

Nonspecific Adherence by *Actinobacillus actinomycetemcomitans* Requires Genes Widespread in *Bacteria* and *Archaea*

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The gram-negative coccobacillus, *Actinobacillus actinomycetemcomitans*, is the putative agent for localized juvenile periodontitis, a particularly destructive form of periodontal disease in adolescents. This bacterium has also been isolated from a variety of other infections, notably endocarditis. Fresh clinical isolates of *A. actinomycetemcomitans* form tenacious biofilms, a property likely to be critical for colonization of teeth and other surfaces. Here we report the identification of a locus of seven genes required for nonspecific adherence of *A. actinomycetemcomitans* to surfaces. The recently developed transposon IS903 ϕ kan was used to isolate mutants of the rough clinical isolate CU1000 that are defective in tight adherence to surfaces (Tad⁻). Unlike wild-type cells, Tad⁻ mutant cells adhere poorly to surfaces, fail to form large autoaggregates, and lack long, bundled fibrils. Nucleotide sequencing and genetic complementation analysis revealed a 6.7-kb region of the genome with seven adjacent genes (*tadABCDEFG*) required for tight adherence. The predicted TadA polypeptide is similar to VirB11, an ATPase involved in macromolecular transport. The predicted amino acid sequences of the other Tad polypeptides indicate membrane localization but no obvious functions. We suggest that the *tad* genes are involved in secretion of factors required for tight adherence of *A. actinomycetemcomitans*. Remarkably, complete and highly conserved *tad* gene clusters are present in the genomes of the bubonic plague bacillus *Yersinia pestis* and the human and animal pathogen *Pasteurella multocida*. Partial *tad* loci also occur in strikingly diverse *Bacteria* and *Archaea*. Our results show that the *tad* genes are required for tight adherence of *A. actinomycetemcomitans* to surfaces and are therefore likely to be essential for colonization and pathogenesis. The occurrence of similar genes in a wide array of microorganisms indicates that they have important functions. We propose that *tad*-like genes have a significant role in microbial colonization.

Actinobacillus actinomycetemcomitans is a gram-negative bacterium associated with several human diseases (8, 36, 47). The most predominant of these is known as localized juvenile periodontitis, a severe disease of adolescents that is characterized by bone and tissue destruction and ultimately loss of teeth if untreated. *A. actinomycetemcomitans* is also a member of a clinically important group of bacteria implicated in infective endocarditis (39). These bacteria are referred to as the HACEK group (*Haemophilus aphrophilus*, *A. actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella kingae*) (9). Vegetative growths and inflammation of the heart valves caused by the HACEK bacteria result in serious complications owing to the formation of bacterial masses on the valves.

A. actinomycetemcomitans expresses several potential virulence factors (8), but only the RTX-type leukotoxin has been studied in detail at the molecular and genetic levels (14, 24). Other possible factors include a cytolethal distending toxin, iron and heme binding proteins, a trypsin-like protease, an OmpA family member, capsular polysaccharide biosynthetic proteins, catalase, and a GroEL-like protein (5, 12, 13, 23, 26, 28, 35, 41, 43, 46). However, their potential roles in pathogenesis are unknown. *A. actinomycetemcomitans* is also able to

invade epithelial cells, and the bacteria can be transferred between cells (8, 27).

A striking and characteristic property of fresh clinical isolates of *A. actinomycetemcomitans* is their ability to form tenacious biofilms on solid surfaces, including glass, plastics, and hydroxyapatite (6, 21, 30). This property is very likely required for pathogenesis by allowing for colonization of teeth in an environment of continuous salivary flow. Clinical isolates form rough-appearing colonies, autoaggregate, and express bundles of fimbria-like structures that may be important for adherence and colonization (18, 30, 34).

Genetic analysis of rough, adherent strains has proven to be difficult. The distinctive adherence property of clinical strains is easily lost, as nonadherent, smooth-colony variants readily emerge during subculture (7, 45). In addition, while DNA can be introduced into *A. actinomycetemcomitans* by transformation (37) or conjugation (11), the efficiency of DNA transfer into rough, adherent strains is too low for standard transposon mutagenesis protocols involving suicide vectors. Recently we reported the development of transposon IS903 ϕ kan, which carries a cryptic kanamycin resistance gene that can be activated upon insertion of the transposon into an expressed gene, and we have demonstrated its utility in the direct selection of random insertions in genetically recalcitrant bacteria, such as *A. actinomycetemcomitans* (38). Here we report the use of IS903 ϕ kan to obtain adherence-defective mutants of *A. actinomycetemcomitans* CU1000, a well-characterized rough clinical isolate (7, 20, 30). Genetic and nucleotide sequence anal-

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yses of these mutants have allowed us to identify a locus of seven novel genes that are required for tenacious adherence, autoaggregation, and the production of bundled fibers. Examination of genome sequences of *Bacteria* and *Archaea* revealed that surprisingly diverse microorganisms are predicted to carry *tad*-related genes. We discuss the likely function of these genes and the significance of their widespread occurrence.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *A. actinomycetemcomitans* CU1000N is a spontaneous nalidixic acid-resistant (Nal^r) derivative of the rough clinical isolate CU1000 (6). CU1060N is a Nal^r derivative of CU1060 (6), a spontaneous smooth-colony variant of CU1000. *A. actinomycetemcomitans* strains were stored and cultured as previously described (6). Bacteria were streaked from -70°C frozen stocks onto agar plates containing *A. actinomycetemcomitans* growth medium (AAGM) (11) and incubated in a sealed chamber enriched with 5% CO₂ at 37°C for several days. Individual colonies were inoculated into AAGM broth (11) in sealed, screw-cap tubes and incubated at 37°C. For light microscopy, bacteria were grown in broth for 1 day (CU1060N) or 2 days (CU1000N). For CU1000N, the cells were scraped from the walls and resuspended before preparation of wet mounts. To assay adherence of cells onto the surface of culture tubes, 2-day cultures were subjected to vortex mixing and the tube contents were discarded. An equal volume of 5-µg/ml ethidium bromide solution was added to the tubes, left for 10 min at room temperature, and then poured off, and the tubes were washed with water. The tubes were photographed in UV light.

IS903 ϕ kan mutagenesis of *A. actinomycetemcomitans*. Plasmid pVJT128, which carries a chloramphenicol resistance (Cm^r) gene and the cryptic IS903 ϕ kan transposon, was mobilized into rough strain CU1000N by conjugation with an *Escherichia coli* donor strain (SK338) that contains pVJT128 and an *oriT*-defective derivative of RK2, pRK21761 (38). CU1000N(pVJT128) transconjugants were selected on AAGM medium containing nalidixic acid (20 µg/ml) and chloramphenicol (4 µg/ml) and screened for sensitivity to kanamycin to confirm that pRK21761 had not transferred and that transposition of IS903 ϕ kan had not occurred. One such CU1000N(pVJT128) transconjugant was grown in AAGM broth containing chloramphenicol and then plated onto medium containing 4 µg of chloramphenicol per ml and 2 mM IPTG (isopropyl- β -D-thiogalactopyranoside) (to induce transposition of IS903 ϕ kan), and colonies were grown for 3 days. Several pools of colonies were each plated onto solid medium containing 20 or 40 µg of kanamycin per ml. Because the kanamycin-resistant (Km^r) colonies were small on this medium, the colonies from each pool were mixed and replated onto nonselective medium, where colony morphology was easily distinguished. To ensure that the mutants were independent isolates, only one smooth-colony mutant was kept from each original pool. Mutants were grown in broth in the absence of chloramphenicol to allow loss of the resident pVJT128 plasmid, as described previously (38).

