Natural Transformation and DNA Uptake Signal Sequences in *Actinobacillus actinomycetemcomitans*

Ying Wang,[1] Steve D. Goodman,[1] Rosemary J. Redfield,[2] and Casey Chen[1]*

University of Southern California School of Dentistry, Los Angeles, California 90089,[1] and Department of Zoology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z4[2]

Actinobacillus actinomycetemcomitans is a member of the family *Pasteurellaceae* and a major causative agent of periodontitis. While several genera from this family are known to be competent for transformation, *A. actinomycetemcomitans* has yet to be fully characterized. Here we show that the competence of *A. actinomycetemcomitans* is remarkably similar to that of *Haemophilus influenzae*. In addition to having a similar frequency of transformation as *H. influenzae*, *A. actinomycetemcomitans* competence could also be induced at least 100-fold by cyclic AMP, suggesting that, as in *H. influenzae*, at least some competence genes are regulated by catabolite repression. Even more intriguing was the discovery of a putative *A. actinomycetemcomitans* DNA uptake signal sequence (USS) virtually identical to the USS of *H. influenzae*. Moreover, we provide evidence that this sequence functions in the same capacity as that from *H. influenzae*; the sequence appears to be required and sufficient for DNA uptake in a variety of assays. Finally, we have taken advantage of this system to develop a simple, highly efficient competence-based method for generating site-directed mutations in the wild-type fimbriated *A. actinomycetemcomitans*.

Actinobacillus actinomycetemcomitans, a gram-negative, facultative, nonmotile coccobacillus, is a major causative agent of localized juvenile periodontitis and other forms of chronic periodontitis (36). Moreover, *A. actinomycetemcomitans* is transmissible among family members and may persist for long periods (4). *A. actinomycetemcomitans* can also spread from the oral cavity, causing a variety of nonoral infections (31, 36). Indeed, the genomic DNA of *A. actinomycetemcomitans* was identified in atherosclerotic lesions (14), suggesting a possible role of this bacterium in cardiovascular disease.

Some *A. actinomycetemcomitans* strains are known to be naturally competent for transformation (23, 30). In many bacteria, transformation occurs when cells enter the state of competence. This enables them to take up DNA and incorporate it into their genomes (8, 18). In the best-studied naturally competent gram-negative bacteria, *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Neisseria gonorrhoeae*, and *Neisseria meningitidis*, double-stranded donor DNA fragments are preferentially bound into a DNase-resistant state if they contain a short recognition sequence abundant in their own genomes, the uptake signal sequence (USS) (10, 11). Although uptake has been demonstrated elsewhere for *A. actinomycetemcomitans* in two preliminary studies (23, 30), a quantitative analysis has yet to be performed. In particular, neither of these studies examined any of the requirements for the induction of competence. Nor was a specific DNA uptake sequence requirement for transformation investigated.

Because the function of the USS has been assumed to be exclusion of DNAs from other species, different genera were expected to use different USSs. However, only USSs from the very distantly related genera *Haemophilus* and *Neisseria* have been characterized; these use completely unrelated USSs. *H. influenzae* and *A. actinomycetemcomitans* are both members of the family *Pasteurellaceae*. In this report, we identified a highly competent, fimbriated wild-type *A. actinomycetemcomitans* clinical isolate and found that its transformation properties resemble those of *H. influenzae*. We also identified a USS that was remarkably similar to the *H. influenzae* USS. With this knowledge we have subsequently been able to develop a highly efficient method for gene replacement in wild-type *A. actinomycetemcomitans* via natural transformation.

MATERIALS AND METHODS

Bacteria and culture conditions. *A. actinomycetemcomitans* strains HK1651 (ATCC 700685) (15), ATCC 33384, and Y4 (ATCC 43718) were purchased from the American Type Culture Collection, Manassas, Va. *A. actinomycetemcomitans* strain JP-2 was a gift from S. Asikainen of Umea University, Umea, Sweden. Clinical isolates of *A. actinomycetemcomitans* strains 29R, 29SS, H1S, H5P, D3S, D7S, D9S, D11S, D15S, D17S, D18P, D28S, and D37S were part of the collection of oral clinical isolates of *A. actinomycetemcomitans* in our laboratory. The *A. actinomycetemcomitans* clinical isolates were identified from subgingival plaque samples by the culture method as described previously (24) and further confirmed by a 16S rRNA-based PCR method (3). Spontaneous nonfimbriated smooth-colony mutants of *A. actinomycetemcomitans* were isolated by repeatedly transferring the bacteria into fresh medium until increased turbidity was noted and streaking the bacteria on agar medium for isolation of smooth-colony isolates. *Escherichia coli* plasmids were propagated in *E. coli* DH5α as previously described (32).

A. actinomycetemcomitans was routinely cultured on Trypticase soy broth supplemented with 0.1% yeast extract, 5% heat-inactivated horse serum, and 1.5% agar (sTSB agar) at 37°C in 5% CO_2. When indicated, the medium was supplemented with spectinomycin (50 μg/ml), nalidixic acid (16 to 24 μg/ml), or rifampin (25 to 50 μg/ml). Additional media used for transformation included sTSB broth (same as above but without agar), TSB agar, Columbia agar (Difco), and Luria-Bertani (LB) agar. Bacteria were grown in broth without shaking at 37°C in 5% CO_2.

