Tight-adherence genes of *Actinobacillus actinomycetemcomitans* are required for virulence in a rat model

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***Actinobacillus actinomycetemcomitans* is a Gram-negative coccobacillus that has been associated with localized aggressive periodontitis and infections of the heart, brain, and urinary tract. Wild-type clinical isolates have the remarkable ability to adhere tenaciously and nonspecifically to solid surfaces such as glass, plastic, and hydroxyapatite. Adherence by *A. actinomycetemcomitans* is mediated by the tight-adherence (*tad*) gene locus, which consists of 14 genes (*flp-1–flp-2–tadV–rcpCAB–tadZABCDEFG*). All but 2 of the genes have been shown to be required for the secretion and assembly of long, bundled Flp1 fibrils. To test whether the *tad* locus is required for colonization and disease, we developed a rat model for periodontal disease. To mimic the natural route of infection, Sprague–Dawley rats were inoculated orally by adding bacteria directly to their food for 8 days. After inoculation with wild-type or mutant strains defective in adherence (*flp-1* and *tadA*), the rats were assessed for colonization of the oral cavity and pathogenesis. Wild-type *A. actinomycetemcomitans* was able to colonize and persist for at least 12 weeks in the oral cavity, elicit a humoral immune response, and cause significant bone loss in rats. In contrast, rats fed *flp-1* or *tadA* mutant strains showed no bone loss and their immune responses were indistinguishable from those of the uninoculated controls. These results demonstrate the critical importance of the *tad* locus in the colonization and pathogenesis of *A. actinomycetemcomitans*.**

nonspecific adherence | *tad* genes | pathogenesis | localized juvenile periodontitis

Animal models are crucial to the study of bacterial pathogenesis (1). To classify a gene product as a "virulence factor," one must obtain evidence *in vivo* that the protein contributes to the disease process. Ideally this is accomplished by comparing disease provoked by a wild-type isolate to that of an isolate lacking or harboring a mutation in a putative virulence factor. This study utilizes a rat model that our group has developed for colonization and persistence of the human pathogen *Actinobacillus actinomycetemcomitans* (2). In the current study we compare wild-type and isogenic mutant strains of *A. actinomycetemcomitans* and the relationship of these strains to bone loss and periodontal disease.

A. actinomycetemcomitans is a Gram-negative coccobacillus that causes infections in humans (3), most notably localized juvenile periodontitis (LJP) (4), recently renamed localized aggressive periodontitis (LAP) (5, 6). LAP is a destructive disease of the oral cavity that affects the first molars and central incisors and causes rapid bone and tooth loss. The prevalence of LAP in the general adolescent population in the U.S. is ≈0.5%; it is 15 times more prevalent in African-American populations (7). *A. actinomycetemcomitans* has also been isolated from patients with infections of the heart (8, 9), urinary tract (10), and brain (11). The bacterium is a member of the HACEK group of bacteria associated with infective endocarditis (12, 13). Whereas the presence and significance of *A. actinomycetemcomitans* in LAP and infective endocarditis have been firmly established, little is known about the interactions between the pathogen and the host and the roles of putative virulence factors in disease.

We are interested in understanding the mechanisms by which *A. actinomycetemcomitans* colonizes the host. A distinguishing feature of *A. actinomycetemcomitans* is that fresh clinical isolates from LAP patients exhibit a nonspecific tight-adherence phenotype (14). Cells adhere avidly to solid surfaces, including plastic, glass, and hydroxyapatite (14–16). Clinical isolates also autoaggregate and produce bundled pili that mediate nonspecific adherence (16–19). The tight-adherence phenotype is thought to be important for colonization of host surfaces, and recently we reported that an adherent clinical isolate was able to colonize, survive, and persist in the oral cavity of rats (2). Using random transposon mutagenesis, we recently identified a *t*ight-*ad*herence (*tad*) locus that contains 14 genes (*flp-1–flp-2–tadV–rcpCAB–tadZABCDEFG*), of which at least 12 are essential for tight nonspecific adherence, autoaggregation, and pili formation (16, 20). The genes appear to be arranged in an operon. Our results show that the *tad* locus encodes a secretion system responsible for the secretion and assembly of bundled pili (18, 21). Remarkably, *tad*-related loci are present in diverse Bacteria and Archaea, and we have proposed that the role of *tad*-like loci in other microbes is to mediate adherence in different environments (16). Recently, we have shown that the *tad* locus has a complex history that includes several horizontal transfer events between distant relatives, indicating that the region is a mobile genomic island, which we designated the Widespread Colonization Island (WCI) (20). In the case of *A. actinomycetemcomitans* and several other organisms, including *Pasteurella multocida* and *Haemophilus ducreyi*, the WCI appears to be a pathogenicity island (20).