DNA procedures. Preparation of plasmid DNA from *E. coli* was done by the alkaline lysis protocol (1). Agarose gel electrophoresis has been described previously (33). DNA manipulations with restriction endonucleases and T4 DNA ligase were done according to the manufacturers' recommendations. Amplification of DNA by PCR was done with *Taq* DNA polymerase (32). All cloned PCR products were confirmed by nucleotide sequencing. Transformation of *E. coli* was done by the method of Cohen et al. (4). Inverse PCR with IS903 ϕ kan insertion mutants was done as previously described (38). Approximately 10 to 20 µg of genomic DNA was digested with *Eco*RI (which does not cleave IS903 ϕ kan), followed by ethanol precipitation, dilution, and ligation to circularize genomic fragments. PCR amplification was carried out for 30 cycles using primers directed outward from the ends of IS903 ϕ kan. Amplified products were cloned into pCR2.1 (Invitrogen, Carlsbad, Calif.) or pT7blue-3 (Novagen, Madison, Wis.) for sequencing. Nucleotide sequence determination was done by the Columbia University DNA Sequencing Facility using a Perkin-Elmer Applied Biosystems Automated DNA sequencer 373A.

Genetic complementation analysis. The desired open reading frame (ORF) was amplified by PCR, cloned into pCR2.1, sequenced, and then subcloned into the IncQ expression vector pJAK16 (a gift of J. Kornacki), a derivative of the *tacp* expression vector pMMB67 (10). The pJAK16 derivatives were mobilized by conjugation from *E. coli* donors to the appropriate *A. actinomycetemcomitans* strains (38). Because the *tacp* promoter is leaky even in the presence of functional LacI repressor, complementation did not require induction by IPTG.

Electron microscopy. Colonies were resuspended in 0.1 M Tris-HCl (pH 7.5), and a drop of the suspension was placed on a Formvar- and carbon-coated copper grid (EM Sciences, Fort Washington, Pa.) and left for approximately 10 min. Grids were immersed in a drop of 1% uranyl acetate and removed immediately; the excess stain was wicked away. Grids were viewed in a JEOL 1200EX transmission electron microscope at 80.0 kV.

Sequence analysis and similarity searching. Nucleotide sequences potentially coding for proteins similar to the *tad* gene products were identified using the BLAST alignment program to search both GenBank and the Unfinished Genomes Database available on the National Center for Biotechnology Information web page (<http://www.ncbi.nlm.nih.gov/BLAST/unfinishedgenome.html>). De-

fault settings of the basic BLAST program were used. All reported sequences significantly exceeded the default criteria and were among the best hits when compared to the corresponding Tad polypeptides. Contiguity and sequential order of *tad*-related ORFs were established using the ORF finder program to characterize the region around each *tad*-like sequence. The putative products of all neighboring ORFs were compared to sequences in GenBank using the pBlast function. Preliminary sequence data for *Methanococcus jannaschii*, *Archeoglobus fulgidus*, and *Chlorobium tepidum* were obtained from The Institute for Genomic Research website at <http://www.tigr.org>. *Bordetella pertussis* and *Yersinia pestis* sequences were produced by the respective Sanger Centre sequencing groups (<http://www.sanger.ac.uk/>). *Pyrococcus horikoshii* sequences were produced by NITE (<http://www.nite.go.jp/>), the *Pyrococcus abyssi* sequence was produced by Genoscope (<http://www.genoscope.cns.fr/>), the *Pyrococcus furiosus* sequence was produced by the Utah Genome Center (<http://www.genome.utah.edu/>), the *Pseudomonas aeruginosa* sequence was produced by the *Pseudomonas* Genome Project (<http://www.pseudomonas.com/>), the *Methanobacterium thermoautotrophicum* sequence was produced by the Genome Therapeutics Center (<http://www.genomecorp.com/>), and the *Pasteurella multocida* sequence was produced by the University of Minnesota *P. multocida* Genome Project (<http://www.cbc.umn.edu/ResearchProjects/AGAC/Pm/index.html>). A compilation of Intein sequences is found in InBase, the New England BioLabs Intein Database (<http://www.neb.com/inteins>). Accession numbers for *tadA*- and *tadB*-related sequences, respectively, in other organisms are as follows: *M. jannaschii*, Q58191 and Q58189; *P. abyssi*, CAB50295 and CAB50294; *P. horikoshii*, BAA29741 and BAA29744; *A. fulgidus*, AAB90582 and AAB90583; and *M. thermoautotrophicum*, AAB86174 and AAB85476. The GenBank accession number for plasmid pC1 from *Chlorobium limicola* is U77780, that for K1bA (TrbB) is C44020, and that for VirB11 is AAA88655.

Nucleotide sequence accession number. The nucleotide sequence of the 6,704-bp *tad* region has been submitted to GenBank (accession no. AF152598).

RESULTS AND DISCUSSION

Isolation of adherence-defective mutants by transposon mutagenesis. *A. actinomycetemcomitans* strain CU1000 was originally isolated from a 13-year-old, African-American female with localized juvenile periodontitis (7). To facilitate the study and genetic manipulation of CU1000, we isolated a spontaneous nalidixic acid-resistant variant, CU1000N. Like its parent, CU1000N exhibits the characteristic rough, opaque colony morphology typical of fresh clinical isolates (Fig. 1a). In broth, CU1000N adheres tightly to the walls of the culture vessels (Fig. 1d). The biofilm is resistant to vortex mixing or vigorous agitation, and the bacteria must be physically scraped from the walls. The rough colony phenotype can be maintained indefinitely during subcultures on solid medium. Rough *A. actinomycetemcomitans* strains give rise to nonadherent, planktonic variants, whose colonies on solid medium are smooth, translucent, and larger than those of rough strains (7, 20, 30, 45) (Fig. 1e and h). One such derivative of CU1000 is CU1060 (7), and its Nal^r derivative is CU1060N. Cells of the smooth, nonadherent variants, including CU1060N, do not display the striking bundles of parallel, fimbria-like fibers normally present in abundance on cells of rough strains (Fig. 1c and 2). It has been suggested that these fibrils mediate adherence (30, 34). Cells of the rough strains and smooth variants also differ dramatically in their ability to autoaggregate (Fig. 1b and f).

