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Genetic Basis for Conversion of Rough-to-Smooth Colony Morphology in *Actinobacillus actinomycetemcomitans*

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The basis of the rough-to-smooth conversion of *Actinobacillus actinomycetemcomitans* was examined. Smooth variants often contained mutations at the *flp* promoter region. Replacing the mutated *flp* promoter with the wild-type promoter restored the rough phenotype. The expression level of the *flp* promoter was ~100-fold lower in smooth than in rough strains. Mutations of the *flp* promoter are a cause of the rough-to-smooth conversion.

Gram-negative, facultatively anaerobic *Actinobacillus actinomycetemcomitans* is a major periodontal pathogen (1, 20). Fresh oral isolates of *A. actinomycetemcomitans* are invariably fimbriated and form small (~1-mm), rough-surface, translucent colonies with an internal star-shaped structure (2, 9, 15, 20). After repeated in vitro passages, the rough-colony morphotype may yield nonfimbriated smooth-colony variants that grow as large, round, opaque colonies on agar (2, 9, 15, 16). Occasionally, the rough-to-smooth transition goes through an intermediate phase in which the colonies are translucent but smooth surfaced (9). Genes for the fimbria biogenesis of *A. actinomycetemcomitans* reside in a 12-kb *flp* operon that contains 14 genes, *flp-1–flp-2–tadV–rcpCAB–tadZABCDEFG* (6, 8–12, 14). The transcription-initiation points of the operon were located at 101 and 102 nucleotides upstream of *flp-1* (5). Two consensus elements, −10 (TATAAT) and −35 (TTGC AT), separated by 16 nucleotides, of the canonical σ^{70} promoter sequence were identified upstream of the transcription-initiation points (5).

Many pathogenic bacteria are capable of phase variation and colonial morphology shift, which depends on the expression of surface proteins (3, 13, 18). While the rough-to-smooth conversion of *A. actinomycetemcomitans* occurs spontaneously, the reverse smooth-to-rough conversion has not been substantiated. We postulated that the rough-smooth conversion in *A. actinomycetemcomitans* is due not to a phase variation mechanism of the fimbria expression but to some mutational event of the *flp* operon. This study aimed to determine whether mutations at the promoter region of the *flp* operon might explain the rough-to-smooth conversion of *A. actinomycetemcomitans*.

Eighteen *A. actinomycetemcomitans* strains were examined (Table 1). The culture media and conditions for *A. actinomycetemcomitans* were as described previously (16). The sequences of the *flp* promoter of these strains were determined by direct sequencing of the PCR products amplified from this region. A 1-kb DNA fragment encompassing the *flp-1–flp-2* genes and 300 bp upstream of *flp-1* was amplified with the forward primer F-Ev24 (5′-TCGCGATATCTCTAAATCCA CACA; an EcoRV site is underlined), which is located 300 bp upstream of *flp-1* (see Fig. 1 for its location). Three different reverse primers were used. Most of the study strains were amplified with the reverse primer orfB-X, 5′-TATCTAGAAC GGAATAATGGCGAATA (an XbaI site incorporated) located in *orfB* (Fig. 1). Two other reverse primers, orfB-B1, 5′-CAGGATCCAGCAGCGAGAGCGTTAT, and orfC-R, 5′-GCACTGAAATGATCAAGAGC, were used for strains that failed to be amplified with the primer orfB-X. PCR was carried out for 30 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min. PCR products were purified by the PCR purification columns (QIAGEN) and sequenced by the USC School of Medicine Microchemical Core Facility.

Figure 2 is a summary of the sequencing results of the −35

TABLE 1. *A. actinomycetemcomitans* strains used in this study

Strain	Serotype	Colony morphotype[a]	Source
Reference strains			
Y4	b	S	ATCC[c] (oral)
JP2	b	S	S. Asikainen (oral)
HK1651	b	R	ATCC (oral)
ATCC29523	a	S	ATCC (blood)
ATCC29522	b	S	ATCC (mandibular abscess)
ATCC29524	b	S	ATCC (chest aspirate)
Clinical isolates and variants			
D7S	a	R	Oral clinical isolate
D7S-SA	a	S	Derived from D7S[b]
D7S-SB	a	S	Derived from D7S[b]
D7S-SC	a	S	Derived from D7S[b]
D7S-ID	a	I	Derived from D7S[b]
D9	b	R	Oral clinical isolate
D9-SA	b	S	Derived from D9
D28	b	R	Oral clinical isolate
D28-SA	b	S	Derived from D28[b]
D28-IA	b	I	Derived from D28[b]
29R	f	R	Oral clinical isolate
29-SS	f	S	Derived from 29R

[a] R, rough; S, smooth; I, intermediate.
[b] These derivative strains were generated independently from different in vitro passages of their parental strains.
[c] ATCC, American Type Culture Collection.