In this study, we sought to determine whether genes of the *tad* locus are required for colonization and pathogenesis of *A. actinomycetemcomitans*. Using a rat model, we compared the wild-type strain to mutant strains defective in *flp-1* or *tadA*. Flp1 is the major pilus subunit (18, 21), and TadA is an ATP hydrolase that is likely to energize or serve as a chaperone in the secretion and assembly of Flp1 (22). Our results show that this model system can be used to study disease resembling LAP of humans. In addition, we show that genes for adherence of *A. actinomycetemcomitans* are essential for colonization and disease.

Materials and Methods

Bacterial Strains and Preparation of Inocula. Spontaneous rifampicin (Rif)-resistant variants of the clinical isolate CU1000N (14)

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Abbreviations: LAP, localized aggressive periodontitis; Rif, rifampicin; cfu, colony-forming units.

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and nonadherent mutant strains (*tadA*::IS*903ϕkan* and *flp-1*::IS*903ϕkan*) (15, 18) were grown in 100 ml of AAGM medium (23) containing 70 μg/ml Rif in tissue culture flasks for 2 days in a 37°C incubator containing a 10% CO_2/90% air atmosphere. Bacteria for feeding to the rats were prepared in the following way. For the adherent clinical isolate, CU1000N Rif, culture flasks were washed three times with PBS, then adherent cells were scraped into a solution of PBS and 3% sucrose. The nonadherent strains were pelleted by centrifugation at 2,000 × *g* in a Sorvall SS-34 rotor at 4,000 rpm for 8 min, and the pellet was resuspended in PBS with 3% sucrose. Cells were suspended by Vortex mixing for 30 sec and adjusted to $\approx 10^8$ cells per ml (OD_{560} = 0.80).

Inoculation of Rats. Pathogen-free, Sprague–Dawley male rats 6–8 weeks old and weighing 150–250 g were purchased from Taconic (Germantown, NY). The rats were housed in separate cages and fed Laboratory Rodent Meal Diet 5001 (Purina Mills Feeds, St. Louis) with 3% sucrose (24, 25). To depress the resident flora, rats received in their water a daily dose of kanamycin (20 mg) and ampicillin (20 mg) for 4 days (26). During the last 2 days of antibiotic treatment, the oral cavities of the rats were swabbed with a 0.12% chlorhexidine gluconate rinse (Peridex, Procter and Gamble, Cincinnati). After a subsequent period of 3 days without antibiotic treatment, 24 rats were divided into four groups of 6. Group 1 was fed wild-type strain CU1000N Rif, group 2 was fed an isogenic *tadA* mutant (*tadA*::IS*903ϕkan*), group 3 was fed a *flp-1* isogenic mutant (*flp-1*::IS*903ϕkan*), and group 4 served as the uninoculated control.