To investigate the molecular mechanism of tight, nonspecific adherence of *A. actinomycetemcomitans* to surfaces, we first set out to identify the genetic determinants involved. We exploited the apparent correlation of smooth colony morphology with nonadherence to isolate transposon insertion mutants that are defective in adherence. We induced transposition of IS903 ϕ kan in the rough strain CU1000N, and insertion mutants were selected by plating on medium containing kanamycin. Several independent Km^r mutants exhibiting a smooth colony phenotype were chosen for study. Southern blot analysis revealed a single IS903 ϕ kan insertion in the chromosome of each smooth mutant (data not shown).

On solid medium, the mutants form smooth-appearing colonies (Table 1; Fig. 3a), although the colony size was routinely smaller than that observed for the spontaneous smooth strain

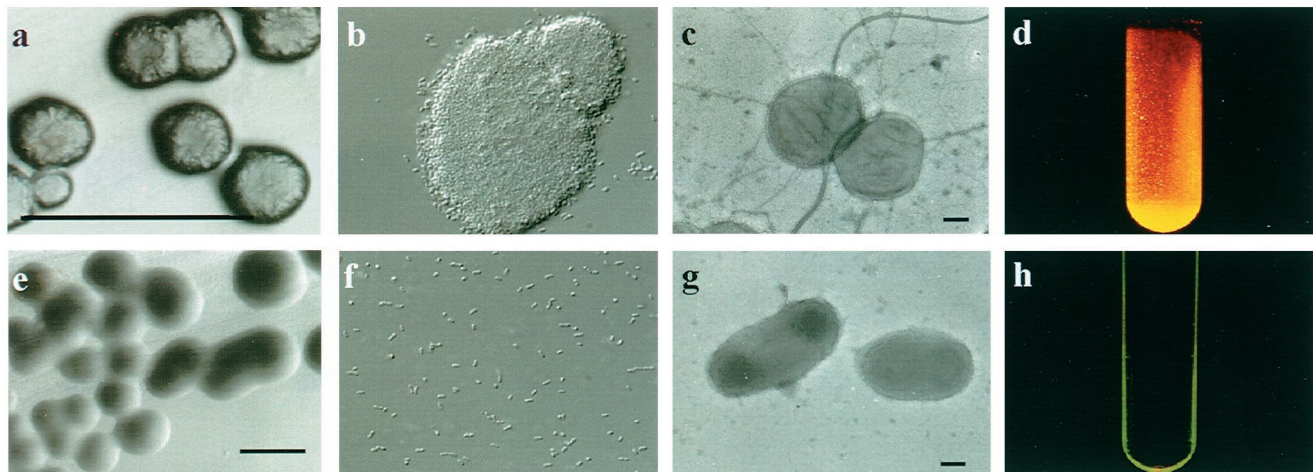


FIG. 1. Phenotypes of isogenic rough- and smooth-colony strains of *A. actinomycetemcomitans*. Top panels, rough-colony strain CU1000N; bottom panels: smooth-colony variant CU1060N. (a and e) Images from stereomicroscopy of 3-day-old colonies. Bars, 1.0 mm. (b and f) Images from light microscopy of bacterial cells using differential interference contrast optics. (c and g) Images from transmission electron microscopy of negatively stained cells. Bars, 0.2 μ m. (d and h) Adherence of cells to the culture tube surface, as visualized by fluorescence of ethidium bromide-stained cells in UV light.

CU1060N. The mutants showed obvious defects in surface adherence in broth culture (Table 1). All mutants grew in loose association with the walls of the culture tubes, and cells were easily dispersed into suspension upon vortex mixing. This phenotype differs markedly from the tight adherence (*Tad*⁺) phenotype of the parental rough strain CU1000N, and it is also

distinct from the complete nonadherence phenotype of the spontaneous smooth isolate CU1060N, in which the bacteria show no association with the walls of the culture tube. Light microscopy of the mutants revealed that the ability of the cells to autoaggregate was greatly reduced, but not abolished, relative to that of the rough strain (data not shown). The dimin-

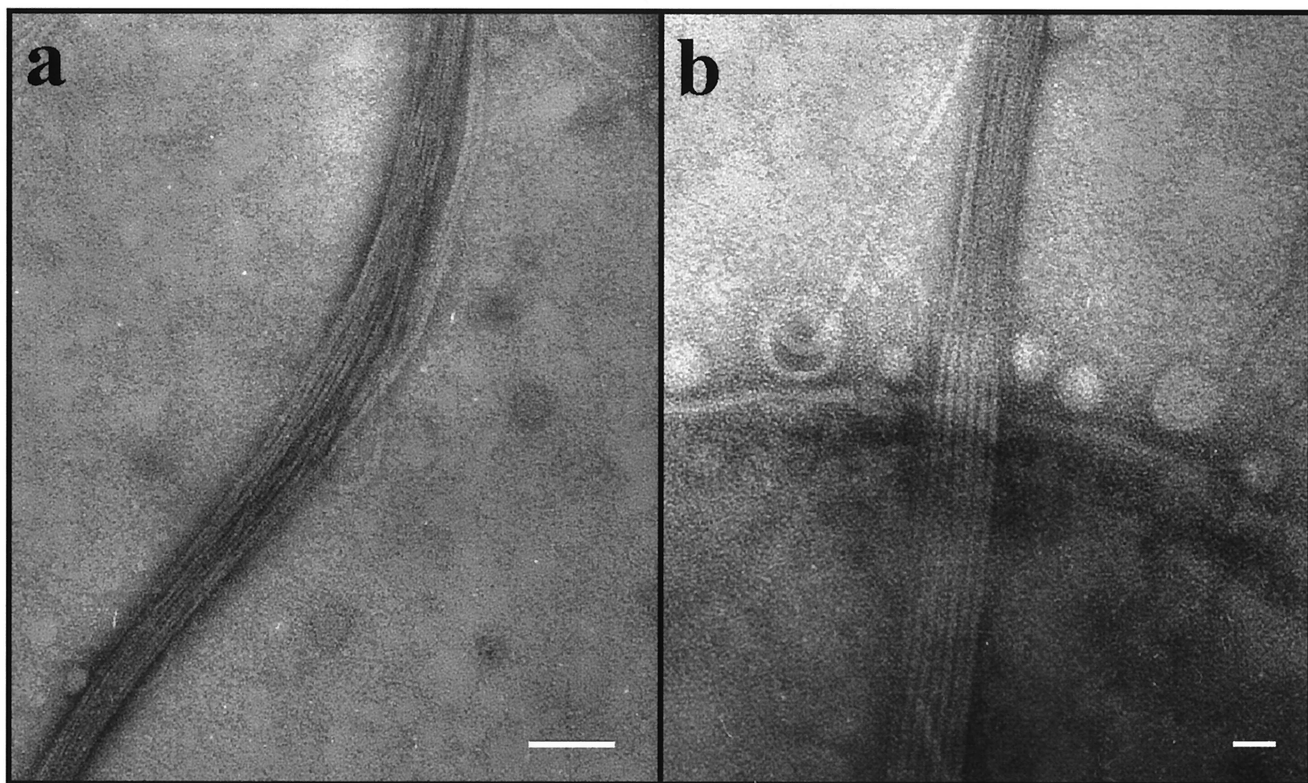


FIG. 2. Electron micrograph of fibril bundles produced by the rough strain CU1000N. (a) Fibril bundle showing individual strands separating from the bundle at several places. Bar, 50 nm. (b) Parallel array of six or seven fibrils passing over the edge of a bacterial cell. The spherical structures at the bacterial cell surface are likely to be membranous vesicles, which are commonly seen in preparations of rough strains of *A. actinomycetemcomitans* (30). Bar, 20 nm.