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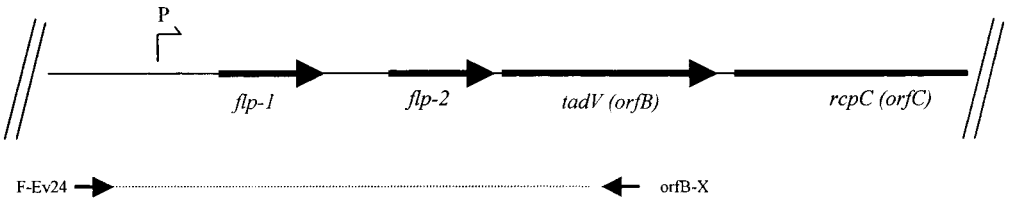

FIG. 1. Genetic map of the 5′ end of the *flp* operon including ~300 bp upstream of *flp-1*. The locations of the primer annealing sites for PCR are depicted. The region between the primer F-Ev24/orfB-X annealing sites was amplified from *A. actinomycetemcomitans* study strains and sequenced.

site, the spacer, and the −10 site. More detailed information on the entire sequenced regions may be accessed through GenBank. All four rough clinical isolates (D7S, D9, D28, and 29R) had a promoter essentially identical to that identified by Haase et al. (5). Two published *flp* sequences from strain CU1000 (GenBank AY157714) (12) and strain 310a (GenBank D83053) (10) also contain the same promoter sequence. Therefore, this sequence was designated as the consensus sequence. The rough-colony strain HK1651 exhibited a one-base variation in a semiconserved base at the −10 site (Fig. 2). In broth culture, strain HK1651 formed less compact aggregates than did other rough clinical strains. Presumably, this base variation of strain HK1651 may slightly reduce the *flp* promoter strength. The flanking regions of the promoter exhibited a greater sequence variation among strains, but the variations did not correlate with the rough-smooth conversion (data not shown).

Five of the eight smooth- or intermediate-colony derivative strains and three of the six smooth-colony reference strains exhibited sequence variations in the conserved −10 site. Six of these variations were due to a single-nucleotide substitution, and the remaining two variations were due to a single-nucleotide deletion. A frequent mutation was the transition of the most conserved T residue of the −10 site. This base was often called "invariable" T, as it was conserved in 97% of *Escherichia coli* promoters among 112 promoter sequences compiled, and mutations of this base were demonstrated to severely affect a number of promoters' activities in *E. coli* (7, 19). Strain JP2 exhibited a single-nucleotide deletion in the spacer region between the conserved −35 and −10 sites. Strain ATCC 29524 showed a deletion of the entire −35 and part of the spacer region. The promoter sequences of the remaining three derivative strains (strains D7S-ID, D28-IA, and 29-SS) and the smooth reference strain ATCC 29523 were identical to the consensus sequence or the sequence of the parental strain (i.e., strain 29R).

The bacterial morphology was examined by transmission electron microscopy (TEM) by a previously described protocol (17). As expected, the smooth-colony strain D7S-SA was nonfimbriated, in contrast to its parental rough-colony strain D7S (Fig. 3). We have examined 15 other smooth-derivative strains from clinical isolates and have not detected the presence of fimbriae (data not shown). However, TEM of strain ATCC 29523 showed a few thin fibrils that resembled fimbriae (Fig. 3). It is interesting that strain ATCC 29523 has a wild-type *flp* promoter. We also noted a tendency of strain 29523 to aggregate in broth cultures. The low expression of fimbria may explain the aggregation of ATCC 29523 in broth.