Before feedings with the bacteria or control food mix, the rats were not fed for 3–4 h. The inoculum consisted of 10^8 cells of bacteria in 1 ml of PBS with 3% sucrose mixed with 1 g of food. The control rats received 1 g of food mixed with 1 ml of PBS with 3% sucrose. To confirm that the inoculated food was eaten, the mixture was placed on specially designed feeder trays that fit over the bedding in the cages. We modified the trays used in our previous study (2) so that they were partially disposable. Trays were made from 21.5-cm × 43-cm × 0.6-cm sheets of poly(vinyl chloride) foam plastic with corners rounded so that they would fit inside of the cage and rest on the bedding. The tray was covered with a thin polyethylene plastic sheet and a sheet of filter paper with a small tissue culture dish attached to hold the food/bacteria mixture. After 1 h, we removed the trays and noted whether the food/bacteria mixture had been eaten. The used paper, thin plastic sheets, and tissue culture dishes were discarded. The feeding/inoculation regimen was repeated daily for 8 days, after which uninoculated food was given to all experimental groups for the remainder of the study. The rats were switched to powdered food without added sucrose after the bacterial inoculation was completed. At 8 days after inoculation, the rats were switched to hard pellet food to prevent overgrowth of the incisor teeth.

Sampling of Rat Oral Flora. The oral flora of the rats were sampled at 2, 6, and 12 weeks after the final food/bacteria inoculation to assess colonization by the bacteria. For sampling, the rats were anesthetized i.p. with 40–60 mg/kg ketamine and 1–2 mg/kg acepromazine. The tongue and soft tissues of the mouths were swabbed for 30 sec with a sterile cotton-tipped swab moistened with PBS. The swab was then placed into a tube containing 1 ml of PBS for the soft tissue sample. Plaque was collected from the maxillary molars with a balsa wood toothpick (Stimudent, Johnson & Johnson). The Stimudent was placed into a second sample tube containing 1 ml of PBS for the hard tissue sample. The samples were subjected to Vortex mixing for 30 sec before 10-fold serial dilutions were made for plating on solid medium.

Samples were diluted and plated in duplicate on trypticase soy agar with 5% sheep blood (BBL no. 4321261, Becton Dickinson, Sparks, MD) for total anaerobic colony-forming units (cfu)/ml of sample. Tenfold and 100-fold dilutions of the sample were plated in duplicate on AAGM plus 70 μg/ml Rif and 100 units/liter nystatin to select for individual colonies of *A. actinomycetemcomitans*. Total anaerobic-count plates were incubated in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) at 37°C for 5–7 days. The *A. actinomycetemcomitans* selective plates were incubated for 2–3 days at 37°C in an atmosphere of 10% CO_2. Numbers of bacteria were determined from cfu/ml. *A. actinomycetemcomitans* colonies were counted manually and were verified by PCR amplification of the leukotoxin gene (27). Anaerobic counts were done using a CASBA 4 automated plate counter (Spiral Systems, Bethesda), and the ratio of *A. actinomycetemcomitans* cfu/ml to total anaerobic bacteria cfu/ml was determined.

Assay for Bone Loss. At the 12-week sampling, the rats were killed with sodium pentobarbital (100 mg/kg i.p.). The heads were removed and stored at −70°C. Rat maxillae were cleaned and defleshed by autoclaving for 10 min. Radiographs were taken with Kodak Insight film at 90-kV, 15-mA setting and 3/60-sec exposure. Maxillae were oriented perpendicular to the x-ray source by mounting with soft wax. The cone of the x-ray emitter was placed flush to the table supporting the maxillae so that the distance from the emitter to maxillae would be constant. The radiographs were assigned random code numbers to allow the analysis to be performed without bias.

To measure vertical bone loss, the coded radiographs were projected against a white wall to produce an image 17 times magnified. The area between the cemental–enamel junction (CEJ) and specific areas of the alveolar bone crest (ABC) surrounding the first, second, and third molars were labeled as follows. The areas of bone between furcations of the roots of molar one were labeled A and B, the area between furcations of molar two was labeled D, and that of molar three was labeled F (Fig. 1*A*). The area between the first and second molar was labeled C and the area between the second and third molar was labeled area E (Fig. 1*A*). These areas were traced from the projected radiograph onto paper. The tracings were scanned into digital files by using a Microtek Scan Maker III (Microtek, Hsincho, Taiwan, Republic of China), and the area of bone loss was calculated by using CANVAS (Deneba Software, Miami). Two investigators who were blind to the group assignments of the rat radiographs independently assessed bone loss and achieved a correlation coefficient of $r^2 = 0.868$. Only data from one of the assessors was presented. The significance of bone loss at different sites for the four groups of rats was tested by using a two-factor ANOVA test with Super ANOVA software (Abacus Concepts, Berkeley, CA). The significance of bone loss at an individual site versus the groups was analyzed with a one-factor ANOVA test. Comparison of total bone loss between the groups was by one-factor ANOVA.