Scanning electron microscopy. Scanning electron microscopy was used to examine the cell morphology of *A. actinomycetemcomitans* growing on coverslips. A starter culture of *A. actinomycetemcomitans* was prepared by growing the bacteria in sTSB broth overnight. The overnight culture (20 μl) was transferred

* Corresponding author. Mailing address: Division of Primary Oral Health Care, USC School of Dentistry, 925 W. 34th St., Room 4382, Los Angeles, CA 90089-0641. Phone: (213) 740-1075. Fax: (213) 740-6778. E-mail: ccchen@hsc.usc.edu.

to a tube containing 3 ml of sTSB broth and a piece of coverslip, and the bacteria were cultured for an additional 15 h. The coverslip was then gently rinsed once with cacodylate buffer (0.1 M cacodylate buffer with 0.02% CaCl₂) to remove the nonadherent bacteria. The coverslip samples were then fixed for 20 min in fixative (2.5% glutaraldehyde, 2% paraformaldehyde in cacodylate buffer), rinsed three times with cacodylate buffer, and dehydrated in a graded ethanol series. The coverslip was then immersed in hexamethyldisilazane and air dried. After gold coating, the preparations were observed with a Cambridge 360 scanning electron microscope.

Isolation of antibiotic-resistant mutants. *A. actinomycetemcomitans* cells were plated on sTSB agar containing rifampin (50 μg/ml) at ~2 × 10⁸ CFU per plate. Rif^r variants exhibiting normal colony size were isolated for further use. Nal^r Rif^r double mutants were identified by plating Rif^r mutants on agar containing nalidixic acid (24 μg/ml) and subsequently culturing them for 3 to 4 days.

DNA method and plasmids. Chromosomal DNA was prepared as previously described (33). Plasmids and PCR products were purified with spin columns (Qiagen, Inc.). DNA sequencing and oligonucleotide synthesis were performed by the USC School of Medicine Microchemical Core Facility.

Plasmid pPK1 was a generous gift from P. Fives-Taylor. It is a 3.6-kb Spe^r *E. coli-A. actinomycetemcomitans* shuttle plasmid constructed from pUC19 and pVT736-1 (28). pPK1 used in experiments was always propagated in *E. coli*. The spectinomycin resistance cassette plasmid, pK-Spe, was constructed by replacing the 0.3-kb *Eco*RI fragment on pBSL237 (2) with the 1.2-kb *Sph*I Spe^r gene from pVT1461 (a gift from P. Fives-Taylor) by standard recombinant DNA techniques (22).

Transformation assays. Transformation assays were routinely performed on agar. Recipient bacteria were grown on sTSB agar overnight (approximately 15 to 20 h) and resuspended in TSB at 1 × 10⁹ CFU/ml for the fimbriated wild-type strains or 5 × 10⁹ CFU/ml for the nonfimbriated mutants. A 20-μl aliquot of the suspension was spotted onto a prewarmed sTSB agar plate and spread in a small area (diameter of ~10 mm). After incubation for 2 h, 10 μl of donor DNA at a concentration of ~100 μg/ml was added and mixed with the bacterial cells by using a loop. The mixture was incubated for an additional 6 h (for the fimbriated strain) or 5 h (for nonfimbriated mutants). The bacteria were scraped up, resuspended in LB broth, and plated onto selective medium for 2 to 7 days. Ten microliters of DNase I solution (200 μg/ml) was added simultaneously with DNA as a negative control or at designated times to quench DNA uptake. Unmarked chromosomal DNA from the wild-type strain D7S or Tris-EDTA buffer was also used as a negative control. Transformation frequency was calculated as the number of transformants per CFU, and transformation efficiency was calculated as the number of transformants per microgram of DNA.

Competition assays. Transformation in the presence of competing DNA was performed on agar surfaces as described above. Transforming DNA (0.2 μg for each sample) and competing DNA (1.8 μg) were mixed in 10 μl of Tris-EDTA buffer and added to the competent bacteria. DNase I was added after 20 min, and the mixture was incubated for an additional 6 h for the wild-type strain or 5 h for the nonfimbriated strain. The relative transformation efficiency was the ratio of the number of Nal^r transformants obtained from 0.2 μg of transforming DNA without competitor (the control) to the number of transformants obtained with the mixed DNA. Competing DNA included genomic DNA from *A. actinomycetemcomitans* strains D7S and HK1651, from *E. coli* strains DH5α and E44, and from *H. influenzae* Rd and two DNA fragments produced by PCR, designated pcr-USS-pilA (874 bp) and pcr-pilA (868 bp). Both pcr-USS-pilA and pcr-pilA contained a copy of the *pilA* gene, but they differed by the presence of a putative *A. actinomycetemcomitans* USS (which shares the 9-base USS of *H. influenzae*; pcr-USS-pilA) or a 5-base partial USS (pcr-pilA) at one end. Amplification used the cloned *pilA* gene of *A. actinomycetemcomitans* (unpublished data) and, for pcr-USS-pilA, primers pilB-B1, 5′-TTTCGTCGACTTTTTCGACGCGC, and USS-pilA, 5′-AA<u>AAGTGCGGT</u>GGAATTTTAC (the 9-base core USS is underlined). Amplification of pcr-pilA used primers pilB-B1 and pilA, 5′-<u>GCGG TGGAATTTTAC</u>GGTGT.

