils (S.C. Kachlany *et al.*, unpublished). However, adherence to abiotic surfaces could be significantly prevented and disrupted by periodate, a carbohydrate-oxidizing reagent[d]. Furthermore, scanning electron microscopy of *A. actinomycetemcomitans* attached to hydroxyapatite demonstrated polymer-coated cells in an arrangement characteristic of biofilms[d]. Hence, the search is on for other components, such as polysaccharide, that might interact with Flp1 fibrils and play a role in *A. actinomycetemcomitans* adherence and biofilm formation.

References

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Box 2. Flp pili and the widespread *flp* subfamily

Flp prepilins share several features with other type IV pilins: (1) a three-domain structure composed of an amino-terminal leader sequence followed by a region of approximately 20 hydrophobic amino acid residues and a variable carboxy-terminal region; (2) an invariably conserved glycine residue just preceding the putative site of leader peptide cleavage; and (3) a conserved glutamate residue exactly five amino acid residues from the amino terminus of the mature peptide (Fig. I).

Based on phylogenetic evidence, *A. actinomycetemcomitans flp-1* and *flp-2* genes were shown to belong to a distinct subfamily of the type IVB pilin subunits[a]. The predicted protein products of members of the *flp* subfamily share the following characteristics that distinguish them from other type IV prepilins: (1) relatively small size (smaller than 90 amino acids long); (2) a shorter carboxy-terminal domain than those of other type IV prepilins; and (3) an invariant tyrosine residue immediately following the conserved glutamate.

Further searching has revealed the presence of *flp*-like genes closely linked to almost every *tad* locus in both Gram-negative and Gram-positive organisms. The widespread occurrence of *flp* family member genes in association with *tad* loci suggests that these regions encode proteins that act to assemble and/or export a Flp pilus in diverse organisms.

The phylogeny of *flp* family members also showed a tendency for duplication and maintenance of *flp* alleles in bacteria. Many organisms harbor more than one copy of a *flp* gene, and these copies are not always the most closely related. This result suggests that duplications in ancestors have been maintained through and after speciation events, and it raises questions about the biological function and significance of duplicate *flp* alleles.

The functional significance of *flp-2* in *A. actinomyctemcomitans* is currently unknown, and preliminary work in our laboratory suggests that the gene might not be expressed. However, when the upstream region of *flp-1* (containing a Shine-Dalgarno sequence) was fused to *flp-2*, the protein was expressed in *E. coli*. Future studies should help define the potential role of Flp2 in *A. actinomyctemcomitans* and could lead to further insights into the function of duplicate *flp* genes in other organisms.

Reference

a Kachlany, S.C. *et al.* (2001) *flp-1*, the first representative of a new pilin gene subfamily, is required for nonspecific adherence of *Actinobacillus actinomycetemcomitans*. *Mol. Microbiol.* 40, 542–554

Fig. I. Alignment of predicted Flp proteins. Roman numerals refer to loci numbers in Figure 3. *Shigella flexneri* 2a *flp* is from the *she* pathogenicity island (AF200692). *Bradyrhizobium japonicum flp* is from sequence fragment BJU79771. Preliminary sequences for *Sinorhizobium meliloti* were produced by Stanford University (http://cmgm.stanford.edu/~mbarnett/genome.html); *Bordetella bronchiseptica,* by the Sanger Centre sequencing groups (http://www.sanger.ac.uk/). All other sequence references can be found in the legend to Fig. 3.

A. actinomycetemcomitans to produce mutations in genes of interest. This problem was particularly severe with the rough, adherent strains, which proved to be even less efficient in broad host-range, incompatibility group P plasmid-mediated conjugation[55] and various methods for transformation[56,57] than the smooth laboratory variants. Although several interesting phages of *A. actinomycetemcomitans* have been identified[58–60], their potential utility as high-proficiency suicide vectors has unfortunately not yet been

exploited. Recently, however, as a result of a very productive collaboration with the Keith Derbyshire laboratory, we reported a novel transposon, IS*903*φ*kan* for high-frequency mutagenesis of *A. actinomycetemcomitans* and other Gram-negative bacteria[30]. The method does not rely on a suicide vector, and thus high frequencies of conjugation or transformation are not required. Instead, transposition of IS*903*φ*kan* is inducible and its kanamycin-resistance gene is activated only upon insertion of the transposon into an expressed, non-essential gene. The transposon and its IPTG-inducible transposase gene is resident on a broad-host-range IncQ plasmid. Once the plasmid is introduced into the strain, however inefficiently, the cells are grown, and the transposon is induced. Thousands of random insertion mutants are readily selected by plating the bacteria on medium containing kanamycin.