The mutated *flp* promoters of several smooth-colony variants were replaced with a wild-type promoter. A 1-kb PCR

	-35	-10	mRNA
***A. Actinomycetemcomitans* consensus**	TTGCAT	TTTAATTTTTCAGGAC TATAAT	GCCACTCGTTTATTT
Rough clinical isolates:			
D7S, D9, D28	------	----------	------
29R	------	----------T--	------
Rough reference strain:			
HK1651	------	----------	----G-
Intermediate or smooth derivatives of clinical isolates:			
D7S-SA, D7S-SB, D9-SA, D28-SA	------	----------	-----C
D7S-SC	------	----------	-----Δ
D7S-ID, D28-IA	------	----------	------
29SS	------	----------T--	------
Smooth reference strains:			
ATCC29523	------	----------	------
ATCC29522, ATCC33384	------	----------	-----C
Y4	------	----------	----Δ-
JP2	------	---------Δ--	------
ATCC29524	ΔΔΔΔΔΔΔΔΔΔΔΔ	----------	------
***E. coli* consensus**	TTGACa	tnntnnnnnnntntgn TAtAaT	

FIG. 2. The *flp* promoter sequences of the *A. actinomycetemcomitans* study strains. The entire sequenced regions can be accessed through GenBank: *flp* locus of strain D7S, AY262277; D9, AY460681; D28, AY460682; 29R, AY460683; JP2, AY460684; Y4, AY460685; ATCC 29522, AY460686; ATCC 29523, AY460687; ATCC 29524, AY460688. The consensus sequence of the *E. coli* promoter (7) is provided for comparison (uppercase, >60% conservation; lowercase, >43%). The sequence for ATCC 33384 was from GenBank (T. Tanimoto, unpublished data; accession number AB071167). Flag symbol, the transcription-initiation point; −, nucleotide identical to the parent; Δ, deletion.

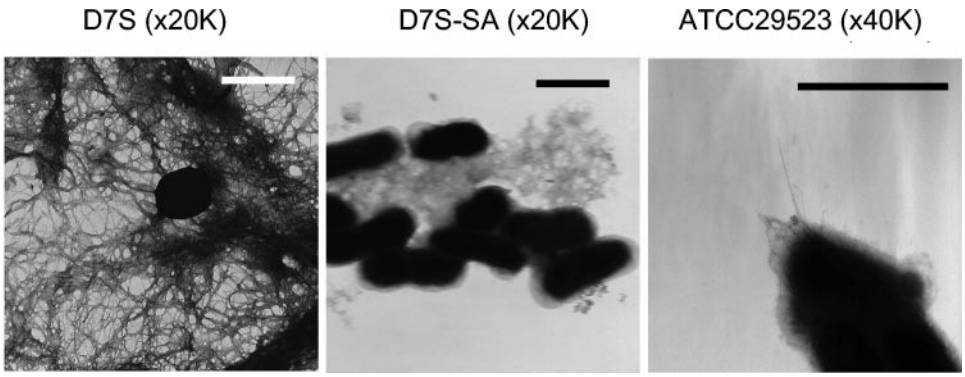

FIG. 3. Transmission electron micrographs of the wild-type *A. actinomycetemcomitans* strain D7S, smooth-derivative strain D7S-SA, and reference strain ATCC 29523. Note that a few strands of fimbriae were present on the surface of strain ATCC 29523. Bars, 1 μm.

DNA fragment encompassing *flp-1–flp-2* and the promoter was amplified from rough strain D7S using primers F-Ev24 and orfB-X (Fig. 1 shows primer locations). The DNA was then digested with EcoRV and XbaI, ligated to pTc-USS at the same sites, and transformed into smooth-colony strains D7S-SA and D7S-SB by a natural transformation protocol described previously (16). The plasmid pTc-USS is a pBluescript II KS (Stratagene) derivative that does not replicate in *A. actinomycetemcomitans* (17). Therefore, the Tc^r transformants should have the plasmid containing the wild-type *flp* promoter integrated in the chromosome by a single crossover. If the point mutation in the *flp* promoter accounted for the rough-to-smooth conversion in strains D7S-SA and D7S-SB, insertion of a promoter (presumably any well-expressed promoter) upstream of the *flp* operon should restore the rough-colony phenotype. As expected, the transformants were predominantly of rough-colony type (an example is shown in Fig. 4). The integration of the plasmid in the chromosome was verified in selected rough colonies by PCR using two pairs of primers, F-Ev24/Umer and orfC-R/Rmer (data not shown). We also replaced the promoter region of the smooth variant D7S-SA with the corresponding promoter region from different *A. actinomycetemcomitans* strains. Again, the 1-kb promoter-*flp-1–flp-2* region was amplified from the rough strains HK1651 and 29R and smooth strains ATCC 29523 and ATCC 33384. Each PCR amplicon DNA was ligated to pTc-USS and transformed into strain D7S-SA. The results showed that no rough transformants were obtained with the donor DNA from strain ATCC 33384, while most transformants with donor DNA from the other three strains were predominantly rough-colony type (e.g., 75% of the transformants were rough-colony type with donor DNA from strain ATCC 29523). The results indicated that random mutations in the promoter of the *flp* operon are a mechanism for the rough-to-smooth conversion of *A. actinomycetemcomitans.*