Transformation in the presence of cAMP. *A. actinomycetemcomitans* strains D7S and D7S-smooth were inoculated from overnight plate cultures into sTSB broth at an optical density value at 600 nm of 0.1 and incubated without shaking at 37°C in 5% CO₂. After 30 min of incubation, cyclic AMP (cAMP) was added to the cultures to a 2 mM concentration, and incubation continued for an additional 50 min, followed by the addition of donor DNA to a final concentration of 2 μg/ml. After 20 min, DNA uptake was quenched by the addition of DNase I (50 μg/ml). Transformants were selected as described above.

Competence-based allelic replacement. The competence-based allelic replacement strategy depends on homologous recombination of a chromosomal target gene with a recombinant DNA fragment consisting of a selectable marker inserted between two flanking regions of the target gene. The fragment was constructed either in *E. coli* by standard methods or by a PCR method developed in our laboratory. More details of this method will be published elsewhere. Briefly, the *flp-rcp-tad* gene cluster, which contains 14 genes coding for fimbria biogenesis and tight adherence (13, 16, 17), was deleted and replaced with the Spe^r cassette. A 1.1-kb DNA upstream of the *flp* gene was amplified with primers flp-UE (5′-CTGAATTCTCGCTCAGATACGGA) and flp-B1 (5′-AA<u>GGATC</u>CGACCGGTACTATAGTCCT; a *Bam*HI site is underlined) with HK1651 DNA as template. Similarly, primers tadG-B2 (5′-CA<u>GGATCC</u>ATACTATAC AAACCCGT) and tadG-R (5′-GATTATGAGTCGACTGCTCT) were used to amplify a 1-kb DNA downstream of the *tadG* gene. Both of these PCR DNAs contain a copy of the USS. These two amplicons were purified through a spin column (Qiagen), digested with *Bam*HI, and partially filled in with dGTP and dATP with the *E. coli* DNA polymerase Klenow fragment. pK-Spe was cleaved with *Sal*I to produce a 2.7-kb vector and a 1.2-kb Spe^r cassette. The *Sal*I-digested DNA was partially filled in with dTTP and dCTP without purification. Both PCR DNA and the marker DNA were combined, ethanol precipitated, and ligated at 15°C overnight. The ligation mixture was directly applied to competent bacteria on an agar plate. Transformants were isolated by selection for the inserted Spe marker.

All mutants were confirmed by PCRs to verify (i) the deletion of the target gene by using primers within the target gene, (ii) the insertion of the antibiotic cassette by using primers flanking the target site, and (iii) the direction of the inserted antibiotic cassette by using a primer upstream of the target site and a forward or reverse primer within the cassette.

RESULTS

Identification of naturally transformable *A. actinomycetemcomitans*. *A. actinomycetemcomitans* strains ATCC 33384, JP-2, HK1651, and Y4 and 13 clinical isolates were examined by the agar-based transformation assay. The donor DNA was the genomic DNA of a Nal^r Rif^r mutant of *A. actinomycetemcomitans* strain D17S. Among 17 strains examined, only *A. actinomycetemcomitans* strain D7S was found to be transformable under the test conditions. The strain D7S was recovered from a deep periodontal pocket of an upper central incisor of a female African-American patient with aggressive periodontitis. D7S was found to be of serotype a.

Transformation frequencies for both chromosomal markers (Rif^r and Nal^r) were similar at (1.1 ± 0.8) × 10⁻³ transformants/CFU. However, since cells of strain D7S formed aggregates, the calculated transformation frequency is likely to be overestimated. Preliminary studies showed that the transformation frequency was higher with sTSB agar than with sTSB broth, TSB agar, Columbia agar, or LB agar (data not shown). Subsequent characterization of *A. actinomycetemcomitans* strain D7S and its derivatives was therefore routinely performed on sTSB agar. Transformation was also performed with DNA from a Rif^r transformant of D7S. The transformation frequencies were similar whether the genomic DNA from strain D7S or that from D17S was used. The subsequent experiments were routinely performed with marked genomic DNA from strain D17S.

Characterization of natural transformation. Figure 1 shows the development of competence by strain D7S (asterisks). Bacteria grown overnight on sTSB agar were transferred onto fresh agar. Transformation was initiated by adding DNA to the cells on the plate at the times indicated and stopped by addition of DNase I after 20 min of incubation with DNA. Although cells were immediately competent, the transformation frequency increased 100-fold after 2 h of incubation and then slowly decreased. Transformants were not detected (transformation frequency of <10⁻⁸/CFU) when DNA was added to overnight cultures.
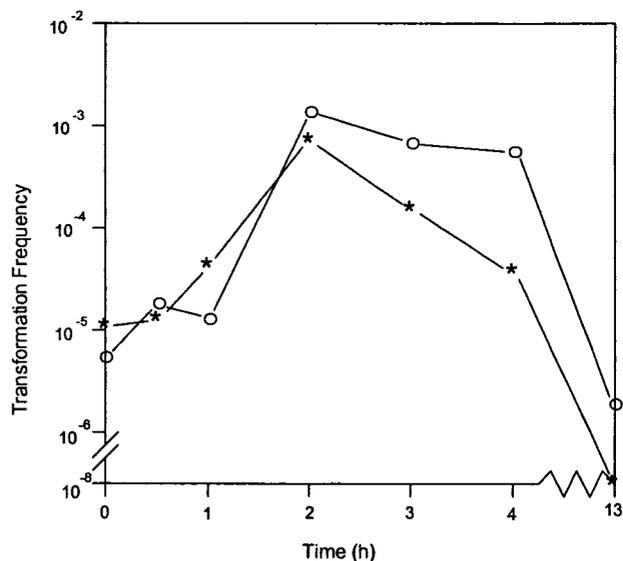
FIG. 1. Effect of incubation time on transformation frequency of *A. actinomycetemcomitans*. DNA (1 µg) was added at the indicated time after bacteria were placed on agar, and uptake was terminated by addition of DNase I after 20 min. Transformation frequency was defined as the number of Nal^r transformants per CFU of recipient cells. Data are the averages of three experiments. ∗, D7S; ○, D7S-smooth.