Genetics of tight adherence: identification of the *tad* locus

To identify genes required for tight adherence, pools of random IS*903*φ*kan* insertion mutants were generated in the well-documented, rough, adherent strain CU1000 derived from a patient with LJP (Ref. 45). Because of the previously established correlation between smooth colony phenotype and non-adherence, the IS*903*φ*kan* insertion mutants were plated on solid medium, and smooth-appearing colonies were selected for further study[44].

Nucleotide sequence analysis of the transposon insertion sites and intervening regions revealed that insertion mutations occurred in a cluster of seven novel genes, which were named *tadABCDEFG* for 'tight adherence'[44]. Like the spontaneous smooth mutants, mutants with insertions in any of the *tad* genes failed to adhere, were unable to autoaggregate and showed no evidence of pili or fibrils[44]. The mutations were complementable *in trans*, indicating for the first time that the multiple phenotypes of adherence, autoaggregation and fibril production can be affected by a mutation in a single gene.

The *tadABCDEFG* genes are arranged in an operon-like structure with a potential transcriptional terminator immediately downstream of *tadG*. Kachlany *et al.*[44] were able to link the upstream region of *tadA* to two other genome segments whose sequences had been reported previously[51,61]. The additional upstream genes and open reading frames (ORFs) appeared to be part of the *tad* operon. This region of 5 kb upstream of *tadA* has a 36% G+C content, which is identical to that of the *tadABCDEFG* cluster and different from the G+C content of typical housekeeping genes, the overall genome of *A. actinomycetemcomitans* and the flanking regions (approximately 48%)[62]. The first indication that this upstream region is involved in tight adherence came from complementation analysis of the spontaneous non-adherent variants that arise during growth *in vitro*. Complementation of two independent variants was not successful with the *tadABCDEFG* region expressed in *trans*. However, complementation to tight adherence was successful when the upstream region was also included[44].

Significantly, the first gene of this upstream, low G+C region is *flp-1*. From biochemical studies, the Flp1 protein is predicted to be the major subunit of the bundled pili[51]. If this is true and if *flp-1* is part of the *tadABCDEFG* gene complex, then a knockout of *flp-1* might be expected to have a phenotype similar to that of the *tad* mutants. Kachlany *et al.*[50] have recently isolated two classes of IS*903*φ*kan* insertion mutants of *flp-1*. Insertions early in the gene abolish protein expression. Like *tad* mutants, these *flp-1* mutants fail to adhere and do not express pili. Because they are complementable *in trans*, the mutations are not polar on expression of the downstream *tad* genes. Thus, the phenotypes result from the defect in *flp-1*. An insertion at the 3′-end of *flp-1* resulted in the production of a Flp1 protein with an altered carboxyl terminus. This protein allowed the production of individual, unbundled pili of altered morphology, and the bacteria were able to adhere weakly. A similar result was obtained when an 11-amino acid epitope was added to the carboxyl terminus of Flp1 (Ref. 50). Thus, the carboxyl terminus of Flp1, predicted from sequence to be hydrophilic, is crucial for tight adherence (Box 2).

The upstream *flp-1–tadA* region also contains the known genes *rcpA* and *rcpB*. These genes were reported by Haase *et al.*[61] to encode outer membrane proteins that are expressed specifically in rough, adherent bacteria but not in spontaneous smooth, non-adherent variants. Their functions have not been determined. However, mutations in *rcpA* result in a non-adherent phenotype like that of *tad* mutants (P.J. Planet *et al.*, unpublished).

Function of the *tad* locus: a novel secretion system for Flp pili?

From these studies, it is clear that the *tad* locus required for the production of Flp pili and tight non-specific adherence of *A. actinomycetemcomitans* extends from *flp-1* to *tadG*. Apart from *flp-1*, which encodes the major pilin subunit, what do we know about the functions of these genes?

