Sortase-Catalyzed Assembly of Distinct Heteromeric Fimbriae in *Actinomyces naeslundii*[▽]

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Received 22 December 2006/Accepted 25 January 2007

Two types of adhesive fimbriae are expressed by *Actinomyces*; however, the architecture and the mechanism of assembly of these structures remain poorly understood. In this study we characterized two fimbrial gene clusters present in the genome of *Actinomyces naeslundii* strain MG-1. By using immunoelectron microscopy and biochemical analysis, we showed that the *fimQ-fimP-srtC1-fimR* gene cluster encodes a fimbrial structure (designated type 1) that contains a major subunit, FimP, forming the shaft and a minor subunit, FimQ, located primarily at the tip. Similarly, the *fimB-fimA-srtC2* gene cluster encodes a distinct fimbrial structure (designated type 2) composed of a shaft protein, FimA, and a tip protein, FimB. By using allelic exchange, we constructed an in-frame deletion mutant that lacks the SrtC2 sortase. This mutant produces abundant type 1 fimbriae and expresses the monomeric FimA and FimB proteins, but it does not assemble type 2 fimbriae. Thus, SrtC2 is a fimbria-specific sortase that is essential for assembly of the type 2 fimbriae. Together, our experiments pave the way for several lines of molecular investigation that are necessary to elucidate the fimbrial assembly pathways in *Actinomyces* and their function in the pathogenesis of different biofilm-related oral diseases.

Over 30 years ago, Girard and Jacius described “fibril-like” appendages on the surface of *Actinomyces naeslundii* (17). In subsequent years, fimbriae or surface fibrils were found on several strains of this prominent gram-positive oral species (3, 14). Genospecies 2 strains of *A. naeslundii* (19), such as strain T14V, are believed to assemble two distinct types of fimbriae (41). The so-called type 1 fimbriae mediate adhesion of actinomyces to salivary proline-rich proteins that coat the tooth enamel (16), whereas type 2 fimbriae are responsible for the binding of these bacteria to oral streptococci and various host cells, including erythrocytes, epithelial cells, and polymorphonuclear leukocytes (22, 41). The abilities of actinomyces to adhere to the tooth surface, to form mixed-species biofilms with other potentially infectious bacterial partners, and to specifically interact with host cell receptors may be important steps in the pathogenesis of certain oral diseases, including root surface caries (26) and gingivitis (23). Consequently, a detailed molecular understanding of *A. naeslundii* fimbriae biogenesis and function would improve our understanding of these and other biofilm-related oral diseases and might suggest novel strategies for the prevention and control of such infections.

Our current knowledge of *A. naeslundii* fimbriae is based largely on the biochemical and reverse-genetic identification of specific fimbrial antigens and the corresponding genes by Yeung, Cisar, and coworkers (41). By screening cosmid gene libraries of the *A. naeslundii* T14V genome in *Escherichia coli*

and monitoring the expression of specific fimbrial antigens, these authors identified FimP and FimA as the major structural subunits of *A. naeslundii* type 1 and type 2 fimbriae, respectively (10, 42). Moreover, by selecting for bacteria that failed to react with antibodies against either or both fimbrial antigens, these investigators isolated a set of spontaneous mutants that lack type 1 fimbriae, type 2 fimbriae, or both types of fimbriae (4, 13). Further cloning and sequence characterization of the chromosomal regions that encode FimA and FimP led to identification of the corresponding gene clusters for fimbria production. The type 1 gene cluster of strain T14V was predicted to contain seven open reading frames with the gene order *orf3-orf2-orf1-fimP-orf4-orf5-orf6* (45). Two of these genes, *orf1* and *fimP*, encode proteins whose primary sequences exhibit striking similarity to those of the well-characterized cell wall-anchored surface proteins of *Staphylococcus aureus* and other gram-positive bacteria (36). Insertional mutations were introduced into each of these predicted genes to identify the genes that are essential for type 1 fimbria production. While mutations in *orf3* and *orf4* did not eliminate synthesis of the 65-kDa fimbrial antigen, the antigen was affected by insertions in *orf1*, *orf2*, and *fimP*. Each of these insertions, however, eliminated adhesion to proline-rich proteins (45).