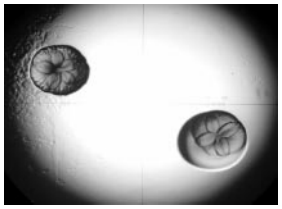

FIG. 4. Colonies of *A. actinomycetemcomitans* strain D7S-SA transformed with pTc-USS carrying the wild-type *flp* promoter DNA. Colonies were 5 days old and ~1 mm in size. The picture was taken under a light microscope with a 4× objective. Left, wild-type-like rough colony; right, chimeric colony, in which the smooth portion appeared after 3 days of culture.

Conversely, we replaced the *flp* promoter of the rough-colony strain D7S with the promoter obtained from smooth-colony variants. A 2.3-kb DNA fragment that included the *flp* promoter region was amplified by PCR from each of the smooth-colony strains JP2, Y4, and ATCC 29524 with primers orfC-R and flp-UE (5′-CTGAATTCTCGCTCAGATAC GGA, which is located at 1.1 kb upstream of the promoter region) and directly used as the donor DNA to transform strain D7S. To enrich the nonfimbriated bacteria, the transformed bacteria were briefly incubated in broth. Fimbriated D7S bacteria formed aggregates that either settled to the bottom of the culture tube or adhered to the tube, while the nonfimbriated smooth-colony variants would grow as a single-cell suspension. The top portion of the undisturbed broth culture of the transformed D7S was collected and plated on agar. The results showed that the colonies were predominantly smooth. The *flp* promoters of four smooth-colony variants (two were transformed with the *flp* promoter from strain JP2 and one each was transformed with the *flp* promoter from strains Y4 and ATCC 29524) were amplified by PCR and sequenced. The results showed that each of the *flp* promoters of these smooth-colony variants carried the specific sequence variation of the *flp* promoter found in the corresponding donor strain JP2, Y4, or ATCC 29524. The smooth-colony transformant derived from transformation with donor DNA of strain ATCC 29524 displayed an additional insertion of two nucleotides downstream of the −10 site, which may have arisen from PCR

TABLE 2. Luciferase activities of different *A. actinomycetemcomitans* strains

Strain	Luciferase activity[a]
D7S (Δ*rcpB*::*luc*-Spe)	14,421 ± 5,856
D7S-SA (Δ*rcpB*::*luc*-Spe)	181 ± 20
D7S-SC (Δ*rcpB*::*luc*-Spe)	108 ± 18
ATCC 29523 (Δ*rcpB*::*luc*-Spe)	3,524 ± 942

[a] Mean ± standard deviation.