Immediately downstream of *flp-1* is another gene (*flp-2*) whose predicted product is 51% identical to Flp1. Phylogenetic analysis of Flp2 and other predicted Flp proteins from other bacterial genomes indicates that it is a homolog of Flp1 (Ref. 50). The phenotype of the *flp-1* mutant indicates that *flp-2* is not expressed or has a different function from *flp-1* (Box 2). The predicted amino acid sequence of the next ORF, OrfB, is similar (41%) to prepilin peptidase[51,61], which removes the leader peptide from prepilin for assembly into pili. The product of the *rcpA* gene located downstream appears to be related to the GspD/OutD/PulD family of proteins that are components of type II secretion systems of Gram-negative bacteria[61]. These proteins are located in the outer membrane and form channels for the secretion of protein substrates[63–65].

Table 1. Prokaryotes that harbor the *tad* locus

Organism	Human/animal pathogen?	Disease(s) caused	Habitat[a]	Number of loci	Location of loci
Bacteria					
Actinobacillus actinomycetemcomitans	Yes	Periodontitis, endocarditis	Oral cavity	1[b]	Chromosome
Acidithiobacillus ferrooxidans	No	N/A	Soil, acidic environments	1[b]	Chromosome
Bordetella bronchiseptica	Yes	Respiratory infections	Respiratory tract	2[b]	Chromosome
Bordetella parapertussis	Yes	Respiratory infections	Respiratory tract	2[b]	Chromosome
Bordetella pertussis	Yes	Whooping cough	Respiratory tract	2[b]	Chromosome
Caulobacter crescentus	No	N/A	Aquatic, soil	1	Chromosome
Chlorobium limicola	No	N/A	Aquatic, sulfur springs	1[b]	Plasmid
Chlorobium tepidum	No	N/A	Aquatic, sulfur springs	1[b]	Chromosome
Corynebacterium diphtheriae	Yes	Diphtheria	Skin, nasopharynx	1[b]	Chromosome
Haemophilus ducreyi	Yes	Chancroid	Skin	1[b]	Chromosome
Mesorhizobium loti	No	N/A	Soil, root nodules	3	Chromosome
Mycobacterium bovis	Yes	Tuberculosis (cattle)	Respiratory tract	1[b]	Chromosome
Mycobacterium tuberculosis	Yes	Tuberculosis	Pulmonary tree	1[b]	Chromosome
Pasteurella multocida	Yes	Fowl cholera, hemorrhagic septicemia, atrophic rhinitis, systemic infections	Oral cavity	1	Chromosome
Pseudomonas aeruginosa	Yes	Respiratory infections	Respiratory tract, skin	1	Chromosome
Pseudomonas syringae	No	N/A	Soil, plants	1[b]	Chromosome
Sinorhizobium meliloti	No	N/A	Soil, root nodules	1[b]	Plasmid
Streptomyces coelicolor	No	N/A	Soil, composts	3[b]	Chromosome
Yersinia enterocolitica	Yes	Gastroenteritis	Gastrointestinal tract	1[b]	Chromosome
Yersinia pestis	Yes	Bubonic and pneumonic plague	Flea proventriculus, lymphatic system	1	Chromosome
Yersinia pseudotuberculosis	Yes	Gastroenteritis, respiratory infections	Gastrointestinal tract	1[b]	Chromosome
Archaea					
Aeropyrum pernix	No	N/A	Solfataric thermal vents	2[c]	Chromosome
Archaeoglobus fulgidus	No	N/A	Marine hydrothermal vents	4[c]	Chromosome
Halobacterium sp. NRC-1	No	N/A	Salt lakes, marine salterns	3[c]	Chromosome
Methanococcus jannaschii	No	N/A	High-temperature salt marshes	2[c]	Chromosome
Methanothermobacter thermautotrophicus	No	N/A	Flooded soils, sediments	2[c]	Chromosome
Pyrococcus abyssi	No	N/A	Geothermally-heated marine sediments	2[c]	Chromosome
Pyrococcus furiosus	No	N/A	Geothermally-heated marine sediments	2[b,c]	Chromosome
Pyrococcus horikoshii	No	N/A	Geothermally-heated marine sediments	2[c]	Chromosome
Sulfolobus solfataricus	No	N/A	Solfatara fields	2[c]	Chromosome
Thermoplasma acidophilum	No	N/A	Self-heating coal refuse piles	2[c]	Chromosome
Thermoplasma volcanicum	No	N/A	Self-heating coal refuse piles	2[c]	Chromosome

[a]Although many of the organisms are found in multiple habitats, the most relevant are cited.
[b]Sequencing of the genome is either incomplete or not available; the number of loci might be higher.
[c]The *flaIJ* locus is included in this number because of the similarity to *tadA* and *tadB* (Ref. 66).

