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NOTE

Amended Description of the Genes for Synthesis of *Actinomyces naeslundii* T14V Type 1 Fimbriae and Associated Adhesin▽†

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The type 1 fimbriae of *Actinomyces naeslundii* T14V mediate adhesion of this gram-positive species to the tooth surface. The present findings show that the locus for type 1 fimbria production in this strain includes three genes, *fimQ* for a minor fimbrial subunit that appears to be an adhesin, *fimP* for the major structural subunit, and *srtC1* for a type 1 fimbria-specific sortase involved in the assembly of these structures.

Studies of the prominent oral microorganism *Actinomyces naeslundii* have provided important insights into both the properties of fimbriae or pili on gram-positive bacteria (16, 18) and the underlying mechanisms of dental plaque formation (4). The fimbriae of *A. naeslundii*, which were among the first observed for a gram-positive species (11), consist of two functionally distinct types (5). The type 1 fimbriae mediate adhesion of this species to the tooth surface through the binding of adsorbed salivary proline-rich proteins (PRPs) (7, 10, 13), whereas the type 2 fimbriae promote biofilm formation (14) through recognition of hostlike saccharide motifs in the surface polysaccharides of early colonizing streptococci (3). The major subunits of type 1 and type 2 fimbriae, encoded by the genes *fimP* (19) and *fimA* (20), respectively, are similar in size (56 kDa) and have sequences that are approximately 40% identical. The gene *fimA* of *A. naeslundii* T14V occurs between open reading frame 977 (ORF 977), which may encode the type 2 fimbria-associated adhesin (12), and ORF 365 for a sortase that is required for the covalent polymerization of FimA monomers (20). In contrast, *fimP* of this strain appears to be flanked by three essential upstream ORFs (i.e., ORF3-ORF2-ORF1) of unknown function and two essential downstream ORFs (i.e., ORF4-ORF5) (22), which include the partial coding sequence of a putative sortase (9). Moreover, the genes that reportedly flank *fimP* in strain T14V differ dramatically from those that flank this gene in type 1-fimbriated *Actinomyces viscosus* 19246 (13).

To verify the genes for type 1 fimbria production in *A. naeslundii* T14V, we amplified and sequenced a series of overlapping PCR products from genomic DNA of this strain by using primers designed with the previously published sequence of this region (22). Annotation of the resulting sequence revealed *fimP* as previously described (19). However, this gene in our amended sequence is flanked by one upstream gene (i.e., *fimQ*) rather than three (i.e., ORF3-ORF2-ORF1) and by two downstream genes (i.e., *srtC1* and *orfC*) that differ from those (i.e., ORF4-ORF5-ORF6) previously described (22). For the most part, these differences appeared to reflect the existence of sequencing errors, introduced previously by manual sequencing of GC-rich regions. Importantly, the presently described genes in strain T14V (i.e., *fimQ*, *fimP*, *srtC1*, and *orfC*) are comparable to those previously identified (13) in *A. viscosus* 19246 (i.e., *orfA*, *fimP*, *orfB* and *orfC*), as well as those in the whole genome sequence of *A. naeslundii* MG1, which is available at The Institute for Genomic Research website (http://cmr.tigr.org).

The gene *fimQ* of *A. naeslundii* T14V (Fig. 1) and *orfA* in *A. viscosus* 19246 (13) encode 149-kDa proteins with sequences that are 83% identical. Both proteins have typical 45-amino-acid gram-positive leader sequences, predicted by the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/), identical E box sequences (17), similar to the E box sequence present in FimP, and cell wall sorting motifs (LPLSG). These features raised the possibility that FimQ represented a previously unidentified type 1 fimbria-associated protein. Support for this possibility was gained from proteomic analysis of immunoaffinity-purified type 1 fimbriae, isolated by elution from a coupled FimP-specific monoclonal antibody (MAb) (2). Eluted fimbriae were digested either with pepsin or by dilute-acid hydrolysis, and the resulting peptides were analyzed by liquid chromatography-tandem mass spectrometry. Not surprisingly, most of the identified peptides (i.e., greater than 95%) had