Analysis of Antibody to A. actinomycetemcomitans. IgG antibody reactive with *A. actinomycetemcomitans* was assessed by ELISA (28, 29) using Nunc-ImmunoPlate with MaxiSorp surface microtiter dishes. Preinoculation orbital bleeds were taken from a subset of rats in group 1 and rats in group 4. Blood was collected by cardiac puncture from all of the rats at the final 12-week sampling and stored at −70°C. To prepare bacterial lysates, 3-day cultures of the bacteria (adjusted to 10^8 bacteria per ml, OD_{560} = 0.8) were centrifuged in 1.5-ml culture tubes. The pellets were resuspended in 1.2 ml of TEN buffer (28). Sixty-five microliters of 10% SDS was added to the supernatant and pellet samples, and the tubes were incubated for 10 min at 37°C. The samples were then stored frozen at −20°C until used for the assay. To coat the wells of the microtiter dish with the primary

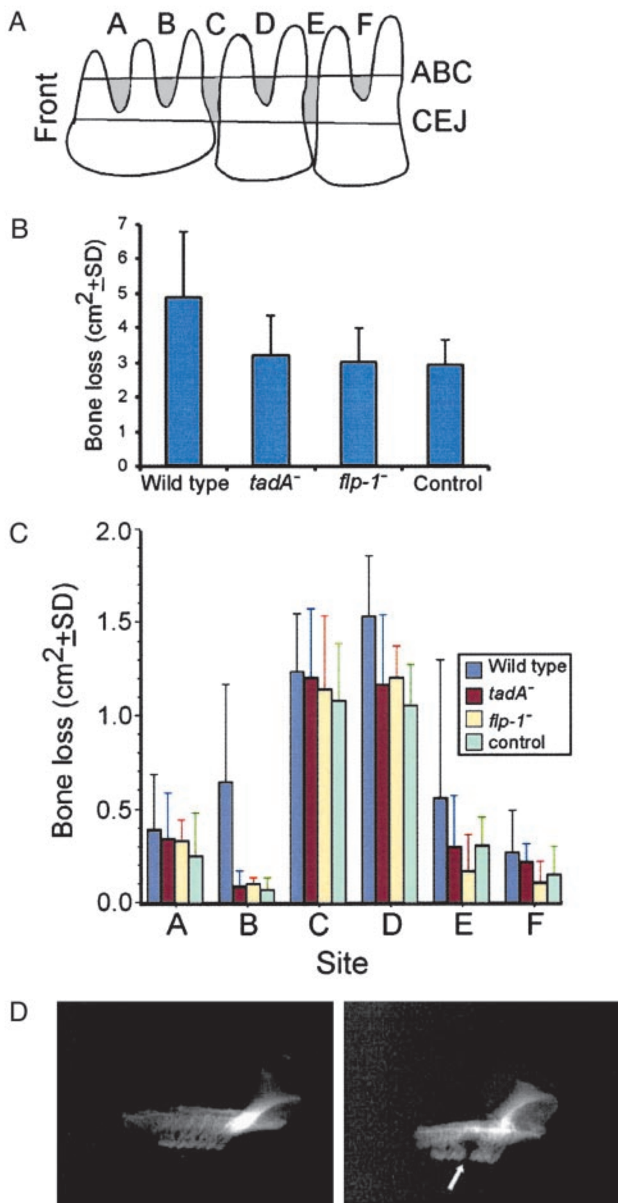

Fig. 1. Bone loss in the rat oral cavity. (*A*) Schematic of the molars of the left rat maxilla viewed buccal to lingual. "Front" denotes the area closest to the front of the mouth (anterior). The solid line labeled ABC represents the alveolar bone crest in the diseased animals. Shaded area below the line indicates the area evaluated for bone loss; the solid line labeled CEJ represents the cemental–enamel junction. Letters A–F indicate the sites that were evaluated for bone loss (shaded areas). (*B*) Mean bone loss from all sites by rat group. Bar heights represent the combined area bone loss per rat group as cm^2 in the projected radiograph image (see *Materials and Methods*). Error bars represent SD. The wild-type strain produces significantly greater ($P = 0.046$) total bone loss as determined by ANOVA. (*C*) Average bone loss at different sites in the evaluated left rat maxillae. Letters correspond to those regions presented in Fig. *A*. Bar heights represent area of bone loss (cm^2 as measured from the projected radiograph; see *Materials and Methods*). Error bars represent SD. The wild-type strain produces significantly greater ($P = 0.004$) bone loss at site B by ANOVA. (*D*) Radiograph of the left maxilla of a rat from the uninoculated control group (*Left*) and from the group that was fed wild-type *A. actinomycetemcomitans* (*Right*). The amount of bone loss indicated by the arrow was not typical of all samples, and represents an obvious case of bone loss.