In contrast to the apparent complexity of the type 1 gene cluster in *A. naeslundii* T14V, the type 2 gene cluster of this strain, located elsewhere on the chromosome, is predicted to encode three proteins and to have the gene order *orf977-fimA-orf365* (18, 43). An insertion mutation in *orf365*, predicted to encode a sortase, a transpeptidase required for cell wall anchoring of gram-positive surface proteins (36), eliminated the formation of fimbriae but not the synthesis of the 59-kDa fimbrial antigen (43). By contrast, an insertion mutation in *fimA* eliminated the synthesis of this antigen. The role of

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[▽] Published ahead of print on 2 February 2007.

TABLE 1. Bacterial strains and plasmids used

Bacterial strain or plasmid	Relevant characteristics	Reference
Strains		
<i>A. naeslundii</i> MG-1	Wild-type strain, expresses type 1 and 2 fimbriae	9
<i>A. naeslundii</i> AR1	Δ srtC2, derivative of MG-1	This study
<i>A. naeslundii</i> AR2	Δ srtC2 containing plasmid pSrtC2	This study
<i>A. naeslundii</i> T14V	Wild-type strain, expresses type 1 and 2 fimbriae	4
<i>A. naeslundii</i> 5519	Expresses only type 1 fimbriae	4
<i>A. naeslundii</i> 5951	Expresses only type 2 fimbriae	4
Plasmids		
pHTT177	Derivative of pUC19, Kan ^r	This study
pSrtC2	pJRD215 containing wild-type SrtC2 from MG-1	This study
pJRD215	<i>A. naeslundii</i> replicating vector, Mob ⁺ Kan ^r	8

orf777, which exhibits homology to the gene encoding a known streptococcal adhesin, has not been addressed (18).

By analyzing a different strain of actinomyces (*Actinomyces viscosus* ATCC 19246), isolated from a human actinomycotic lesion, Stromberg and coworkers identified a fimbrial gene cluster that exhibits 81% nucleotide sequence identity to the type 1 fimbrial operon of strain T14V. This cluster consists of four genes, designated *orfA*, *fimP*, *orfB*, and *orfC* (20). Curiously, the nucleotide sequence of *A. viscosus orfA* is ~80% identical to that of T14V *orf3*, *orf2*, and *orf1* combined. Sequencing errors have recently been found to account for the three different open reading frames in T14V corresponding to *orfA* (Kai Leung, personal communication). OrfA has an N-terminal signal sequence and a C-terminal sorting signal with the conserved LPXTG motif (36). Similar to OrfA, the FimP protein of strain ATCC 19246 exhibits ~85% amino acid identity to the FimP homolog of strain T14V (20); it also has a signal peptide sequence and the C-terminal sorting signal. Significantly, both FimP and FimA contain a pilin motif and an E box, which are common features of gram-positive bacterial major pilin subunits (see Fig. 2) (38). The pilin motif is required for the polymerization of the pilus structures, whereas the E box is necessary for the incorporation of a minor pilin into a major pilus shaft (37). The presence of these conserved motifs suggests that the assembly of *A. naeslundii* fimbriae may occur through the sortase-mediated pilus assembly pathway proposed for many gram-positive organisms (21, 34, 38). Consistent with this notion, *orfB* of strain ATCC 19246 encodes a putative sortase (20). Interestingly, *orfC*, the last gene in the type 1 cluster of *A. viscosus*, is predicted to encode a prepilin peptidase-like protein, and this gene exhibits ~88% nucleotide sequence identity to *orf6* of T14V (20), which does not appear to be required for type 1 fimbria production (45).