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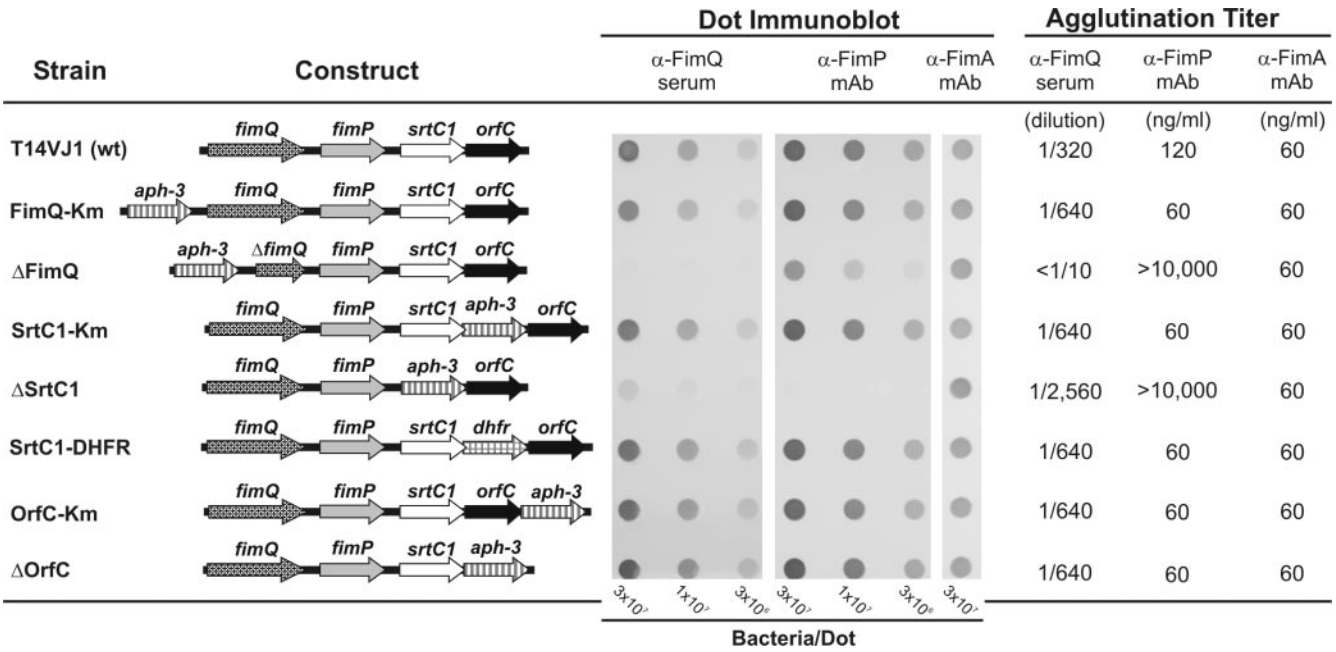

FIG. 1. ORF diagrams of the type 1 gene clusters in *A. naeslundii* T14V and related mutant strains based on the amended sequence of this region obtained in the present study. Each strain was examined for surface expression of fimbria-associated proteins by dot immunoblotting and bacterial agglutination assays performed with rabbit anti-FimQ serum raised by immunization with a DNA vaccine, mouse MAb 8A (2) against FimP, the structural subunit of type 1 fimbriae, and MAb 5A against FimA (1), the structural subunit of type 2 fimbriae. For dot immunoblotting, nitrocellulose membranes were spotted with decreasing numbers of bacteria, incubated with anti-FimQ serum (1/400) or mouse MAb (250 ng/ml), followed by peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin G, and developed using a metal enhanced diaminobenzidine substrate kit (Pierce). Agglutination titers are expressed as the minimum concentration of antibody to achieve macroscopic agglutination of 5×10^8 bacteria/ml based on assays performed with serial twofold dilutions of antiserum (α-FimQ) or purified MAb (α-FimP or α-FimA).

sequences identical to regions of FimP. However, several peptides with FimQ-like sequences were also identified, including the five described in Table 1 and Fig. S1 in the supplemental material. Representative tandem mass spectrometry spectra of two such peptides, VVRNSDGTF derived by pepsin digestion and PSTPGAKPLTD from dilute-acid hydrolysis of type 1 fimbriae, respectively, are shown in Fig. S2 and S3 in the supplemental material. These findings support the notion that FimQ is a minor type 1 fimbria-associated protein.