FIG. 3. Transformation efficiencies of *A. actinomycetemcomitans* strains D7S and D7S-smooth with different concentrations of DNA. Donor DNA (0.002 to 2 µg) was added to the competent bacteria on 2-h plate cultures and incubated for 30 min before addition of DNase I. Nal^r transformants were selected. ∗, D7S; ○, D7S-smooth. Note that total CFU for D7S was ∼5 × 10^8 and that for D7S-smooth was ∼5 × 10^9.

A. actinomycetemcomitans strain D7S exhibited rough colony morphology and was fimbriated (Fig. 2A). To determine whether fimbriae participate in DNA uptake, as they do in many other gram-negative bacteria, a spontaneous nonfimbriated smooth-colony mutant of strain D7S (designated D7S-smooth [Fig. 2B]) was isolated and its competence was examined (Fig. 1, open circles). The time course of competence development was similar to that of strain D7S, but cells remained competent longer. Transformants of strain D7S-smooth could be detected even when DNA was added after overnight growth. This extension of competence for the smooth phenotype may be indicative of the physical exclusion of the transforming DNA by the accumulation of extracellular matrix produced by the wild-type D7S strain as the cells age. Alternatively, there may be other pleiotropic effects linked to the smooth phenotype that are as yet unknown.

Uptake of DNA is saturable in most other naturally competent bacteria, and where it has been investigated, it has been found to be an absolute saturation (cells cannot take up any more DNA) rather than a kinetic one (cells cannot take up DNA any faster). Figure 3 shows that amounts of marked chromosomal DNA less than 0.2 µg are limiting for transformation and confirms that the 1.0 µg of DNA used in the standard transformation assay is saturating. Figure 4 shows that uptake of DNA by the nonfimbriated D7S-smooth became absolutely saturated within 20 min after the addition of the donor DNA. Cells were given 1.0 µg of DNA, and uptake was assayed by conversion of DNA to a state of DNase I resistance. In contrast, the transformation frequency for the wild-type stain D7S continued to increase for at least 30 min. The dense cell aggregations typical of D7S may physically hinder interaction of DNA with cells, so that prolonged incubation is needed for donor DNA to reach competent cells. It should be noted that the degree of aggregation of strain D7S varied between experiments. It was difficult to compare experiments conducted on different occasions. This may explain the unexpected differences in the number of transformants of strain D7S in Fig. 3 and Fig. 4.

Identification of the putative USS and competition assays.
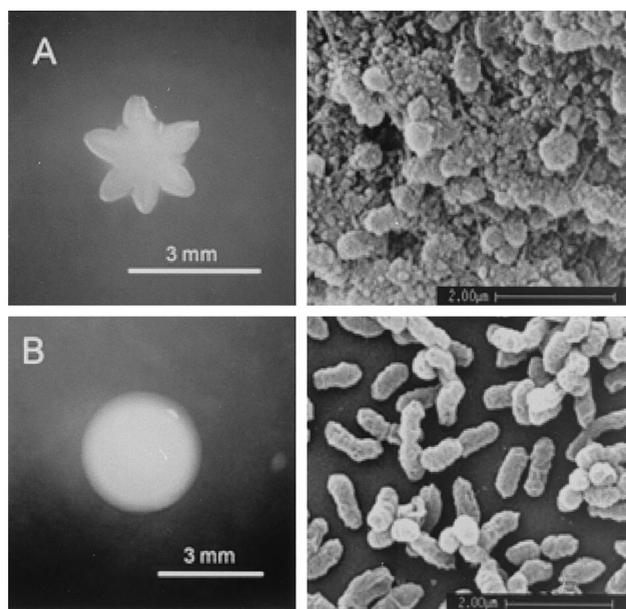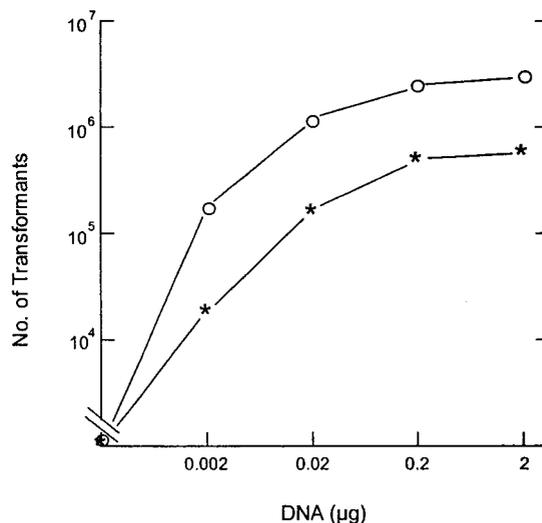

