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Mutation analysis of the *flp* operon in *Actinobacillus actinomycetemcomitans*

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Abstract

Fresh clinical isolates of the periodontal pathogen *Actinobacillus actinomycetemcomitans* live as autoaggregates, in which cells are densely packed and embedded in an extracellular matrix composed of bundled fimbriae, exopolymers, and vesicles. The expression of fimbriae is known to be determined by the *flp* operon of 14 genes, *flp-1-flp-2-tadV-rcpCAB-tadZABCDEFG*. We generated mutations of each gene of this operon in *A. actinomycetemcomitans* strain D7S. All mutants expressed some changes in the production of extracellular matrix materials that include vesicles, exopolymers, and fimbriae. The expression of fimbriae required the function of *flp-1*, *rcpA*, *rcpB*, *tadB*, *tadD*, *tadE*, and *tadF*. Mutants of *flp-2*, *tadZ*, *tadA*, *tadC*, and *tadG* expressed reduced levels of fimbriae, or fimbriae that had different gross appearance. Importantly, the expression of the non-fimbrial matrix materials was affected by all mutations, suggesting that the *flp* operon was involved in production of these materials. The *flp* locus apparently plays a central role in autoaggregation of *A. actinomycetemcomitans,* which may be the primary survival strategy of this bacterium in vivo.

Keywords: Biofilm; Aggregation; Pili; Gene deletion

1. Introduction

Gram-negative, facultatively anaerobic *Actinobacillus actinomycetemcomitans* is implicated as a major causative agent in aggressive periodontitis (Zambon, 1985; Slots and Ting, 1999). Fresh oral isolates of *A. actinomycetemcomitans* are invariably fimbriated, and form rough-surface translucent colonies embedded in agar with an internal star-shape structure (Preus et al., 1988; Rosan et al., 1988; Inouye et al., 1990; Fine et al., 1999b). Repeated in vitro subculturing of this bacterium gives rise to non-fimbriated, smooth-surface opaque colonies, or intermediate-type colonies (Inouye et al., 1990). The broth cultures of *A. actinomycetemcomitans* exhibit distinct features that are correlated to their colony morphotypes on agar. In broth cultures, the rough-colony-type bacteria form compact aggregates adhering to the side or the bottom of the culture vessel, and the broth remains clear (Kachlany et al., 2001a). The smooth-colony variants grow as homogeneous non-aggregating planktonic cells and appear as a turbid suspension in broth. The intermediate morphotypes grow in broth as loose aggregates and the broth appears slightly turbid.

The genes for fimbria biogenesis were found to reside in a 12-kb region containing 14 genes, *flp-1-flp-2-tadV-rcpCAB-tadZABCDEFG*. The *flp-1* gene encodes the major fimbrial subunit Flp1 (fimbrial low-molecular-weight protein) (Ishihara et al., 1997; Inoue et al., 1998; Kachlany et

Abbreviations: aa, amino acid(s); *flp*, gene encoding the fimbrial low-molecular-weight protein; CFU, colony forming unit; mTSB, modified TSB; Orf, open reading frame; PCR, polymerase chain reaction; ^{R}, resistance; SEM, scanning electron microscopy; Spe, spectinomycin; sTSB, serum supplemented TSB; TEM, transmission electron microscopy; Tet, tetracycline; TSB, trypticase soy broth; USS, uptake signal sequence; Δ, deletion; ∷, novel junction (fusion or insertion).

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al., 2001a). RcpAB (rough-colony proteins) were identified as rough-colony-specific outer membrane proteins that are probably involved in fimbria expression (Haase et al., 1999). The *tad* genes (tight adherence) were found to be required for adherence and fimbria production by transposon mutagenesis (Kachlany et al., 2000). Transcriptional analysis by Haase et al. (2003) indicated that this gene cluster likely constitutes an operon, and a canonical σ^{70} promoter was identified at ~120-bp upstream of the *flp-1* gene. Transposon insertions in 11 of these 14 genes, except *flp-2*, *tadV*, and *rcpB*, were obtained by selection of smooth-colony mutants (Planet et al., 2003). These mutants were reported to produce no fibrils, and it was proposed that this 14-gene cluster encodes a secretion system for the export and assembly of fimbriae, which mediate adherence and aggregation, and confer the rough-colony phenotype of *A. actinomycetemcomitans* (Planet et al., 2003).