To further examine the possibility described above, we prepared antiserum against the central region of FimQ (amino acid residues 529 to 951) by immunization of rabbits (Aldevron, LLC, Fargo, ND) with the corresponding coding sequence cloned into the mammalian cell expression vector pJW4303, which was kindly provided by James I. Mullans (Department of Microbiology, University of Washington, Seattle).

TABLE 1. FimQ peptides identified from type 1 fimbriae after pepsin and diluted-acid hydrolysis

m/z	Charge	Mol wt		Sequence (positions)
		Exptl	Calculated	
497.80	2	993.58	993.49	VVRNSDGTF (938–946)[a]
419.93	3	1,256.77	1,256.62	AAAPRAGADGGSRT (1023–1036)[a]
575.83	2	1,149.64	1,149.54	TVRENTPGYN (1044–1053)[a]
399.28	2	796.54	796.37	GSYRLSD (1167–1173)[b]
542.37	2	1,082.73	1,082.56	PSTPGAKPLTD (1277–1287)[b]

[a] Peptide obtained from pepsin digestion of purified *A. naeslundii* T14V type 1 fimbriae.
[b] Peptide obtained from diluted-acid hydrolysis of the isolated type 1 fimbriae.

The anti-FimQ serum obtained labeled *A. naeslundii* T14V in dot immunoblot analysis and caused agglutination of this strain (Fig. 1). It did not, however, identify any bands on Western blots of mutanolysin digests prepared from this strain or any other strain described in this study. In other experiments (results not shown), the anti-FimQ antibody reacted with *A. naeslundii* 5519 cells, which has type 1 but not type 2 fimbriae, but did not react with *A. naeslundii* 5951 cells, which has type 2 but not type 1 fimbriae (5). Results obtained from immunogold labeling of type 1-fimbriated *A. naeslundii* 5519 (5) with rabbit anti-FimQ and mouse anti-FimP antibodies showed surface labeling of this strain with both antibodies (results not shown) but did not provide clear evidence for the localization of each antibody along individual fimbrial structures.

We then constructed the *kan*-containing control mutant, strain FimQ-Km, and the corresponding *fimQ* deletion mutant, strain ΔFimQ (Fig. 1), by allelic exchange. This involved electrotransformation (21) of *A. naeslundii* T14V with plasmids p3FimQ and p8FimQ, respectively (Table 2), and the growth of transformants on kanamycin-containing medium. Strains FimQ-Km and ΔFimQ contained the *kan* marker at the same position (i.e., 317 base pairs upstream of the putative ATG translation start codon of *fimQ*). However, strain ΔFimQ lacked the first 1,063 nucleotides of *fimQ*, which introduced a frame shift in the remaining *fimQ* sequence. The steps and primers used to prepare these and other plasmids are summarized in Table 2 in this paper and in Table S1 in the supplemental material. The integrity of all plasmid constructs listed in Table 2 was verified by restriction enzyme digestions and se-