FIG. 2. Morphology of *A. actinomycetemcomitans* strain D7S and its smooth-colony mutant. Left side, colonies on sTSB agar; right side, scanning electron micrographs of bacteria on the coverslip surface. (A) D7S; (B) D7S-smooth.
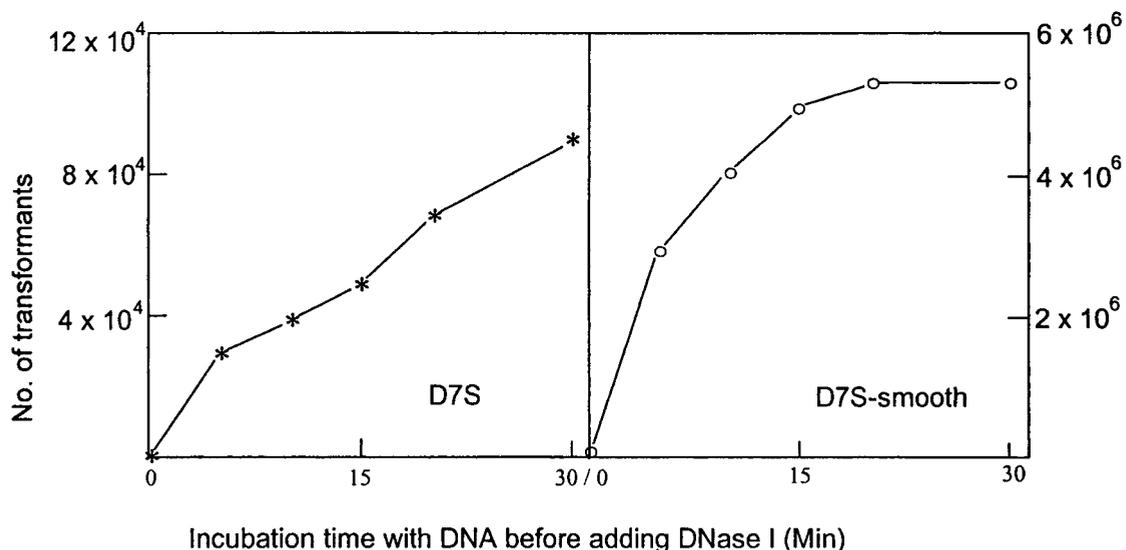
FIG. 4. Transformation efficiency versus time of incubation with donor DNA. DNA uptake was terminated by addition of DNase I at the indicated time. Nal[r] transformants were selected.

Competent cells of *H. influenzae* (like *A. actinomycetemcomitans*, a member of the family *Pasteurellaceae*) preferentially take up DNA fragments containing a 9-bp sequence which is abundant in *H. influenzae* DNA (6, 25). Interestingly, DNA of *A. actinomycetemcomitans* has been reported elsewhere to compete efficiently with DNA of *H. influenzae* in *H. influenzae* transformation experiments (1). This suggests that the two genomes may share the same uptake sequence. We examined 133 kb of randomly chosen *A. actinomycetemcomitans* genomic sequence (downloaded from www.genome.ou.edu/act.html) and found 52 occurrences of the *H. influenzae* USS or its complement, a frequency of 0.4/kb, much higher than the frequency of 6×10^{-3} copies per kb expected for a random sequence of this base composition. Alignment of the putative USS of *A. actinomycetemcomitans*, shown in Fig. 5, revealed a flanking consensus very similar to that seen in *H. influenzae*.

We assessed the specificity requirements of transforming DNA by performing competition experiments. We tested whether the uptake of *A. actinomycetemcomitans* chromosomal markers could out-compete homologous and heterologous sources of DNA. Figure 6 shows that both *A. actinomycetemcomitans* and *H. influenzae* DNA competed for uptake with genetically marked *A. actinomycetemcomitans* DNA but that *E. coli* DNA did not. Furthermore, an 874-bp PCR fragment containing the *H. influenzae* USS (pcr-USS-pilA) competed for uptake, while a nearly identical fragment lacking the USS (pcr-pilA) did not. This is the first clear evidence not only that an *A. actinomycetemcomitans* USS exists but also that it is likely highly similar to, if not indistinguishable from, the USS of *H. influenzae*.