In this study, which was performed to obtain a better picture of the architecture and assembly of *Actinomyces* fimbriae, we took advantage of a recently sequenced strain, *A. naeslundii* MG-1. Originally isolated from a gingivitis patient (9), this strain appears to be more amenable to genetic manipulation than strain T14V (44). We identified and characterized the two fimbrial gene clusters present in the MG-1 genome. By generating specific antisera against the predicted fimbrial subunits, we found that this strain does have two distinct types of fim-

TABLE 2. Primers used in this study

Primer	Sequence ^a
Ab-FimA-5	AACATATGGAACGCCTAACTACGGCAA
Ab-FimA-3	AAAGATCTCTACTGCTTGGTGTTCCTCATGG
Ab-FimB-5	AAAAGATCTAACCACAACGGCGTCTTTGA
Ab-FimB-3	AAAAGATCTCCGCTTCTCCACCAGGGA
Ab-SrtC2-5	AACATATGAAGGTGACTCCGACTACT
Ab-SrtC2-3	AAGGATCCCTAGTCCTTGGCCGGGGTTCG
Ab-FimP-5	AACATATGCACTCCCTCAACACGGCGCC
Ab-FimP-3	AAGGATCCTTAGCGGAAGCCGGCGTTCTTC
Ab-FimQ-5	AAGGATCCAATAACTACTGGACTGACTATG
Ab-FimQ-3	AAGGATCCTTAGAAGGTTCTTCCGGTGAG
SrtC2-A	CGCGGATCCCTGCTGATGATCGCCGT
SrtC2-B	CCCATCCACTAACTTAAACAGGGTATGCGT
	GCTTTGCG
SrtC2-C	TGTTTAAGTTTAGTGGATGGGCGGACTCAC
	AGGCTCTAG
SrtC2-D	CGCGGATCCCGCGAGGAGTCAATGAC
pSrtC2-5'	CGCGGATCCCTACGTCCTGGTTGAGACC
pSrtC2-3'	CGCGGATCTTACGGTATCAGGGGCTCCC
Kan-215-5	CGCAACGTTCCAGAGTCCCGCTCAGAA
Kan-215-3	CGCAACGTTCCAAGCTAGCTTCACGCTGC

^a The restriction sites are underlined.

briae and that each type of fimbriae contains not only a major subunit that forms the fimbrial shaft but also a minor fimbrial protein that is located in the tip region. By creating an in-frame gene disruption, we determined that the formation of type 2 fimbriae, but not the formation of type 1 fimbriae, requires a specific sortase (SrtC2) whose gene is located in the type 2 gene cluster.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are listed in Table 1. *A. naeslundii* MG-1 (formerly *A. viscosus* MG-1 [9]), a clinical isolate obtained from a patient with gingivitis, was used as the wild-type parental strain. Plasmid pJRD215 (8) was kindly provided by Kai P. Leung. *E. coli* strain DH5 α was used for standard DNA manipulations. *Actinomyces* strains were grown in heart infusion broth, in Todd-Hewitt broth, or on heart infusion agar (HIA). *E. coli* was grown in Luria-Bertani broth. Kanamycin was added at a concentration of 50 μ g/ml as needed. Reagents were purchased from Sigma unless indicated otherwise.

Generation of rabbit-raised polyclonal antibodies. Appropriate oligonucleotide primers (Ab-primers) (Table 2) and *A. naeslundii* MG-1 chromosomal DNA were used for PCR amplification of coding sequences for FimA, FimP, SrtC2, FimQ, and FimP, omitting signal peptide and sorting signal sequences except for SrtC2. The resulting DNA fragments were cloned between appropriate sites of the expression vector pQE30 (QIAGEN) to produce recombinant proteins with an N-terminal six-histidine tag. The recombinant plasmids were transformed into *E. coli* XL1-Blue. DNA sequencing was performed to verify the recombinant plasmids. The recombinant proteins were purified by affinity chromatography using nickel-nitrilotriacetic acid columns and were injected into rabbits to raise different antibodies, as described previously (15).

Plasmid construction. (i) **Plasmid pHTT177.** Primers Kan-215-5 and Kan-215-3 (Table 2), each of which contained an AclI site (Table 2) for cloning purposes, were used along with pJRD215 template DNA to amplify a DNA segment encompassing the presumed promoter, the 5' untranslated region, and the kanamycin coding sequence in a PCR. The PCR-amplified DNA fragment was cut with AclI and ligated with the cleaved AclI sites of vector pUC19 to generate pHTT177.