TABLE 2. Bacterial strains and plasmids

Plasmid or strain	Description[a]	Source or reference
Plasmids		
pJRD215	Template for PCR amplification (primers 1 and 2) of *kan* (the *aph-3* gene for Km^r without its transcriptional terminator)	8
EZ-TN5<DHFR-1>	Template for PCR amplification of *dhfr* (primers 3 and 4) (the gene for Tm^r without its transcriptional terminator)	Epicentre
pDONR/Zeo	Cloning vector; Zeo^r	Invitrogen
p1FimQ	pDONR/Zeo containing a PCR-amplified (primers 5 and 6) region extending from upstream of strain T14V *fimQ* to the middle of *fimQ*	This study
p2FimQ	p1FimQ with an XbaI site created 317 bp upstream of *fimQ* using a QuikChange site-directed mutagenesis kit[b] and primers 7 and 8	This study
p3FimQ	p2FimQ with *kan* in the XbaI site	This study
p4FimQ	pDONR/Zeo containing a PCR-amplified (primers 5 and 9) region extending from upstream of strain T14V *fimQ* to the ATG starting codon of *fimQ*	This study
p5FimQ	p4FimQ with an XbaI site created at the same position as in p2FimQ by site-directed mutagenesis (primers 7 and 8)	This study
p6FimQ	p5FimQ with *kan* in the XbaI site	This study
p7FimQ	pDONR/Zeo containing a PCR-amplified (primers 10 and 11) region of strain T14V *fimQ* corresponding to nucleotides 1066 to 2348 of the complete gene	This study
p8FimQ	p7FimQ with the NdeI-HpaI fragment replaced with an NdeI-HpaI fragment from p6FimQ, resulting in a 1,063-bp deletion of *fimQ* (from ATG starting codon)	This study
p1Srt	pDONR/Zeo containing a PCR-amplified (primers 12 and 13) *srtC1* region of strain T14V	This study
p2Srt	p1Srt with an XbaI site created 42 bp upstream of the 3′ end of *srtC1* by site-directed mutagenesis (primers 14 and 15)	This study
p3Srt	p2Srt with *kan* in the XbaI site	This study
p4Srt	p2Srt with *srtC1* deleted (all coding sequence except the 42 bp at the 3′ end) by inverse PCR (primers 16 and 17); the resultant plasmid contains an XbaI site at the same position as in p2Srt	This study
p5Srt	p4Srt with *kan* in the XbaI site	This study
p6Srt	p2Srt with *dhfr* in the XbaI site	This study
p1OrfC	pDONR/Zeo containing a PCR-amplified (primers 18 and 19) *orfC* region of *A. naeslundii* T14V	This study
p2OrfC	p1OrfC with an XbaI site created 6 bp downstream of the 3′ end of *orfC* by site-directed mutagenesis (primers 20 and 21)	This study
p3OrfC	p2OrfC with *kan* in the XbaI site	This study
p4OrfC	p2OrfC with *orfC* deleted (all coding sequence except the first 12 bp from the 5′ end) by inverse PCR (primers 22 and 23); the resultant plasmid contains an XbaI site at the same position as in p2OrfC	This study
p5OrfC	p4OrfC with *kan* in the XbaI site	This study
A. naeslundii strains		
T14V	Wild-type strain; Km^s Sm^r	5
FimQ-Km	Km^r transformant of T14V obtained with p3FimQ	This study
ΔFimQ	Km^r transformant of T14V obtained with p8FimQ	This study
SrtC1-Km	Km^r transformant of T14V obtained with p3Srt	This study
ΔSrtC1	Km^r transformant of T14V obtained with p5Srt	This study
SrtC1-DHFR	Tm^r transformant of ΔSrtC1 obtained with p6Srt	This study
OrfC-Km	Km^r transformant of T14V obtained with p3OrfC	This study
ΔOrfC	Km^r transformant of T14V obtained with p5OrfC	This study

[a] The sequences of all primers used in the present study are listed in Table S1 in the supplemental material. Antibiotic abbreviations: Km, kanamycin; Sm, streptomycin; Tm, trimethoprim; Zeo, zeocin.
[b] From Stratagene, La Jolla, CA.

quencing at the junctions of inserted DNA fragments. Likewise, the location and orientation of the allelic-exchange plasmid DNA in each mutant were confirmed by amplification of specific PCR products across the upstream and downstream boundaries of the introduced sequence by using appropriate primers.