TABLE 1. Phenotypes and genetic complementation of *tad* mutants

Strain	Genotype of:		Phenotype		
	Strain	Test plasmid ^a	Colony ^b	Adherence	Fibrils ^c
CU1000N	Wild type rough	None	R++	+	+
CU1060N	Smooth	None	S	–	–
		<i>tadA-G</i>	S	–	–
		<i>flp-tadG</i>	R+	+	+
Aa1360	<i>tadA</i>	Vector	S	–	–
		<i>tadA</i> ⁺	R+	+	+
Aa1332	<i>tadB</i>	Vector	S	–	–
		<i>tadB</i> ⁺	R+	+	+
Aa1359	<i>tadC</i>	Vector	S	–	–
		<i>tadC</i> ⁺	R+	+	+
Aa1577	<i>tadD</i>	Vector	S	–	–
		<i>tadC</i> ⁺ <i>D</i> ⁺	R+	+	+
Aa1347	<i>tadE</i>	Vector	S	–	–
		<i>tadE</i> ⁺	R+	+	+
Aa1512	<i>tadF</i>	Vector	S	–	–
		<i>tadF</i> ⁺	R+	+	+
Aa1561	<i>tadG</i>	Vector	S	–	–
		<i>tadG</i> ⁺	R+	+	+

^a The vector is *tacp* promoter plasmid pJAK16 (see Materials and Methods). Complementation did not require induction by IPTG.

^b R, rough; S, smooth.

^c Determined by electron microscopy as described for Fig. 1.

ished autoaggregation parallels the behavior of the resuspended mutants in broth. Cells of the parental rough strain (CU1000N) settle in clumps within minutes after being scraped from the walls and resuspended, whereas *Tad*[–] mutant cells formed clumps and settled to the bottom of the tube only after several hours. Cells of the spontaneous smooth-colony variant (CU1060N) grow in suspension and do not settle.

Identification of seven *tad* genes. To map the sites of IS903 ϕ kan insertion for several *Tad*[–] mutants, we did inverse PCR on genomic DNA with primers directed outward from the ends of the transposon (38). Using these sequences and partial nucleotide sequences from the ongoing *A. actinomycesetmcomitans* Genome Sequencing Project to design additional PCR primers, we found that all of the insertions mapped to a continuous 7-kb region of the *A. actinomycesetmcomitans* genome. We determined the sequence for both strands of this region for the rough strain CU1000N. The sequence revealed a cluster of seven closely spaced ORFs oriented in the same direction (Fig. 4). At least one IS903 ϕ kan insertion mutant was isolated for each ORF, and all mutants were defective in adherence (Table 1) and autoaggregation (data not shown). Genetic complementation analysis with the appropriate ORFs on plasmids showed that the IS903 ϕ kan insertions are not polar on expression of downstream genes (Table 1). The complemented *Tad*[–] mutants produced rough-appearing colonies on solid medium, although not to the same degree as those of the wild-type rough strain (Fig. 3a, d, and g), presumably due to nonstoichiometric levels of expression from the plasmid-borne genes. However, cells of the complemented mutants clearly regained the tight adherence phenotype of the rough parental strain (Fig. 3c, f, and i) and the ability to autoaggregate (data not shown). All mutants with insertions in different ORFs were examined by electron microscopy, and all were found to lack

fibrils (Fig. 3b), unless the complementing plasmid was present (Table 1, Fig. 3h). Controls with the vector (Fig. 3d, e, and f) or plasmids with noncognate ORFs (Table 1) showed no evidence of complementation for any of the phenotypes. These results confirmed that all seven ORFs are required for tight adherence, and we therefore designated them genes *tadA*, *tadB*, *tadC*, *tadD*, *tadE*, *tadF*, and *tadG*.

Tad proteins may constitute a fibril secretion system. We searched GenBank to identify other proteins that are related to the *tad* gene products. The predicted polypeptide products of the *tadB*, *tadC*, *tadD*, *tadE*, *tadF*, and *tadG* genes showed no significant similarities to any other proteins of known function in the database. However, PSORT (29) and TMPred (17) predicted the presence of six (TadB), five (TadC), and one (TadD, TadE, TadF, and TadG) membrane-spanning regions. Thus, these proteins are likely to be localized to the membrane.

The predicted amino acid sequence of the TadA polypeptide showed significant similarity (32% identity and 51% similarity) to VirB11, a protein involved in the export of macromolecules from bacterial cells (2). VirB11 is required for the transfer of T-DNA from the tumor-inducing bacterium *Agrobacterium tumefaciens* to plant cells (3). Other members of the VirB11 family include TrbB, which is required for the formation of bacterial mating pairs and conjugative transfer of the promiscuous antibiotic resistance plasmid RP4 (25), and PtlH of *B. pertussis*, which is needed for secretion of the pertussis toxin (42). These proteins are cytoplasmic but act at the inner membrane, possibly through interaction with a membrane protein (2). The VirB11 family of proteins all contain a Walker nucleotide-binding motif (40), as does TadA, and they are believed to provide energy for the export apparatus by hydrolysis of ATP (2). However, robust phylogenetic analyses have shown that *tadA* is the prototype of a distinct and novel gene subfamily that does not include *trbB*, *virB11*, or *ptlH* (P. J. Planet, S. C. Kachlany, R. DeSalle, and D. H. Figurski, unpublished results).