Besides fimbriae, *A. actinomycetemcomitans* secretes a large amount of amorphous materials (also called exopolymers that are composed mostly of polysaccharide) and vesicles (Holt et al., 1980; Nowotny et al., 1982), both of which seemed to contribute to aggregation and adherence (Meyer and Fives-Taylor, 1993). Several reports suggested that polysaccharide or glycoconjugates play a key role in autoaggregation and adherence of *A. actinomycetemcomitans*. For example, Fine et al. (1999a) found that treatment of *A. actinomycetemcomitans* with periodate, but not trypsin, reduced the adherence and induced the detachment of the bacteria from the surfaces. Kaplan et al. (2003b) identified a glycosyl hydrolase encoded by *dspB*, which allowed dispersion of bacteria from *A. actinomycetemcomitans* clumps.

The aim of this study was to further examine the function of the *flp* operon. We were able to generate specific mutations of individual ORFs of the *flp* operon in order to probe their functions. Here we describe the phenotypes of these mutants, and propose that this 14-gene *flp* operon is involved in the production of exopolymers, vesicles, and fimbriae, all of which are likely required for the tenacious autoaggregation of *A. actinomycetemcomitans*.

2. Materials and methods

2.1. Bacteria, plasmids, and culture conditions

A. actinomycetemcomitans strains used were D7S (serotype a), and its derivatives D7S-smooth and D7S(Δ*flp-tad*) (Wang et al., 2002). HK1651 (ATCC700685; serotype b) was also used. *A. actinomycetemcomitans* strains were grown in a modified sTSB medium (3% trypticase soy broth, 0.3% yeast extract, and 5% horse serum), or mTSB (3% TSB, and 0.6% yeast extract) at 37 °C in air supplemented with 5% CO_2. For solid media, 1.5% agar was added to the broth. *Escherichia coli* plasmids were propagated in *E. coli* DH5α by standard methods (Sambrook et al., 1989). When necessary, the medium was supplemented with spectinomycin (Spe, 50 μg/ml), or tetracycline (Tet, 6 μg/ml or 10 μg/ml for *E. coli*).

For the Congo Red binding test, the culture medium was made with sTSB supplemented with 1.2% agar, 40 μg/ml Congo Red, and 20 μg/ml Coomassie brilliant blue. Bacteria were collected from fresh plate cultures, and were suspended in PBS to ~10^6 CFU/ml. Two microliters of each samples was spotted on the agar medium and incubated at 37 °C. Colonies were photographed after 48 and 72 h of incubation, and the amount of the Congo Red binding was evaluated based on visual examination of the photos.

The spectinomycin-resistance (Spe) cassette plasmid, pK-Spe, has been described previously (Wang et al., 2002). The sequence of this 1.2-kb Spe cassette gene is identical to the original Spe gene (LeBlanc et al., 1991) in the promoter and the coding region, but contains a deletion in the inverted repeat sequence downstream of the Spe ORF (unpublished sequencing data). Therefore, there is no transcription attenuation downstream of this Spe cassette. Plasmid pTet-KS (4.17 kb) was constructed from pBluescript KS (Stratagene) by replacing the ampicillin marker with the *tetO* gene (Wang and Taylor, 1991). pTc-USS is identical to pTet-KS except that a USS site was incorporated at the upstream sequence of the *tetO* gene.

2.2. Cloning and sequencing of the flp locus DNA

The DNA region encompassing *flp-1* to *rcpA* was PCR-amplified from strain D7S. The resultant amplicons were directly sequenced after purification through PCR purification columns (QIAGEN). The rest of the operon was sequenced by cloning the target region in an *E. coli* host. Briefly, the *rcpC* gene of strain D7S was first replaced with the *E. coli* plasmid pTet-KS by the following protocol. The *Sal*I-digested pTet-KS was ligated to two *Bam*HI-cleaved PCR DNAs of ~1 kb each, which were derived from the flanking sequences of *rcpC*, by the previously described method (Wang et al., 2002). The ligation mix was used to transform strain D7S. Chromosomal DNA was then isolated from one of the Tet^R transformants. Subsequently, a 17-kb plasmid was obtained by *Kpn*I digestion of this DNA, self-ligation, and transformation of *E. coli* DH5α. Sequence analysis revealed that this plasmid (named pC-Kpn1) contained the *rcpA-tadG* genes exactly as expected (see Fig. 1 for the gene organization). The DNA upstream of *flp* was also cloned by a similar method. A 13-kb *Cla*I-fragment upstream of *flp-1* was cloned after inserting pTc-USS at the *flp-1* site in strain D7S and followed by plasmid-rescue in *E. coli.* Sequence analysis was performed for only 1.3-kb upstream of *flp-1*, and the sequence was found to be 98% identical to that of HK1651 (University of Oklahoma Genome sequencing Center).