Unlike control strain FimQ-Km, strain ΔFimQ was unreactive with anti-FimQ antibody in both dot immunoblotting and bacterial agglutination assays (Fig. 1). The anti-FimP immunoreactivity of strain ΔFimQ in dot immunoblotting was also lower than that of strain FimQ-Km. Importantly, strain ΔFimQ agglutinated in the presence of anti-FimA MAb 5A against type 2 fimbriae but not in the presence of anti-FimP MAb 8A against type 1 fimbriae. The absence of typical type 1 fimbriae on strain ΔFimQ was further suggested by the different patterns of anti-FimP reactive bands seen in Western blots of mutanolysin digests prepared from strains FimQ-Km and ΔFimQ (Fig. 2). The percentages of adherence of bacteria to saliva-treated hydroxyapatite (SHA) were similar for wild-type strain T14V and control strain FimQ-Km. In contrast, the value of approximately 20% adherence for strain ΔFimQ was comparable to values obtained previously for strains that lacked type 1 fimbriae (5). Thus, the deletion in *fimQ* abolished type 1 fimbria-mediated adhesion.

The genes designated *srtC1* in *A. naeslundii* T14V (Fig. 1) and *orfB* in *A. viscosus* 19246 (13) encode putative class C

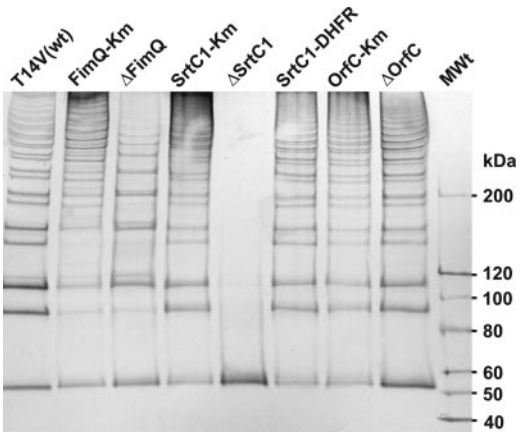

FIG. 2. Western blot analyses of cell surface proteins extracted from *A. naeslundii* wild-type and mutant strains. Cell surface proteins were extracted by mutanolysin digestion of bacteria that were osmotically stabilized in the presence of 26% melizitose, separated on a NuPAGE Tris-acetate gradient gel (3 to 8%), transferred to a nitrocellulose membrane, probed with MAb 8A against an epitope of FimP, and developed with a chromogenic Western blot immunodetection kit (Invitrogen). Lane MWt, 10 μl of MagicMark XP Western protein standard (Invitrogen).

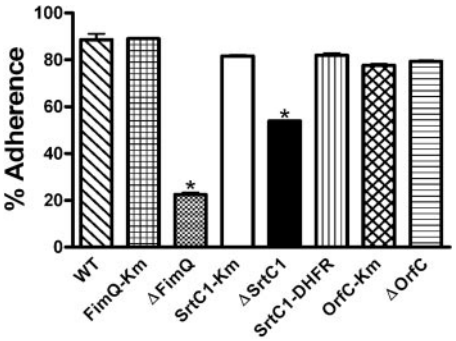

FIG. 3. Adherence of *A. naeslundii* strains to beads (5 mg) of SHA determined by using the adsorption assay described by Clark et al. (6). A *t* test was used for comparison of adhesion levels between strains ΔFimQ and ΔSrtC1. The single asterisks indicate a statistically significant difference in adherence between strains ΔFimQ and ΔSrtC1 ($P < 0.0001$).

sortases (9) that are 76% identical. To assess the involvement of *srtC1* in type 1 fimbria production, we constructed the *kan*-containing control strain SrtC1-Km and the corresponding SrtC1 deletion mutant, strain ΔSrtC1 (Fig. 1), by an allelic-exchange strategy similar to that described above and outlined in Table 2. This involved the creation of a unique XbaI site 14 codons upstream from the 3′ end of *srtC1* in p2Srt. This engineered site was used for the insertion of antibiotic markers alone or linked to appropriate *srtC1* constructs. These insertions created a premature stop codon in *srtC1* and fused the last 14 codons of this gene with the 3′ end of *kan*, thus preserving the putative ribosome binding site of downstream *orfC*.