Analysis of the sequences of the polypeptide products of the *tadABCDEFG* region of the *tad* locus revealed that all but TadA have putative transmembrane domains indicative of inner membrane proteins. However, only TadA is similar to proteins of known function. The predicted TadA polypeptide contains a canonical Walker-type nucleotide-binding motif[66] and has similarity with the amino acid sequences of well-known ATP-hydrolyzing proteins required for the conjugative transfer of DNA, such as TrbB of plasmid RK2 (Ref. 67) and VirB11 of Ti plasmids[68], or secretion of protein substrates, such as PtlH of *Bordetella pertussis*[69] and HP0525 of *Helicobacter pylori*[70]. In these systems, ATP hydrolysis is thought to provide energy for the assembly of the secretion complexes or secretion of the substrate. Recently, an epitope-tagged derivative of TadA (TadA–T7) was purified by affinity chromatography and found to hydrolyze ATP[71]. TadA–T7 was localized to both the cytoplasm and the inner membrane of *A. actinomycetemcomitans*, suggesting that it is a peripheral inner membrane protein like other secretion ATPases[68]. Electron microscopy of purified TadA–T7 revealed structures resembling the hexameric rings observed for other secretion ATPases[70].

These relationships strongly indicate that the Tad proteins form part of a secretion apparatus. There is recent direct evidence for this hypothesis. Immunoelectron microscopy of an epitope-tagged version of Flp1 showed that the protein can be incorporated into fibrils, and western blot analysis