antigen, 50 μl of a 1/40 dilution of the bacterial lysate was adsorbed to the wells overnight and a standard ELISA protocol was followed. For the primary antibody, 50 μl of 1/500 dilutions of the serum samples was added to wells coated with the bacterial lysates. Fifty microliters of goat anti-rat IgG conjugated to alkaline phosphatase (Sigma) was used as the secondary antibody. A 1 mg/ml solution of *p*-nitrophenyl phosphate (PNPP) was used as the substrate (PNPP tablets 0201-01, Southern Biotechnology Associates). After incubation for 1 h, the enzyme product was read on a Bio-Rad Benchmark microplate reader set at 405 nm by using Microplate Manager III Macintosh data analysis software. To identify the level of background reaction to the antigen, antigen from each inoculated bacterial strain used for that group was run against the control rat sera. All assays were done in duplicate. Results were presented as ELISA units of OD_{405}. The statistical significance of the difference between absorbance levels of the different groups was analyzed by a one-factor ANOVA test by using Super ANOVA software.

Results

Colonization of Rats by *A. actinomycetemcomitans*. We found previously that the wild-type adherent clinical isolate CU1000N Rif was able to colonize the oral cavities of rats (2). Bacteria could be recovered up to 7 months after the feeding with inoculated food.[§] In this study, we wished to determine whether *tad* locus genes for tight, nonspecific adherence are required for colonization and persistence. Mutations in *tad* locus genes, including those in *tadA* or *flp-1*, result in a nonadherence phenotype of *A. actinomycetemcomitans* (15, 16, 18). Furthermore, mutants fail to autoaggregate and do not produce bundled pili (15, 18). For each mutation that we have isolated in the *tad* locus, we have successfully complemented the mutant strain with the corresponding wild-type gene, indicating that mutations are not polar on downstream genes (15, 18). For every phenotype that we have been able to assay for, complemented strains are virtually indistinguishable from the wild-type strain.