Competence stimulated by cAMP. Transcription of some *H. influenzae* competence genes depends on the cAMP-cAMP
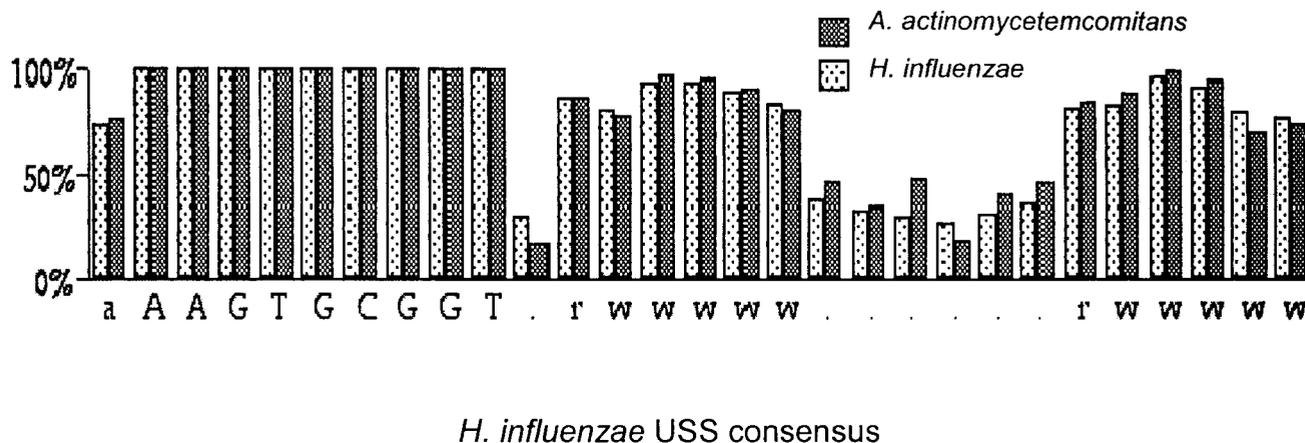

FIG. 5. Comparison of 52 occurrences of the putative USS of *A. actinomycetemcomitans* to the USS consensus of *H. influenzae*, aligned at the 9-bp core. Capital letters are invariant bases; for others, r, A or G; w, A or T.
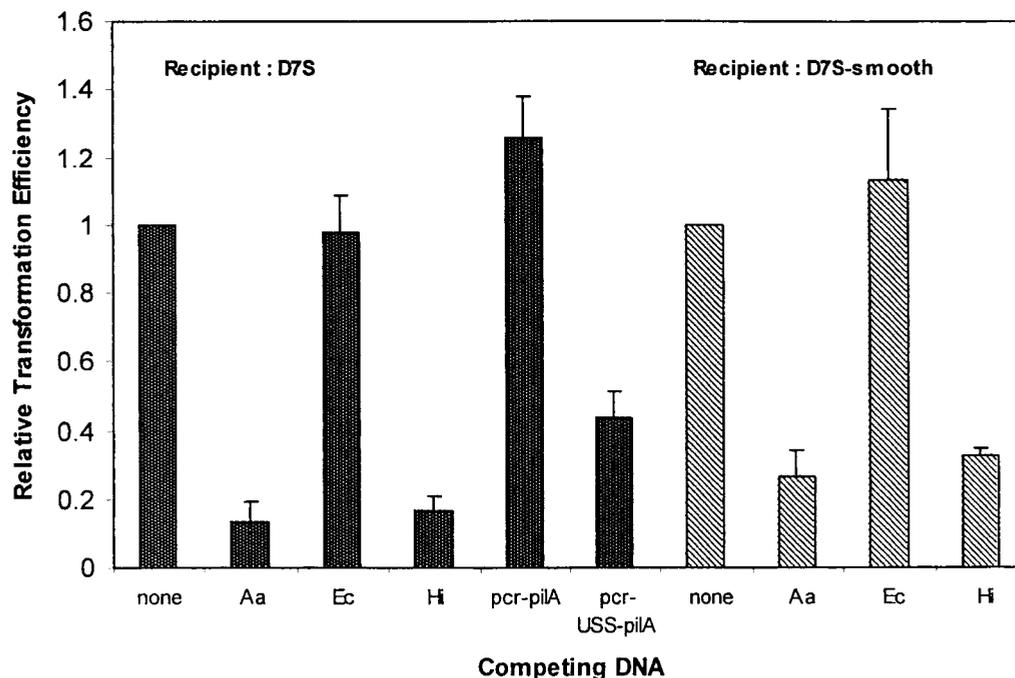
FIG. 6. Relative transformation efficiencies of strains D7S and D7S-smooth in the presence of competing DNA. Competing DNAs used: Aa, *A. actinomycetemcomitans* genomic DNA; Hi, *H. influenzae* Rd genomic DNA; Ec, *E. coli* chromosomal DNA; pcr-pilA, an 868-bp PCR DNA containing *pilA*; pcr-USS-pilA, an 874-bp PCR DNA containing the same sequence as that in pcr-pilA and a USS site at one end. The relative transformation efficiency was expressed as the ratio between the number of transformants of control (no competing DNA) to the number of transformants in the presence of mixed DNA; data are the averages of three experiments ± standard deviations.

receptor protein complex (5, 19), and addition of cAMP to cells in exponential growth phase increases transformation frequencies from the threshold of detectability ($\sim 10^{-8}$) to about 10^{-4}. Therefore, we tested the effect of cAMP on *A. actinomycetemcomitans* competence development in exponential-phase cells grown in broth. The doubling time of the nonfimbriated strain D7S-smooth was approximately 2 h under our culture conditions. Although the culture was less homogenous, the growth phase of the fimbriated wild-type strain D7S was estimated to be exponential phase as well. Figure 7 illustrates that, although the broth cultures of *A. actinomycetemcomitans* strains D7S and D7S-smooth were competent without cAMP, the transformation frequencies increased by approximately 100-fold in the presence of cAMP. Furthermore, addition of cAMP to the plate cultures also increased competence (data not shown). The results indicate that *A. actinomycetemcomitans* competence, like that of *H. influenzae*, is likely regulated by catabolite repression.