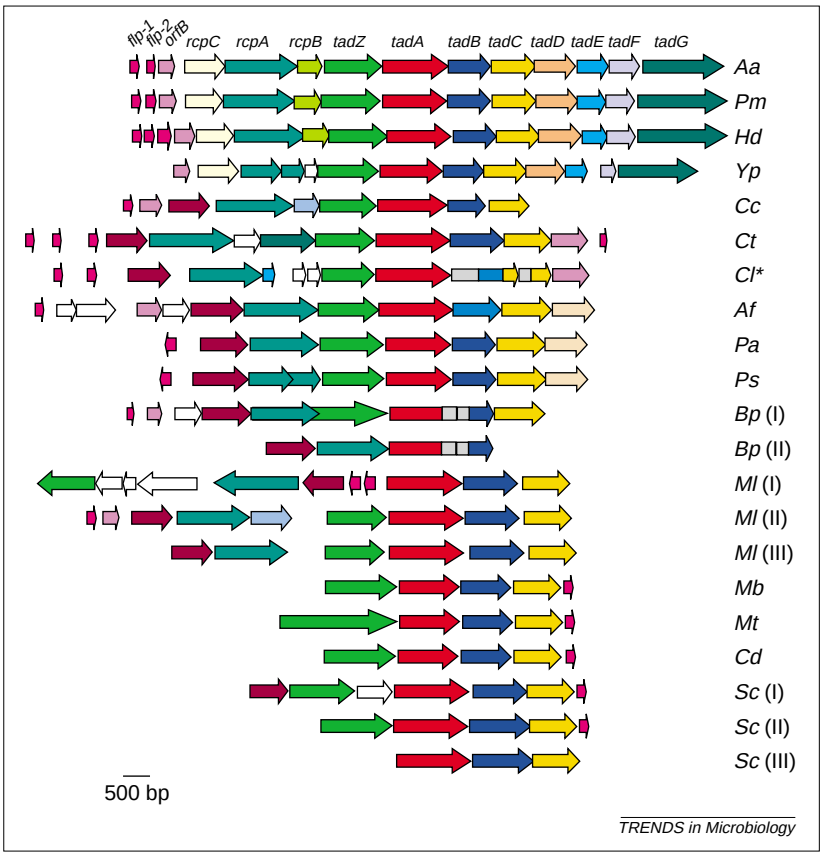

Fig. 3. Widespread *tad* loci. Open reading frames (ORFs) with similar predicted products (as determined by BLAST) have the same color. White ORFs showed no similarity to any other ORFs in the loci listed here. Roman numerals represent different *tad* loci in the same genome. Designations within certain ORFs indicate the ORF name/number given by the corresponding genome sequencing projects. Gray areas designate sequences that continue the similarity of the adjacent ORF with putative homologs, albeit in a different reading frame. It is possible that this results from sequence errors or frame-shift mutations. Aa, *Actinobacillus actinomycetemcomitans* (*tadA–G* AF152598; *flp-1* and *flp-2* AF320002; *rcpC* AF242856; *rcpA* AF139249; *rcpB* AF139250); Pm, *Pasteurella multocida* (AE006123); Hd, *Haemophilus ducreyi*; Yp, *Yersinia pestis*; Cc, *Caulobacter crescentus* (AAF40193); Ct, *Chlorobium tepidum*; Cl, *Chlorobium limicola* (pCL1) (U7780); Af, *Acidithiobacillus ferooxidans* (formerly *Thiobacillus ferrooxidans*); Pa, *Pseudomonas aeruginosa* (AE004846); Ps, *Pseudomonas syringae* pv. tomato; Bp, *Bordetella pertussis*; Ml, *Mesorhizobium loti* (http://www.kazusa.or.jp/rhizobase/java/chromo/cmap.html). The three clusters are indicated by their approximate positions in the chromosome: I = 5285–5300 kb; II = 4485–4496 kb; III = 715–725 kb; Mb, *Mycobacterium bovis*; Mt, *Mycobacterium tuberculosis* (AL022121); Cd, *Corynebacterium diphtheriae*; Sc, *Streptomyces coelicolor* (I = AL356813; II = AL35636; III = 160312). Preliminary sequence data for *C. tepidum*. *P. syringae* and *A. ferrooxidans* were obtained from The Institute for Genomic Research at http://www.tigr.org. *M. bovis, Y. pestis, C. diphtheriae* and *B. pertussis* sequences were produced by the respective Sanger Centre sequencing groups (http://www.sanger.ac.uk/); *A. actinomycetemcomitans*, by the University of Oklahoma's Advanced Center for Genome Technology (http://www.genome.ou.edu) and *H. ducreyi* at (http://www.htsc.washington.edu/hducreyi/info/index.cfm).

established that it is secreted by wild-type and *flp-1* mutant cells. However, as predicted by the model, Flp1 protein is not secreted from *tad* mutants (S.C. Kachlany *et al*., unpublished). Because the phylogenies of *tadA* and *flp* genes suggest that they have experienced an evolutionary history that is independent of other secretion systems[50,66], we propose that the *flp–rcp–tad* region specifies a novel system for the assembly and secretion of Flp pili, which in turn mediate tight adherence of *A. actinomycetemcomitans* to surfaces[44,50].

tad loci are widespread in Bacteria and Archaea

Completed and ongoing microbial genome-sequencing projects were initially searched for genes related to those of the *tad* locus of *A. actinomycetemcomitans.* Surprisingly, similar *tadABCEDFG* gene clusters appeared in the unfinished genomes of the bubonic plague bacterium, *Yersinia pestis*, and the human and animal pathogen, *Pasteurella multocida*. *Haemophilus ducreyi*, the causative agent of chancroid, also contains *tad*-like genes (E. Hansen, pers. commun.; *H. ducreyi* genome sequencing project). All three organisms harbor complete sets of very similar *tadA–tadG* genes in identical order[44]. Continued searches have led to the identification of *tad* loci in a wide variety of organisms from diverse habitats (Table 1). For example, the genomes of the human pathogen *Pseudomonas aeruginosa* and the photosynthetic, green sulfur bacterium *Chlorobium tepidum* contain ORFs very similar to *tadABC*. Furthermore, bacterial *tad* clusters were also closely linked to ORFs similar to genes of the *flp-1–tadA* region of the *A. actinomycetemcomitans tad* locus (Fig. 3). All of the available archaeal genome sequences contain ORFs with similarities to *tadA* and *tadB*. An interesting feature of the putative archaeal TadA proteins is that some contain all or part of an intein. Examples of *tad* loci from various bacteria are shown in Fig. 3.

tadA-like genes retrieved from *tad* loci form a distinct, monophyletic *tadA* subfamily within the type IV family of predicted secretion NTPase genes[66]. The *tadA* subfamily is extremely widespread and includes genes found in the Archaea and both Gram-negative and Gram-positive bacteria. Phylogenetic analysis of putative *flp* genes showed that they form a distinct subfamily of type IV pilin genes[50].