Rats were inoculated with wild-type, *flp-1*, or *tadA* mutant strains by adding bacteria to the food for 8 days. All strains were equally viable in the rat food. This inoculation method resembles most closely the probable natural route of entry for *A. actinomycetemcomitans*. The oral cavities were sampled by scraping and swabbing, as described in *Materials and Methods*. We found that wild-type *A. actinomycetemcomitans* could be recovered from rats for at least 12 weeks after initial feeding (Table 1). *A. actinomycetemcomitans* was not recovered at every time point, very likely because of variation in sampling sites of the rat mouth. In contrast, *A. actinomycetemcomitans* was never recovered from rats fed the *flp-1* and *tadA* mutant strains. As expected, no *A. actinomycetemcomitans* was recovered from the uninoculated control group. Total bacterial counts from the various groups were comparable (Table 1). When total counts for all groups were compared by two-factor ANOVA, the average total numbers by group or by type of tissue sampled were not significantly different ($P = 0.7609$ for group comparisons, $P = 0.7823$ for tissue type). This finding suggests that our sampling was consistent and that failure to recover *A. actinomycetemcomitans* from the groups fed the mutant strains was not the result of reduced bacterial recovery. We conclude that *flp-1* and *tadA* are required for colonization of the rat oral cavity and persistence in it.

Immune Response to *A. actinomycetemcomitans*. To determine whether feeding *A. actinomycetemcomitans* to the rats elicited an immune response, we performed ELISAs with sera collected from rats at the final 12-week time point. Comparison of ELISAs between the groups was done by reacting antiserum from a given rat against the lysate of the bacterial strain that it was fed. Table

[§]Schreiner, H. C., Sinatra, K., Kaplan, J. B., Furgang, D. & Fine, D. H., 30th Annual Meeting of the American Association for Dental Research, March 8–11, 2001, Chicago, p. 66, Abstr. 719.

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Table 1. Colonization by, antibody level against, and bone loss induced by *A. actinomycetemcomitans*

Group	Rat	Colonization, cfu of *A. actinomycetemcomitans* per ml/(total cfu per ml) $\times 10^{-4}$		ELISA OD_{405}*	Bone loss, cm^2
		Hard tissue	Soft tissue		
1 Wild type	1	3.0/2.3	5/3.8	0.465	4.54
	2	50/3.8	0/3.3	0.485	7.54
	3	3.0/2.2	0/28	0.4	3.07
	4	0/3.2	3.0/17	0.484	14.54
	5	3.0/3.0	3.0/1.0	0.397	4.48
Mean		1.2/2.9	2.2/10.6	$0.446 \pm 0.04^{\dagger}$	$6.8 \pm 4.6^{\ddagger}$
2 *tadA* mutant					
Mean		$0/15^{\S}$	$0/6.2^{\S}$	0.096 ± 0.089	3.3 ± 0.9
3 *flp-1* mutant					
Mean		$0/9.8^{\S}$	$0/13^{\S}$	0.177 ± 0.111	3.0 ± 0.77
4 Uninoculated control					
Mean		$0/16^{\S}$	$0/9.2^{\S}$	0.178 ± 0.126	2.9 ± 0.6

*Preimmune $OD_{405} = 0.038 \pm 0.009$.
†$P = 0.0001$ by one-factor ANOVA.
‡$P = 0.046$ by one-factor ANOVA.
§Data for individual rats in groups 2, 3, and 4 were omitted because no *A. actinomycetemcomitans* could be detected.

1 shows the results of ELISAs of individual rats in group 1, the average level of the entire group, and the averages for groups 2, 3, and 4. The mean antibody equivalence level presented as ELISA OD_{405} units of group 1 was significantly different from the other groups and the preimmune sample by one-factor ANOVA ($P = 0.0001$). Antiserum from the rat group that was fed wild-type bacteria reacted strongly with the lysate. In contrast, antisera from the groups that were fed mutant strains did not react with the lysates above background levels.

Bone Loss. A characteristic feature of LAP is significant localized bone loss (30, 31). We therefore asked whether bone loss was evident in the rats colonized by *A. actinomycetemcomitans*. Radiographs of the rat oral cavities were obtained at the final time point. The mean total bone loss was determined for specific sites in the maxilla, as shown schematically in Fig. 1*A*. The mean bone loss from all sites in rats that were fed wild-type bacteria was higher than the bone loss seen in the three other groups of rats ($P = 0.046$) as tested by ANOVA (Fig. 1*B*). The mean bone loss in the rats that were fed the *flp-1* or *tadA* mutant strains was not significantly different from that of the uninoculated control group (Fig. 1*B*). Interestingly, the amount of bone loss varied according to site. When bone loss by site was examined (Fig. 1*C*), loss at site B was significantly greater for the group fed wild-type bacteria than for the other groups as tested by ANOVA ($P = 0.004$). Radiographs comparing the bone loss of a rat that was fed wild-type bacteria to a control uninoculated rat are shown in Fig. 1*D*.