Genes for other proteins specifically expressed by rough *A. actinomycesetmcomitans* strains map near the *tad* gene cluster. Haase et al. (15) recently identified two rough strain-specific outer membrane proteins expressed from adjacent genes, *rcpA* and *rcpB*. We found that these genes map upstream of the *tad* region, separated from *tadA* by an ORF whose function is unknown. At present the *rcp* gene products have not been implicated in adherence, but RcpA is similar to the GspD family of outer membrane proteins involved in secretion and pilus assembly (15, 31). Inoue et al. (19) have identified a 6.5-kDa protein (Flp) as a fibril subunit, and the *flp* gene was also found to map in the vicinity of the *tad* region, upstream of *rcpA*. It has been suggested by others that the fibrils displayed by rough strains of *A. actinomycesetmcomitans* are responsible for surface adherence (18, 30, 34), which is consistent with our finding that all seven *tad* genes are required for adherence and fibril formation (Table 1). We found that a plasmid carrying the complete *tadABCDEFG* region from the rough strain is able to complement mutations in any of the *tad* genes (data not shown), but it is unable to complement the spontaneous smooth strain CU1060N (Table 1). However, a plasmid containing the upstream region, beginning from *flp* and continuing through *tadG*, is fully able to complement the spontaneous smooth strain to a rough, adherent phenotype (Table 1). Thus, it is likely that the entire 12-kb *flp-tadG* region is responsible for the tight adherence properties of *A. actinomycesetmcomitans*. We also have preliminary evidence that *flp* is required for fibril formation and adherence (S. C. Kachlany, P. J. Planet, R. Aussenberg, D. H. Figurski, D. H. Fine, and J. B. Kaplan,

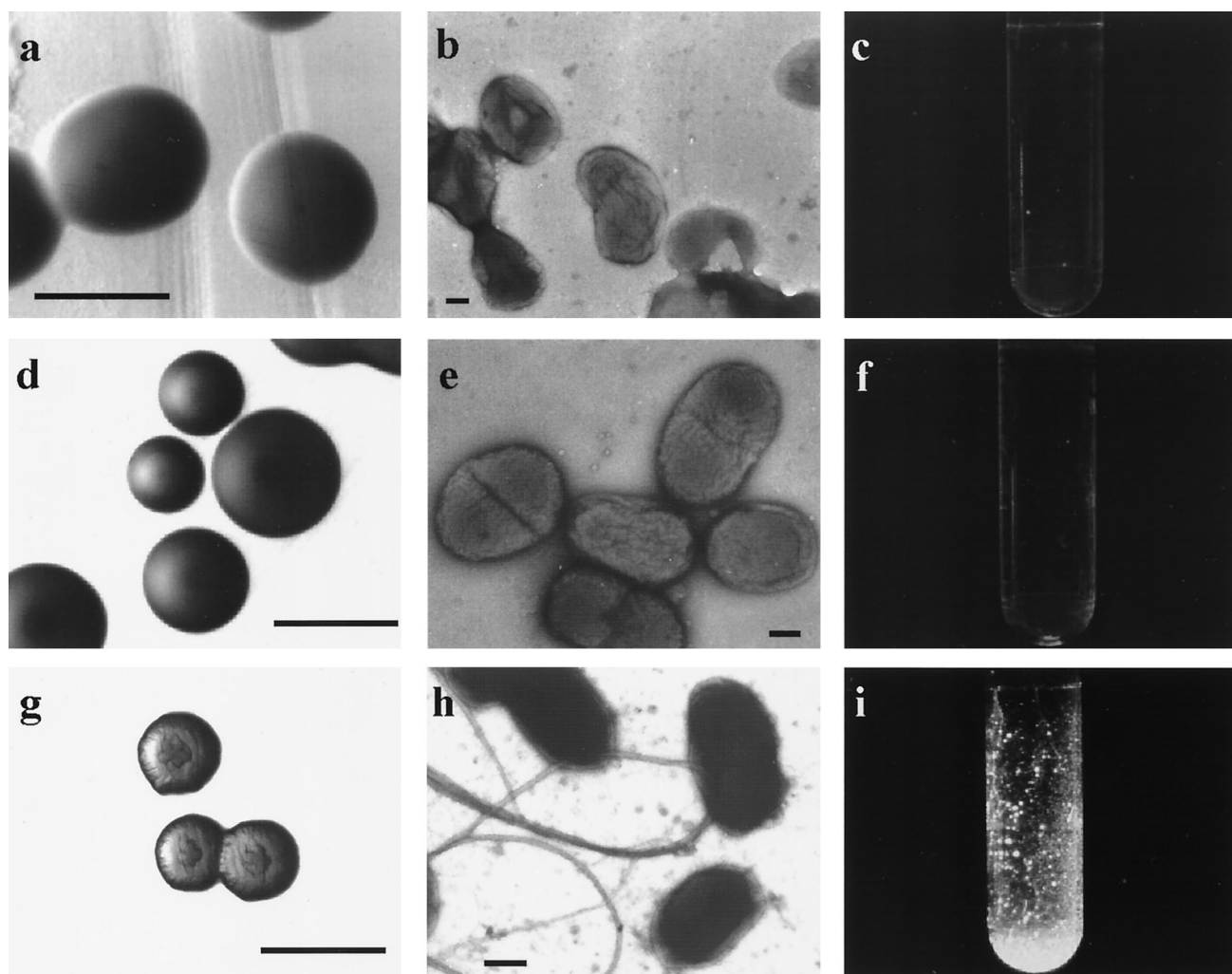


FIG. 3. Phenotypes and genetic complementation of a *tadC* mutant. Top panels, mutant strain Aa1359 (*tadC*::IS903 ϕ kan). Middle panels, strain Aa1359 containing the pJAK16 vector plasmid. Bottom panels, Aa1359 containing pJAK16 with the cloned *tadC* ORF from CU1000N. The methods used are identical to those described for Fig. 1. Results for complementation of the other mutants were essentially the same (Table 1). Bars, 1.0 mm (a, d, and g), 0.2 μ m (b and e), and 0.5 μ m (h).

unpublished data). Furthermore, new evidence implicates a similar locus in fibril formation in the distantly related α -proteobacteria. A region of the *Caulobacter crescentus* genome was found to contain highly similar genes in conserved order with the *flp-tadA* region of *A. actinomycetemcomitans*, and the genes were shown to be required for pili formation (35a). Because *A. actinomycetemcomitans* TadA is related to proteins involved in macromolecular transport and the other Tad proteins are potentially localized to the membrane, we suggest that the Tad proteins constitute a novel system involved in the export of factors (possibly RcpA, RcpB, or Flp) that are required for fibril formation and tight adherence of *A. actinomycetemcomitans*.

***tad* genes are widespread.** We also compared each Tad protein individually to the Unfinished Microbial Genomes Database, and we were surprised to find significant similarities for each of the Tad proteins with predicted proteins of unknown function in *Y. pestis*, the causative agent of bubonic plague, and *P. multocida*, a pathogen that infects humans and animals. The coding regions for the Tad-like proteins in both organisms show all seven ORFs in an arrangement identical to that of the *tadABCDEFG* region of *A. actinomycetemcomitans*. Not only

do the predicted *P. multocida* and *Y. pestis* proteins show significant percent identities with the predicted Tad proteins, but the sizes and isoelectric points (pIs) of the corresponding proteins are strikingly similar (Table 2). Recently, a complete *tad* region coding for putative polypeptides with high similarities to the seven *A. actinomycetemcomitans* *tad* gene products has been identified in the genome of *Haemophilus ducreyi*, the causative agent of chancroid (E. J. Hansen, personal communication).