These phylogenies also indicate the possibility of horizontal transfer of *tadA* and *flp* genes and hint at the transfer of complete *flp–rcp–tad* regions. The low % G+C content of the *flp–rcp–tad* cluster compared with the rest of the genome in *A. actinomycetemcomitans, Y. pestis*, *H. ducreyi* and *P. multocida* is also consistent with the possibility of horizontal transfer of the locus.

It is remarkable that despite the widespread occurrence of *tad*-related loci in diverse bacteria (Table 1), their existence has gone unrecognized until recently. What are the functions of the *tad* loci in diverse microorganisms? In *A. actinomycetemcomitans*, the function is clearly the expression of fibrils responsible for tight adherence to surfaces. There is only one other example where the function of the *tad*-like genes is known. Jeff Skerker and Lucy Shapiro[72] have discovered a novel type of pili expressed by *Caulobacter crescentus,* an organism isolated from pond water. The function of these pili is unknown, but they are used as receptors for bacteriophage ϕCbK. By isolating phage-resistant mutants, these investigators identified a cluster of seven genes required for the expression of pili at the flagellar pole of the swarmer cell. The *C. crescentus* pilus-encoding region contains a pilin gene, *pilA*, which is a member of the *flp* subfamily (Box 2)[50], as well as six other putative homologs of genes of the *flp–rcp–tad* region (Fig. 3). Pilus synthesis

occurs during a short period in the cell cycle, with transcription of *pilA* positively regulated late in the cell cycle by the response regulator CtrA. Their results provide strong additional evidence that the *tad* loci in other bacteria are likely to be involved in the assembly and secretion of novel pili.

The *tad* loci of pathogens might be important for disease. A *tadD* mutant of *P. multocida* was recently reported to be attenuated for virulence in mice[73]. For the bubonic plague bacterium, *Y. pestis*, transmission to the human host occurs from the bite of an infected flea. When the flea bites its host, a blockage of the foregut caused by a large aggregate of *Y. pestis* bacteria will cause the flea to starve unless it regurgitates the aggregate into the bloodstream, thereby infecting the host. The requirements for autoaggregration have not been defined, and it is tempting to speculate that the *tad* locus of *Y. pestis* is involved. Like *A. actinomycetemcomitans*, *Y. pestis* has been reported to adhere to culture-vessel walls and clump when grown in broth[74].

The *tad* loci could be important for colonization by environmental bacteria. At present, there is no direct evidence for the role of *tad* genes in these bacteria, but it is interesting to note their adherence properties. One example, *Acidithiobacillus ferrooxidans*, is an iron- and sulfur-oxidizing bacterium responsible for acid mine drainage, a serious ecological problem[75]. *A. ferrooxidans* adheres tightly to sulfur granules and this adherence is required for growth[76,77]. Another environmental organism that contains the *tad* genes is *Chlorobium limicola*. This green sulfur bacterium is often found within adherent microbial mats, which are layered communities of interacting aquatic microorganisms such as cyanobacteria and diatoms[78]. In these mats, *C. limicola* is often found in aggregated microcolonies that closely associate with cyanobacteria. Therefore, one possibility is that the *tad* genes act in aggregation and adherence in microbial communities.

Outlook

The discovery of the *tad* genes has opened the door to an understanding of the remarkable tight adherence property of *A. actinomycetemcomitans*. Of considerable immediate importance is to determine how the proteins expressed by the *flp–rcp–tad* region result in the synthesis, assembly and secretion of Flp fibrils and how these components mediate non-specific adherence to abiotic surfaces. The surprising and exciting finding that related *tad* loci are widespread among Bacteria and Archaea indicates strongly that these systems provide important functions for the survival of diverse microorganisms, which include a wide range of pathogens and environmental bacteria. Because the *tad* genes of *A. actinomycetemcomitans* are clearly required for adherence and colonization, it seems likely that the *tad* loci of other microorganisms allow them to colonize specific environmental niches. For pathogens, these niches include the tissues of the infected host. We are excited by the possibility that future studies of the *tad* loci might lead to the development of new therapeutic agents to prevent the initiation of infections. Without question, studies of these intriguing loci will inevitably provide a deeper understanding of the ecology and evolution of microorganisms.

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