When comparing the level of bone loss to antibody equivalence levels (as ELISA OD_{405} units) to *A. actinomycetemcomitans* (Table 1), in general animals from group 1 had the highest antibody equivalence levels and the most bone loss compared with other groups. Within the animals in group 1, it is interesting to note that the rats with the highest antibody equivalence levels (as ELISA OD_{405} units) to *A. actinomycetemcomitans* also had the highest levels of bone loss.

Discussion

Colonization of target tissue by pathogens is an essential first step in pathogenesis. A thorough understanding of this stage of host–parasite interaction is critical for the development of therapeutic strategies. In this work we present a rat model for *A. actinomycetemcomitans*-mediated periodontal disease that allows for the study of potential virulence factors of *A. actinomycetemcomitans*. Using this model, we reveal the importance of specific genes associated with adherence and their relationship to pathogenesis.

We previously developed genetic approaches for the study of *A. actinomycetemcomitans* (33) and identified genes involved in tight adherence of the bacterium to surfaces (15). To test the hypothesis that genes from the *tad* locus are required for the virulence of *A. actinomycetemcomitans*, we have developed an animal model for colonization and disease. These results support and extend our previous findings that show a wild-type clinical isolate is able to colonize and persist in the oral cavity of rats (2). In addition, this study showed that colonized rats developed antibody against the pathogen and exhibited maxillary bone loss as seen in humans diagnosed with LAP. In contrast, *flp-1* or *tadA* mutants failed to colonize the oral cavities of rats, elicit immune responses, or cause bone loss. These results show conclusively that *tadA* and *flp-1*, and hence tight adherence, are required for *A. actinomycetemcomitans* colonization as the essential first step in the development of disease.

Over the years little progress has been made toward the development of an animal model for periodontal disease induced by bacteria. Experiments that have been done allowed workers to study the role that the immune system plays in the disease process. Unfortunately, none of these previous studies have examined colonization by *A. actinomycetemcomitans* over time and its affect on disease as manifested by bone loss. Two important features of this model that allow us to test both colonization and disease are (*i*) the method by which rats were inoculated with bacteria and (*ii*) the strain of *A. actinomycetemcomitans* that was used. Simple inoculation of the oral cavity by adding bacteria to food most closely approximates a natural route of infection. In most other animal models for periodontal disease, inoculation is carried out through injection or by forcing large numbers of bacteria into a localized site, and the initial stages of disease (adherence and colonization) are bypassed (33–36). In these models, genes that play a role in this crucial step of disease are overlooked. Furthermore, previous models have used nonadherent laboratory isolates of *A. actinomycetemcomi-*

tans (such as Y4 and JP2), which precludes experiments on colonization from being carried out.

For every animal model of a human disease, there are inherent limitations. Among other important variations that could affect conclusions drawn from animal models, nonhuman animals differ from humans in anatomy, immune response, and lifestyle. For the study of periodontal disease, mice (25, 33, 34, 37–39), rats (36, 40, 41), and nonhuman primates (42–44) have been most frequently used. While primates closely resemble humans with regard to anatomy and physiology, their cost, size, and maintenance requirements prohibit studies with large samples. However, rats and mice have been used for the study of periodontal disease induced by *Porphyromonas gingivalis*.

One limitation of using a nonprimate animal model is that a likely virulence factor of *A. actinomycetemcomitans*, leukotoxin, is inactive against nonprimate cell lines (42, 45). Therefore, either leukotoxin has a lower specificity *in vivo* or the disease that we observe is actually limited because leukotoxin is not contributing to pathogenesis. Further studies are needed to distinguish between these possibilities.