BLAST searches also identified partial *tad* loci in a remarkable variety of organisms among the domains *Bacteria* and *Archaea* (44). Genomic regions containing *tad*-like ORFs in the same order and predicted to code for polypeptides with significant similarities to TadA (>63%), TadB (>41%), and TadC (>46%) were found in several bacterial genomes (Fig. 4), including *P. aeruginosa*, *C. tepidum*, *C. crescentus*, *Thiobacillus ferrooxidans*, *B. pertussis*, and an endogenous plasmid (pCL1) of *C. limicola*. In addition, *Sinorhizobium meliloti* and *Bordetella bronchiseptica* also contain ORFs whose predicted products show strong similarity to TadA, TadB, and TadC, but the contiguous order of the ORFs cannot yet be established because they appear on separate sequence contigs. We have also

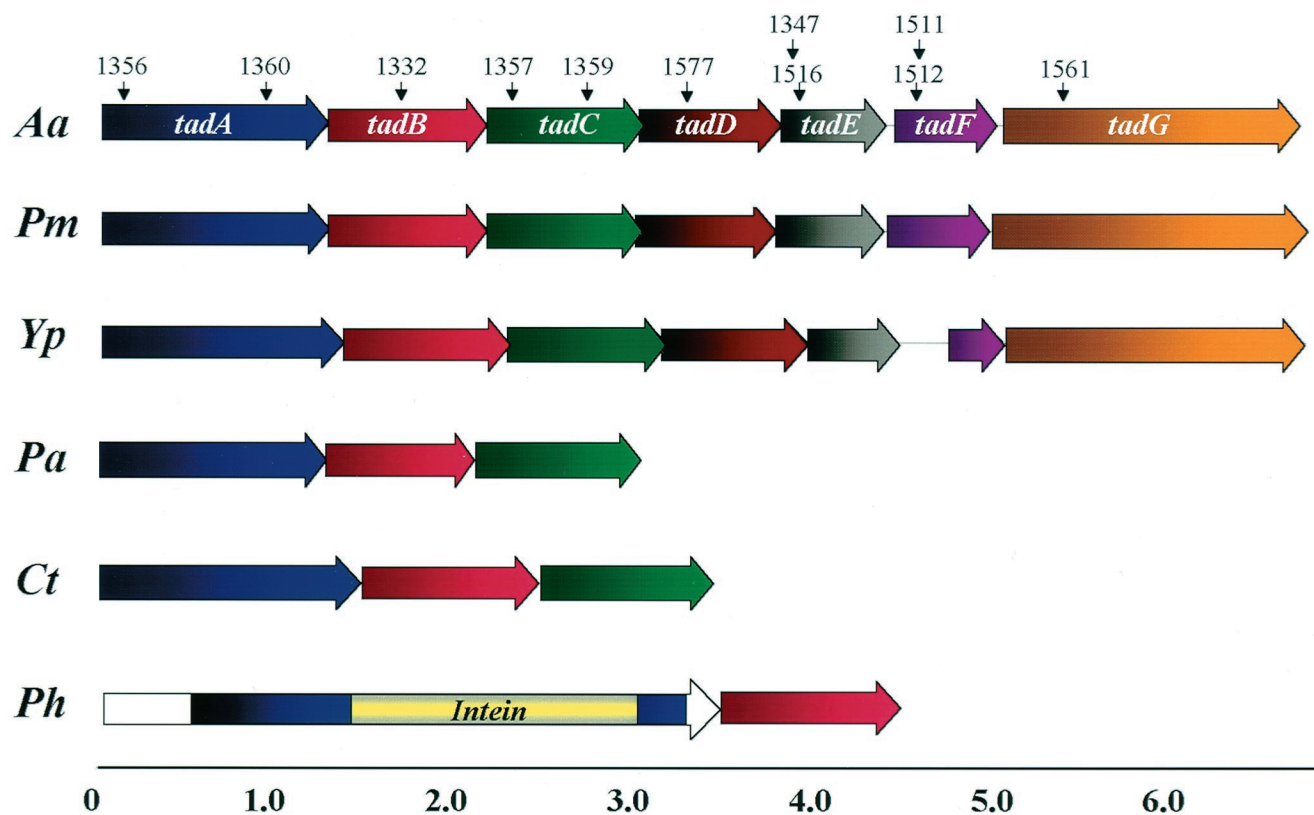


FIG. 4. Arrangement of *tad* genes in *A. actinomycetemcomitans* and other microorganisms. The top line shows the *tadABCDEF* genes of *A. actinomycetemcomitans* (*Aa*) strain CU1000N. The locations of the IS903 ϕ kan insertions in specific mutant strains are indicated by downward arrows. Below are *tad*-related genes from *P. multocida* (*Pm*), *Y. pestis* (*Yp*), *P. aeruginosa* (*Pa*), *C. tepidum* (*Ct*), and *P. horikoshii* (*Ph*). The *tad* sequences for organisms other than *A. actinomycetemcomitans* were obtained from individual genome sequencing projects as described in Materials and Methods. The scale bar at the bottom is in kilobase pairs.

obtained evidence by PCR and nucleotide sequence analysis for the presence of *tad*-related genes in a strain of *H. aphrophilus* originally isolated from a patient with bacterial endocarditis, *Y. enterocolitica*, and *Yersinia pseudotuberculosis* (data not shown). However, we found no evidence of *tad*-like genes in any of several nonpathogenic *Yersinia* species. Remarkably, adjacent ORFs whose predicted polypeptide products have significant similarity to TadA (>58%) and TadB (>38%) exist in nearly all *Archaea* whose genomes have been partly or completely sequenced. These include *P. furiosus*, *P. horikoshii* (Fig. 4), *P. abyssi*, *M. thermoautotrophicum*, *M. jannaschii*, and *A. fulgidus*. Several of the archaeal TadA sequences contain all or part of the KlbA Intein (Fig. 4). The KlbA Intein was first identified in a polypeptide with similarity to the KlbA (TrbB) protein of promiscuous plasmid RK2 (New England BioLabs Intein Database). We found that this polypeptide has even greater similarity to *A. actinomycetemcomitans* TadA (40% identity over 413 amino acids) than to KlbA (35% identity over 297 amino acids) or VirB11 (32% identity over 259 amino acids).

The presence of a complete *tad* region in *A. actinomycetemcomitans*, *Y. pestis*, *P. multocida*, and *H. ducreyi* raises the question of whether these genes are conserved by descent or acquired through horizontal gene transfer. We found that the G+C contents of the complete *tad* regions are similar to each other (35 to 36%) but significantly different from those of the resident genomes for which sequence data exist (48% for *A. actinomycetemcomitans* [22] and 47% for *Y. pestis* [Sanger Centre]). These results are consistent with the hypothesis that the

TABLE 2. Similarities among the *A. actinomycetemcomitans*, *P. multocida*, and *Y. pestis* Tad proteins