Plasmid transformation. DNA uptake pathways that have been characterized in naturally competent bacteria cannot transport closed circular molecules such as intact plasmids into the cytoplasm. Because only single strands of broken or cleaved plasmid molecules enter the cytoplasm, transformation by plasmid DNA depends on either reassociation of strands derived from molecules taken up independently or recombinational repair with a homologous DNA segment already resident in the cell. Consistent with this, in our standard transformation assay the transformation frequency of 1 μg of the supercoiled shuttle plasmid pPK1 was only $\sim 10^{-8}$/CFU in

both strain D7S and strain D7S-smooth. Plasmid isolated from two of the transformants appeared indistinguishable in size from the donor pPK1 isolated from *E. coli*.

Competence-based gene replacement. We exploited the natural competence of *A. actinomycetemcomitans* strain D7S to develop a gene replacement protocol. Transformation by a

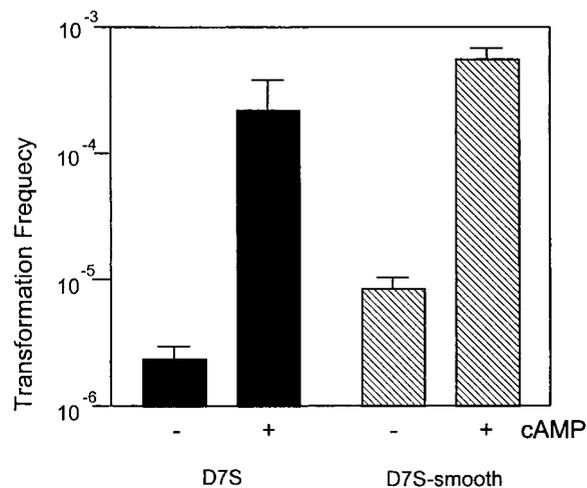
FIG. 7. Transformation frequencies in the presence or absence of cAMP. Data are the averages of two experiments ± standard deviations.

linear DNA molecule containing a disrupted gene was sufficiently efficient such that any cloning steps could be eliminated, and cells were directly transformed with ligation mixtures. The method was simple and typically produced the desired specific deletion mutants in less than 1 week. One example was the deletion of the 11-kb *flp-rcp-tad* gene cluster of 14 genes in D7S. Twenty-three Spe^r transformants were obtained from ~0.6 μg of ligated DNA. All these transformants exhibited the expected smooth-colony phenotype and lacked fimbria. PCR examination of four selected colonies indicated that both *flp* and *tadG* sequences were absent while expected PCR products were amplified from D7S (data not shown), and the *Spe* gene was found to be inserted at either direction in these mutants.

DISCUSSION

Two previous studies have observed natural transformation in *A. actinomycetemcomitans*. Tønjum et al. (30) used a broth-based method and identified two naturally competent strains among five study strains of *A. actinomycetemcomitans* with transformation frequencies of 1.3×10^{-3} and 6.2×10^{-5}. In our present study, we identified a single highly competent *A. actinomycetemcomitans* strain among 17 strains examined. Although this is an apparently lower prevalence of naturally competent *A. actinomycetemcomitans* strains than was reported by Tønjum et al., the transformation frequencies reported in this study were similar.

Sato et al. (23) generated transposon insertional mutants from the *A. actinomycetemcomitans* strain Y4 by natural transformation. The transformation was performed with broth-cultured exponential-phase *A. actinomycetemcomitans* cells. However, strain Y4 was not transformable in our study. While the loss of competence is sometimes a consequence of laboratory propagation, it is also possible that *A. actinomycetemcomitans* strains may spontaneously acquire and lose transformability in their natural habitat, as has been observed previously for *Pseudomonas fluorescens* (7).

Neither of the studies by Tønjum et al. and Sato et al. examined the requirement for the induction of competence or the sequence specificity of DNA uptake. This study showed that competence development in *A. actinomycetemcomitans* resembles that in *H. influenzae*. Moderate levels of competence develop in *H. influenzae* cultures at late exponential phase and early stationary phase, and exponentially growing cultures become fully competent after transfer to a starvation medium (26). Cells growing in colonies on agar plates are also moderately competent (34). *A. actinomycetemcomitans* competence developed within 2 h after ~10^8 cells were plated on a small area of fresh agar. We are not certain whether the growth phase of the competent *A. actinomycetemcomitans* was comparable to that of the competent *H. influenzae*.

We also showed that addition of cAMP induced competence in *A. actinomycetemcomitans*, similar to the effect of cAMP on *H. influenzae* competence (35). This strongly suggests that catabolite repression regulates the development of competence. Consistent with this, *A. actinomycetemcomitans* possesses homologs of the *H. influenzae* genes mediating catabolite repression (20). Recently, Finkel and Kolter (9) have demonstrated that *E. coli* possesses orthologs to *H. influenzae* competence genes and that they appear to function as a means to derive

sustenance from DNA. According to this model, one of the roles of competence is to enable activation of pathways for the utilization of other energy sources when simple sugars are not available. Hence, competence would serve a dual function as either a means to derive energy from DNA or a means of horizontal transfer of genetic information. Our data are consistent with such a model.