In contrast to control strain SrtC1-Km, which was similar to the wild type, strain ΔSrtC1 specifically lacked type 1 fimbriae. This difference was evident from results of dot immunoblotting and agglutination assays performed with anti-FimP and anti-FimA reactive MAbs (Fig. 1). Although the presence of FimP on strain ΔSrtC1 was not detected by surface labeling, monomeric FimP was detected in the mutanolysin digest of this strain. In Western blots, this protein migrated as a sharp band between the 50- and 60-kDa markers (Fig. 2). Interestingly, the deletion of *srtC1* reduced but did not abolish the binding of anti-FimQ antibody in dot immunoblots. Equivalent binding of this antibody to strains SrtC1-Km and ΔSrtC1 required approximately 10-fold more cells of the latter strain (Fig. 1). In addition, the agglutination titer of this antibody was higher in comparable assays performed with strain ΔSrtC1 than in those performed with strain SrtC1-Km. Moreover, the adhesion of strain ΔSrtC1 to SHA, although lower than that of control strain SrtC1-Km, was significantly greater than that observed with strain ΔFimQ (Fig. 3). In genetic complementation studies, the cell surface phenotype of strain ΔSrtC1 was restored to that of wild-type strain T14V by the expression of *srtC1* linked to a selectable *dhfr* marker in strain SrtC1-DHFR (Fig. 1 to 3). The construction of strain SrtC1-DHFR is outlined in Table 2.

The protein encoded by *orfC* in the amended sequence of *A. naeslundii* T14V (Fig. 1) is 90% identical to the putative prepilin peptidase-like protein encoded by this gene in *A. viscosus* 19246 (13). To test the essential role of *orfC* in type 1 fimbria production, we constructed control strain OrfC-Km containing *kan* immediately downstream of *orfC* and strain ΔOrfC containing *kan* in place of this gene (Fig. 1). The strategy used to construct these strains (Table 2) was similar to that described above. The surface phenotypes of strains OrfC-Km and ΔOrfC were the same and indistinguishable from that of wild-type *A. naeslundii* T14V (Fig. 1 to 3). Thus, the production of functional type 1 fimbriae does not require *orfC*.

The present and previous findings (19) identify three essential genes for type 1 fimbria production by *A. naeslundii* T14V: *fimQ* for a minor type 1 fimbria-associated protein, *fimP* for the major subunit, and *srtC1* for a type 1 fimbria-specific sortase. In accordance with the model of pilus assembly in *Corynebacterium diphtheriae* (15, 18), we suspect that the SrtC1-dependent type 1 fimbria production in *A. naeslundii* T14V involves cleavage of FimP cell wall sorting motifs by SrtC1 and linkage of nascent C termini to ε-amino groups of lysine in the pilin motifs of adjacent FimP monomers. In previous studies (2), type 1 fimbria-mediated adhesion of strain T14V to SHA was not blocked by Fab fragments of various FimP-reactive monoclonal and polyclonal antibodies, thereby raising the possibility that the putative PRP-binding adhesin that mediates this interaction is a minor fimbrial component. Clearly, the present findings identify FimQ as an attractive candidate for the long-sought type 1 fimbrial adhesin. In this regard, it is interesting that cells of strain ΔSrtC1, which possess detectable cell surface FimQ but not FimP, were more adherent to SHA than cells of strain ΔFimQ, which possess detectable cell surface FimP but are devoid of FimQ (Fig. 1 and 3). While these findings are consistent with the notion that FimQ mediates adhesion, it is important to note that anti-FimP antibody did not agglutinate strain ΔFimQ, thereby indicating that the polymerized FimP detected from this strain (Fig. 2) does not exist in a form comparable to that of fully assembled fimbriae. Further studies are needed to assess the role of FimQ both in the assembly of type 1 fimbriae and in the adhesion of *A. naeslundii* to adsorbed salivary PRPs.

Nucleotide sequence accession number. The amended sequence of the gene cluster for *A. naeslundii* T14V type 1 fimbria production and flanking regions has been deposited in GenBank under accession number DQ658412.

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