Protein	Source	Molecular mass (kDa)	Theoretical pI	Identity to <i>A. actinomycetemcomitans</i> Tad protein (%)
TadA	<i>A. actinomycetemcomitans</i>	47.1	5.51	
	<i>P. multocida</i>	47.1	5.22	85
	<i>Y. pestis</i>	48.1	5.84	60
TadB	<i>A. actinomycetemcomitans</i>	33.4	9.30	
	<i>P. multocida</i>	34.4	9.86	70
	<i>Y. pestis</i>	33.7	9.70	33
TadC	<i>A. actinomycetemcomitans</i>	32.2	9.13	
	<i>P. multocida</i>	31.9	9.50	73
	<i>Y. pestis</i>	31.6	9.55	29
TadD	<i>A. actinomycetemcomitans</i>	28.4	9.27	
	<i>P. multocida</i>	28.7	9.45	59
	<i>Y. pestis</i>	28.7	7.82	35
TadE	<i>A. actinomycetemcomitans</i>	21.7	9.42	
	<i>P. multocida</i>	22.4	8.87	54
	<i>Y. pestis</i>	17.9	5.91	27
TadF	<i>A. actinomycetemcomitans</i>	22.9	9.52	
	<i>P. multocida</i>	21.7	9.47	47
TadG	<i>A. actinomycetemcomitans</i>	60.4	9.32	
	<i>P. multocida</i>	66.6	8.66	42
	<i>Y. pestis</i>	58.5	5.72	21

tad regions were inserted into these genomes following horizontal gene transfer from an as-yet-unidentified source.

The striking adherence-defective phenotype of the Tad⁻ mutants of *A. actinomycetemcomitans* and the conservation of apparently nonessential *tad* genes in many microorganisms raise the possibility that these genes are important for establishment of the organisms in their environments. Like *A. actinomycetemcomitans*, *Y. pestis* can form crinkled colonies and adhere to the walls of a culture vessel (9). In addition, for *Y. pestis* to be transmitted from the flea vector to the human host, the bacteria must form large clumps which obstruct the proventriculus of the flea (16). The starving flea eventually regurgitates the clumped bacteria into the bloodstream of the host, thereby transmitting plague. The Tad proteins, which we have shown are required for autoaggregation of *A. actinomycetemcomitans*, may likewise be involved in the clumping of *Y. pestis* in the flea. Much less is known about the genetic basis for virulence in *P. multocida*, which causes a variety of animal infections, such as fowl cholera, atrophic rhinitis in pigs, and hemorrhagic septicemia in cattle, resulting in the loss of several hundred million dollars annually in the animal industry (9). *P. multocida* is frequently found in the oral cavities of cats and dogs, and it is the most common organism isolated from human wounds inflicted by bites and scratches of cats and dogs. By analogy with *A. actinomycetemcomitans*, the *tad* genes of *P. multocida* may be important for adherence and colonization of the oral cavity. The elucidation of the specific roles of the Tad proteins in adherence and autoaggregation will be important to understanding *A. actinomycetemcomitans* colonization and pathogenesis. This knowledge may also provide significant clues about the functions of Tad proteins in other microorganisms.

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REFERENCES

- Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1989. Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
- Christie, P. J. 1997. *Agrobacterium tumefaciens* T-complex transport apparatus: a paradigm for a new family of multifunctional transporters in eubacteria. *J. Bacteriol.* **179**:3085–3094.
- Christie, P. J., J. E. Ward, M. P. Gordon, and E. W. Nester. 1989. A gene required for transfer of T-DNA to plants encodes an ATPase with autophosphorylating activity. *Proc. Natl. Acad. Sci. USA* **86**:9677–9681.
- Cohen, S. N., A. C. Y. Chang, and L. Hsu. 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. *Proc. Natl. Acad. Sci. USA* **69**:2110–2114.
- Dongari, A. I., and K. T. Miyasaki. 1991. Sensitivity of *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* to oxidative killing. *Oral Microbiol. Immunol.* **6**:363–372.
- Fine, D. H., D. Furgang, J. Kaplan, J. Charlesworth, and D. H. Figurski. 1999. Tenacious adhesion of *Actinobacillus actinomycetemcomitans* strain CU1000 to salivary coated hydroxyapatite. *Arch. Oral Biol.* **44**:1063–1076.
- Fine, D. H., D. Furgang, H. C. Schreiner, P. Goncharoff, J. Charlesworth, G. Ghazwan, P. Fitzgerald-Bocarsly, and D. H. Figurski. 1999. Phenotypic variation in *Actinobacillus actinomycetemcomitans* during laboratory growth: implications for virulence. *Microbiology* **145**:1335–1347.
- Fives-Taylor, P. M., D. H. Meyer, K. P. Mintz, and C. Brissette. 1999. Virulence factors of *Actinobacillus actinomycetemcomitans*. *Periodontology* **2000** **20**:136–167.
- Forbes, B. A., D. F. Sahn, and A. S. Weissfeld. 1998. Bailey & Scott's diagnostic microbiology. Mosby, Inc., St. Louis, Mo.
- Fürste, J. P., W. Pansegrau, R. Frank, H. Blocker, P. Scholz, M. Bagdasarjan, and E. Lanka. 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. *Gene* **48**:119–131.
- Goncharoff, P., J. K. K. Yip, H. Wang, H. C. Schreiner, J.-A. Pai, D. Furgang, R. H. Stevens, D. H. Figurski, and D. H. Fine. 1993. Conjugal transfer of broad-host-range incompatibility group P and Q plasmids from *Escherichia coli* to *Actinobacillus actinomycetemcomitans*. *Infect. Immun.* **61**:3544–3547.
- Goulhen, F., A. Hafezi, V.-J. Uitto, D. Hinode, R. Nakamura, D. Grenier, and D. Mayrand. 1998. Subcellular localization and cytotoxic activity of the GroEL-like protein isolated from *Actinobacillus actinomycetemcomitans*. *Infect. Immun.* **66**:5307–5313.
- Graber, K. R., L. M. Smoot, and L. A. Actis. 1998. Expression of iron binding proteins and hemin binding activity in the dental pathogen *Actinobacillus actinomycetemcomitans*. *FEMS Microbiol. Lett.* **163**:135–142.
- Guthmiller, J. M., D. Kolodrubetz, and E. Kraig. 1995. Mutational analysis of the putative leukotoxin transport genes in *Actinobacillus actinomycetemcomitans*. *Microb. Pathog.* **18**:307–321.
- Haase, E. M., J. L. Zmuda, and F. A. Scannapieco. 1999. Identification and molecular analysis of rough-colony-specific outer membrane proteins of *Actinobacillus actinomycetemcomitans*. *Infect. Immun.* **67**:2901–2908.
- Hinnebusch, B. J., R. D. Perry, and T. G. Schwan. 1996. Role of the *Yersinia pestis* hemin storage (*hms*) locus in the transition of plague by fleas. *Science* **273**:367–370.
- Hofmann, K., and W. Stoffel. 1993. TMbase—a database of membrane spanning protein segments. *Biol. Chem. Hoppe-Seyler* **347**:166.
- Holt, S. C., A. C. Tanner, and S. S. Socransky. 1980. Morphology and ultrastructure of oral strains of *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*. *Infect. Immun.* **30**:588–600.
- Inoue, T., I. Tanimoto, H. Ohta, K. Kato, Y. Murayama, and K. Fukui. 1998. Molecular characterization of low-molecular-weight component protein, Flp, in *Actinobacillus actinomycetemcomitans* fimbriae. *Microbiol. Immunol.* **42**:253–258.
- Inouye, T., H. Ohta, S. Kokeguchi, K. Fukui, and K. Kato. 1990. Colonial variation and fibrillation of *Actinobacillus actinomycetemcomitans*. *FEMS Microbiol. Lett.* **57**:13–17.
- Kagermeier, A. S., and J. London. 1985. *Actinobacillus actinomycetemcomitans* strains Y4 and N27 adhere to hydroxyapatite by distinctive mechanisms. *Infect. Immun.* **47**:654–658.
- Kaplan, J. B., and D. H. Fine. 1998. Codon usage in *Actinobacillus actinomycetemcomitans*. *FEMS Microbiol. Lett.* **163**:31–36.
- Komatsuzawa, H., T. Kawai, M. E. Wilson, M. A. Taubman, M. Sugai, and H. Suginaka. 1999. Cloning of the gene encoding the *Actinobacillus actinomycetemcomitans* serotype b OmpA-like outer membrane protein. *Infect. Immun.* **67**:942–945.
- Lally, E. T., and I. R. Kieba. 1994. Molecular biology of *Actinobacillus actinomycetemcomitans* leukotoxin, p. 69–82. In R. Genco, S. Hamada, T. Lehner, J. McGhee, and S. Mergenhagen (ed.), *Molecular pathogenesis of periodontal disease*. American Society for Microbiology, Washington, D.C.
- Lessl, M., D. Balzer, W. Pansegrau, and E. Lanka. 1992. Sequence similarities between the RP4 Tra2 and the Ti VirB region strongly support the conjugation model for T-DNA transfer. *J. Biol. Chem.* **267**:20471–20480.
- Mayer, M. P. A., L. C. Bueno, E. J. Hansen, and J. M. DiRienzo. 1999. Identification of a cytolethal distending toxin gene locus and features of a virulence-associated region in *Actinobacillus actinomycetemcomitans*. *Infect. Immun.* **67**:1227–1237.
- Meyer, D. H., P. K. Sreenivasan, and P. M. Fives-Taylor. 1991. Evidence for invasion of a human oral cell line by *Actinobacillus actinomycetemcomitans*. *Infect. Immun.* **59**:2719–2726.
- Miyasaki, K. T., M. E. Wilson, H. S. Reynolds, and R. J. Genco. 1984. Resistance of *Actinobacillus actinomycetemcomitans* and differential susceptibility of oral *Haemophilus* species to the bactericidal effects of hydrogen peroxide. *Infect. Immun.* **46**:644–648.
- Nakai, K., and M. Kanehisa. 1991. Expert system for predicting protein localization sites in Gram-negative bacteria. *Protein Struct. Funct. Genet.* **11**:95–110.
- Rosan, B., J. Slots, R. J. Lamont, M. A. Listgarten, and G. M. Nelson. 1988. *Actinobacillus actinomycetemcomitans* fimbriae. *Oral Microbiol. Immunol.* **3**:58–63.
- Russel, M. 1998. Macromolecular assembly and secretion across the bacterial cell envelope: type II protein secretion systems. *J. Mol. Biol.* **279**:485–499.
- Saiki, R. K., D. H. Gelfand, S. Stoffel, S. I. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**:487–491.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