In humans, colonization of the oral cavity by *A. actinomycetemcomitans* is believed to occur early in life, when the native flora are being established (46–49). In these experiments, we pretreated adult rats with antibiotics to significantly reduce the number of indigenous bacteria, and thus give the mutants the greatest chance of colonizing. The observation that the mutants were unable to colonize even under these favorable conditions where competition with native flora was reduced demonstrates the importance of the adherence phenotype.

To test colonization by food-borne *A. actinomycetemcomitans* and prevent animal-to-animal transmission, each rat was caged separately (see *Materials and Methods*). However, because maternal transmission of *A. actinomycetemcomitans* to offspring can occur (50), the rat model presented here could be used to test animal-to-animal transmission of *A. actinomycetemcomitans*.

The ability to recover *A. actinomycetemcomitans* from soft and hard tissues is an important part of this model. Although the numbers of cfu of *A. actinomycetemcomitans* recovered were often low (Table 1), it is highly significant that bacteria could be recovered 12 weeks after ingestion, considering the noninvasive inoculation route we used. We found that *A. actinomycetemcomitans* varied from 0.02% to 5.0% of the total flora when it was recovered. In a study of human localized juvenile periodontitis subjects, the percentage of *A. actinomycetemcomitans* recovered in subgingival plaque from diseased sites varied from 0.6% to 7% of total flora in one survey and from 0.1% to 114% in a second survey (51).

LAP is characterized by bone loss, typically localized to permanent molars and incisors in children and adolescents (30). It is interesting that bone loss was localized to specific molar sites in the rat, because this is a pattern seen in human disease (Fig. 1). To this point we do not know the mechanism of *A. actinomycetemcomitans*-induced bone loss. However, it is known that *A. actinomycetemcomitans* can cause bone loss directly by means of bacterial enzymes (47, 52) and toxins such as lipopolysaccharide (53), or indirectly by stimulation of host response mediators (40). Recent evidence suggests that *A. actinomycetemcomitans*-sensitized T cells can result in expression of RANK-L (receptor activator of NF-κB ligand) by activated T helper 1 cells, which in turn can induce osteoclastogenesis, osteoclast activation, and bone loss (54). This mechanism of bone loss is compelling because it has been described in a rat model. Because localized bone loss is likely to be directly correlated with bacterial colonization, future experiments should focus on colonization at site B, located at the first molar (Fig. 1*A*). Sampling of specific sites may allow for more consistent and effective recovery of the colonizing *A. actinomycetemcomitans*, because viable counts will not be diluted out by total mouth sampling. This sampling problem has previously been encountered during enumerations of other oral pathogens (55). Additionally, in future studies, it will be interesting to examine other organs, such as the heart and brain, for colonization by *A. actinomycetemcomitans*.

In humans, *A. actinomycetemcomitans* colonizes both soft and hard tissue (56, 57), and this pattern was also observed in the rat model. The ability to adhere to hard and soft tissues probably enhances bacterial survival and persistence in the oral cavity. The Flp fibrils encoded by the *tad* locus are clearly involved in adherence to hard surfaces. Whether they are directly involved in binding to soft tissue or whether colonization of hard tissue is a prerequisite for soft-tissue binding cannot be determined by these experiments. Because *A. actinomycetemcomitans* cannot live for long periods outside of an animal host, its survival depends on transmission to subsequent generations of hosts. On the basis of our results, strains blocked in tight adherence should have difficulty colonizing new hosts and be rapidly eliminated from the population. Such a scenario illustrates the importance of understanding the early stages of colonization.

The success of a pathogen results from expression of a variety of virulence factors. While colonization is critical, undoubtedly other virulence factors are required for disease to occur. This model will help elucidate the role of these determinants in periodontal disease. We also believe that this model will be able to test and identify mutants that are able to colonize but not cause disease. Continued investigations into the adherence properties and virulence factors of *A. actinomycetemcomitans* in this animal model should contribute to a better understanding of the initial stages of LAP and infectious disease in general.

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