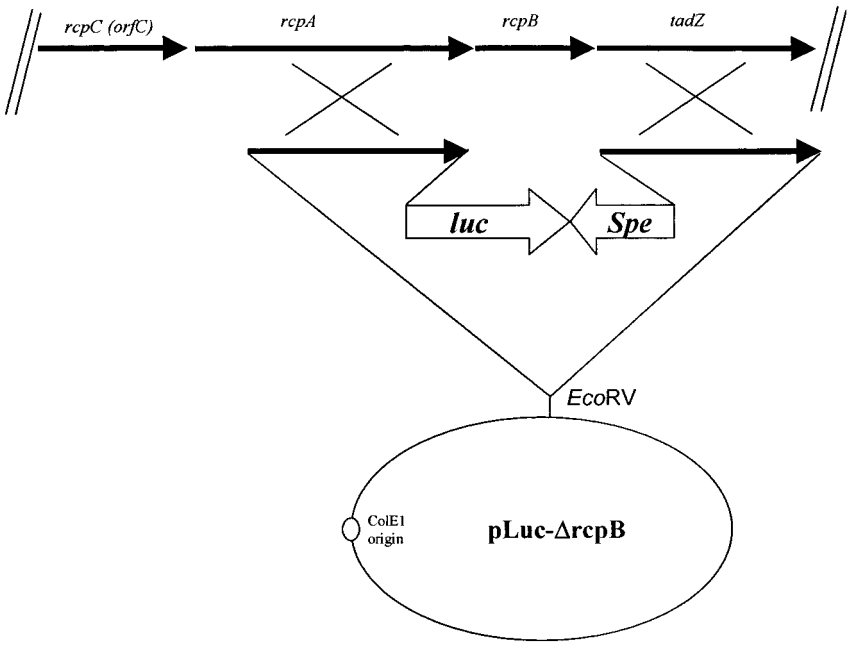

FIG. 5. Genetic map of the plasmid pLuc-Δ*rcpB* (not in exact scale). The plasmid pLuc-Δ*rcpB* contains a 2.85-kb *luc*-Spe cassette and flanking DNA of *rcpB.* The plasmid was used to transform *A. actinomycetemcomitans* strains to generate transformants with *luc*-Spe replacing *rcpB* of the *flp* operon.

amplification or transformation. However, the insertion occurred at the nonconserved area and was not expected to be the cause of the reduced expression of the *flp* operon. The results further substantiated the role of the *flp* promoter mutations in rough-to-smooth conversion.

The expression levels of *flp* promoters in rough- and smooth-colony bacteria were measured by inserting a firefly luciferase gene, *luc*, as the reporter gene at the *rcpB* site of the *flp* operon (Fig. 5). Briefly, 3.2-kb DNA containing the Spe marker and the *rcpB*-flanking DNA was amplified from a previously constructed Δ*rcpB*::Spe mutant of strain D7S (unpublished data) with a primer at 1.1 kb upstream of *rcpB* and another primer at 1 kb downstream of *rcpB*. This 3.2-kb DNA was cloned in pBluescript II KS at the EcoRV site to produce pB-Δ*rcpB*. Separately, a *luc*-Spe cassette plasmid was constructed based on pBluescript II KS and pBRluc (4), and the plasmid was named pLuc-Spe2. A 3-kb *luc*-Spe reporter-marker was amplified from pLuc-Spe2 using the primer Luc-B1, 5′-AGGGATC CTAGGAAGCTTTCCATGGA (the *luc* start codon is underlined), and the primer Spe-USS, 5′-AAAGTGCGGTTTACA CTTACTTTAGTTTT. This *luc*-Spe marker was then used to replace the 1.1-kb Spe marker in pB-Δ*rcpB* to create pLuc-Δ*rcpB* (Fig. 5), which was used to transform *A. actinomycetemcomitans* strains. Transformants were verified by PCR and tested for luciferase production. Briefly, bacteria were grown on serum trypticase soy broth agar overnight and resuspended in tryptic soy broth at an optical density (OD) at 600 nm of 0.5, and luciferase was assayed by mixing 20 μl of luciferin (1 mM in 0.1 M sodium citrate, pH 7.0) with 80 μl bacterial suspension (OD at 600 nm = 0.1 to 0.6) at room temperature for 5 min, and the light was counted twice with the BetaScout Liquid Scintillation Tester (Perkin-Elmer Life Sciences). Enzyme activity was defined as (photon counts in 10 s − background counts)/0.001 OD unit of bacteria. The results (Table 2) showed that the *luc* expression in strain D7S-SA, with a T-to-C transition in the *flp* promoter, was 80-fold lower than that in the wild type and that expression in strain D7S-SC, with a T deletion, was 130-fold lower than that in the wild type. Interestingly, the *luc* activity in strain ATCC 29523 was fourfold lower than that in strain D7S. Perhaps the low-level expression of the *flp* promoter in ATCC 29523 resulted in the scant expression of fimbria seen under TEM.

Our findings suggest that in vitro spontaneous rough-to-smooth conversion of *A. actinomycetemcomitans* commonly occurs due to mutations at the −35 site, the spacer region, or the −10 site of the *flp* promoter. Such mutations are not likely to be reversible at a significant rate and may explain the lack of smooth-to-rough conversion among *A. actinomycetemcomitans* strains. However, several smooth variants had an apparently wild-type *flp* promoter. Mutation of the *flp* promoter is not the only mechanism of the rough-to-smooth conversion of this bacterium.

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