34. Scannapieco, F. A., S. J. Millar, H. S. Reynolds, J. J. Zambon, and M. J. Levine. 1987. Effect of anaerobiosis on the surface ultrastructure and surface proteins of *Actinobacillus actinomycescomitans* (*Haemophilus actinomycescomitans*). *Infect. Immun.* **55**:2320–2323.
35. Shenker, B. J., T. McKay, S. Datar, M. Miller, R. Chowhan, and D. Demuth. 1999. *Actinobacillus actinomycescomitans* immunosuppressive protein is a member of the family of cytolethal distending toxins capable of causing a G₂ arrest in human T cells. *J. Immunol.* **162**:4773–4780.
- 35a. Skerker, J. M., and L. Shapiro. 2000. Identification and cell cycle control of a novel pilus system in *Caulobacter crescentus*. *EMBO J.* **19**:3223–3234.
36. Slots, J., and M. Ting. 1999. *Actinobacillus actinomycescomitans* and *Porphyromonas gingivalis* in human periodontal disease: occurrence and treatment. *Periodontology 2000* **20**:82–121.
37. Sreenivasan, P. K., D. J. LeBlanc, L. N. Lee, and P. Fives-Taylor. 1991. Transformation of *Actinobacillus actinomycescomitans* by electroporation, utilizing constructed shuttle plasmids. *Infect. Immun.* **59**:4621–4627.
38. Thomson, V. J., M. K. Bhattacharjee, D. H. Fine, K. M. Derbyshire, and D. H. Figurski. 1999. Direct selection of IS903 transposon insertions by use of a broad-host-range vector: isolation of catalase-deficient mutants of *Actinobacillus actinomycescomitans*. *J. Bacteriol.* **181**:7298–7307.
39. van Winkelhoff, A. J., and J. Slots. 1999. *Actinobacillus actinomycescomitans* and *Porphyromonas gingivalis* in nonoral infections. *Periodontology 2000* **20**:122–135.
40. Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay. 1982. Distantly related sequences in the α - and β -subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* **1**:945–951.
41. Wang, P.-L., S. Shirasu, M. Shinohara, M. Daito, T. Fujii, Y. Kowashi, and K. Ohura. 1999. Purification and characterization of a trypsin-like protease from the culture supernatant of *Actinobacillus actinomycescomitans* Y4. *Eur. J. Oral Sci.* **107**:147–153.
42. Weiss, A. A., F. D. Johnson, and D. L. Burns. 1993. Molecular characterization of an operon required for pertussis toxin secretion. *Proc. Natl. Acad. Sci. USA* **90**:2970–2974.
43. White, P. A., S. P. Nair, M.-J. Kim, M. Wilson, and B. Henderson. 1998. Molecular characterization of an outer membrane protein of *Actinobacillus actinomycescomitans* belonging to the OmpA family. *Infect. Immun.* **66**:369–372.
44. Woese, C. R., O. Kandler, and M. L. Wheelis. 1990. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proc. Natl. Acad. Sci. USA* **87**:4576–4579.
45. Wyss, C. 1989. Selected low-cohesion variants of *Actinobacillus actinomycescomitans* and *Haemophilus aphrophilus* lack distinct antigens recognized by human antibodies. *Arch. Microbiol.* **151**:133–136.
46. Yoshida, Y., Y. Nakano, Y. Yamashita, and T. Koga. 1998. Identification of a genetic locus essential for serotype b-specific antigen synthesis in *Actinobacillus actinomycescomitans*. *Infect. Immun.* **66**:107–114.
47. Zambon, J. J. 1985. *Actinobacillus actinomycescomitans* in human periodontal disease. *J. Clin. Periodontol.* **12**:1–20.