Fresh clinical isolates of *A. actinomycetemcomitans* invariably express fimbriae and display rough colony morphology (21). The fimbriated strains may yield smooth-colony, nonfimbriated mutants after extended in vitro subculturing (21). In preliminary studies, we noted that the transformation efficiency of the fimbriated wild-type *A. actinomycetemcomitans* was higher if the transformation assay was performed on agar. Furthermore, fimbriated wild-type *A. actinomycetemcomitans* isolates grew poorly in broth, formed microcolonies on the surface of the test tubes, and were generally difficult to manipulate. For the above reasons, we chose to perform transformation assays on agar instead of in broth. However, transformation of the nonfimbriated strain D7S-smooth can be easily performed in broth.

The persistent aggregates formed by wild-type *A. actinomycetemcomitans* interfere with determination of transformation frequencies. However, the number of CFU is a lower limit to the number of viable cells, and so the calculated transformation frequency of D7S is an upper limit. This increases the significance of the finding that the transformation frequency and efficiency of the nonfimbriated *A. actinomycetemcomitans* strain D7S-smooth were higher than those of the fimbriated, wild-type parental strain D7S.

In many naturally competent bacteria, components of type IV pilins are essential for DNA uptake (8, 12). However this study showed that the spontaneous nonfimbriated mutant of *A. actinomycetemcomitans* strain D7S was more competent than was the fimbriated parental wild type. We also noted that the nonfimbriated, *flp*-deletion mutant of *A. actinomycetemcomitans* strain D7S was naturally competent (data not shown). As Inoue et al. have shown that the *A. actinomycetemcomitans flp* gene encodes a fimbrial protein with some similarity to type IV pilins (16), this suggested that *flp*-encoded fimbriae are not required for the natural competence of this bacterium.

H. influenzae Rd preferentially takes up DNA containing the USS. The USS was originally identified as an 11-bp sequence (6), but the 9-bp sequence 5′-AAGTGCGGT (plus orientation) or 5′-ACCGCACTT (minus orientation) was later found to be highly conserved and most important for uptake (10). A total of 1,465 copies of this 9-bp site were found by searching the 1.8 Mb of genome sequence of *H. influenzae* Rd. The alignment of these sequences revealed an extended conserved 29-bp sequence containing the core 9-bp site (25).

A. actinomycetemcomitans genomic DNA anneals poorly to *H. influenzae* DNA in DNA-DNA hybridization experiments (1), and the 16S rRNAs of the two species have only 94% identity (*E. coli* and *Salmonella enterica* serovar Typhimurium have 97%). Nevertheless, the finding that *A. actinomycetemcomitans* DNA competes efficiently with *H. influenzae* DNA for uptake by *H. influenzae* indicated that *A. actinomycetemcomitans* might contain an abundant sequence resembling the *H. influenzae* USS (1). Consistent with this, Thomson et al. found several copies of an 11-bp sequence similar to the *H. influenzae*

USS in intergenic regions near the *katA* gene of *A. actinomycetemcomitans* (29). They further identified 848 copies of this 11-bp sequence in the *A. actinomycetemcomitans* Genome Sequencing Project database and postulated that this sequence may be used as a DNA uptake signal (29). We confirmed these observations by showing that the *A. actinomycetemcomitans* genome does contain a great excess of repeated sequences remarkably similar to the *H. influenzae* USS. Furthermore, we showed that DNA of various sources containing this USS competed for uptake for transformation in *A. actinomycetemcomitans*, suggesting that *A. actinomycetemcomitans* does preferentially take up DNA containing this USS.

These findings call into question the assumed function of the USS. It is unlikely to serve as a genetic barrier between related species, as DNAs of many members of the *Pasteurellaceae* compete for uptake by *H. influenzae*. Nor does it prevent uptake of potentially harmful DNAs: the genome of the *H. influenzae* phage HP1 has a high density of *H. influenzae* USSs. Other functions have been suggested for the USS, but none stand up under close scrutiny. The *H. influenzae* USS is not the Chi sequence (27), and most USSs cannot be transcription terminators, as they lie within coding sequences. Their abundance may simply be a consequence of the molecular drive imposed by the strong sequence bias of the DNA receptor. This bias in turn may result from constraints on DNA binding imposed by the uptake process, perhaps needed to overcome the difficulty of translocating highly charged DNA across the cell membranes.

Our studies of natural transformation in *A. actinomycetemcomitans* provided a new avenue for genetic engineering in this bacterium. Construction of strains with new combinations of existing genes is facilitated by recombinant DNA techniques and natural transformation. Over 30 gene replacement mutants were generated in the strain D7S or D7S-smooth. The PCR DNAs used were between 0.4 and 1.9 kb, and the 1-kb length appears to be optimal. Transformation efficiencies for single-gene deletions in D7S were found in the range of 3×10^2 to 2×10^4/μg of ligated DNA when the PCR DNA contained one or more USSs and about 100-fold less if the DNA did not have USSs (unpublished results). Incorporation of the USS in one of the PCR primers appeared to increase the transformation efficiency. A much higher efficiency was obtained when D7S-smooth was used as the recipient. A detailed study of these conditions is under way in our laboratory.

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