(ii) **Plasmid pSrtC2.** To construct pSrtC2, primers pSrtC2-5' and pSrtC2-3' (Table 2), each of which contained a BamHI site (Table 2) for cloning purposes, were used along with *A. naeslundii* strain MG-1 chromosomal DNA as the template to amplify a DNA segment encompassing the *srtC2* promoter, the 5' untranslated region, and the *srtC2* coding sequence in a PCR. The PCR-amplified DNA fragment was cut with BamHI and ligated with the cleaved BamHI sites of vector pJRD215.

Generation of an in-frame *A. naeslundii* Δ srtC2 deletion mutant. An *A. naeslundii* Δ srtC2 deletion mutant was generated by homologous recombination and verified by PCR and Western and Southern blotting techniques. The gene

deletion cassette was first constructed by crossover PCR (15, 39) and cloned between appropriate restriction sites of pHTT177, and recombinant plasmids were transformed into *E. coli* DH5 α . For crossover PCR, two sets of primers were generated (Table 2). Primers SrtC2-A and SrtC2-B and primers SrtC2-C and SrtC2-D were used to amplify two 300-bp fragments, which were then used as templates for another PCR amplification with primers SrtC2-A and SrtC2-D. As the tail ends of primers SrtC2-B and SrtC2-C annealed to one another, a 600-bp fused PCR product was obtained. After overnight digestion with BamHI, the restricted DNA was purified and cloned into the BamHI site of pHTT177. This *srtC2* deletion construct was then introduced into *A. naeslundii* MG-1 by electroporation (44). The insertion obtained by homologous recombination of the plasmid into the MG-1 chromosome was selected by growth at 37°C in the presence of kanamycin. Kanamycin-resistant colonies representing plasmid integrants were serially passaged twice on solid medium at 37°C. Integrant strains were then serially passaged nine times in heart infusion broth at 37°C in the absence of kanamycin; excision of the plasmid from the chromosome via a second recombination event either completed the allelic exchange or reconstituted the wild-type genotype. Kanamycin-sensitive colonies were identified by replica plating and were screened for the expected deletion mutation by PCR amplification using primers SrtC2-A and SrtC2-D. Candidate deletion mutants were characterized by Western blotting with specific antibodies (anti-SrtC2 and anti-FimA), as well as by Southern hybridization analysis.

Extraction of *A. naeslundii* fimbriae. *A. naeslundii* fimbriae were extracted as previously described (33). Typically, *A. naeslundii* strains were scraped from HIA plates and washed in SMM buffer (0.5 M sucrose, 10 mM MgCl₂, 10 mM maleate; pH 6.8). Next, cell pellets were suspended in the same buffer and treated with lysozyme (1 mg/ml) at 37°C for 6 h or left untreated (mock). An equal amount of bacterial sediment was suspended in 70% formic acid and incubated at 65°C for 30 min. Solubilized fimbriae were isolated from the supernatant after centrifugation at 16,000 $\times$ g, followed by trichloroacetic acid precipitation and acetone washing, and protein samples were dried under a vacuum. Preparations of fimbriae were boiled in sodium dodecyl sulfate (SDS) sample buffer, separated by SDS-polyacrylamide gel electrophoresis (PAGE), subjected to immunoblotting with rabbit antisera (anti-FimA at a 1:10,000 dilution; anti-FimP at a dilution of 1:10,000; anti-FimB at a dilution of 1:5,000; anti-FimQ at a dilution of 1:5,000; and anti-SrtC2 at a dilution of 1:5,000) followed by anti-rabbit horseradish peroxidase-linked immunoglobulin (IgG) antibody, and detected by chemiluminescence.

Electron microscopy and immunogold labeling. Electron microscopy experiments were carried out as previously described (33). Bacterial strains were grown on HIA plates, washed in 0.1 M NaCl, and stained with 1% uranyl acetate. For immunogold labeling, single drops of a bacterial suspension were placed onto carbon grids, washed three times with phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA), and blocked for 1 h in PBS with 0.1% gelatin. Fimbriae were reacted with a primary antibody diluted 1:100 in PBS with 2% BSA for 1 h, followed by washing and blocking. Fimbriae were stained with gold-labeled goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:20 in PBS with 2% BSA for 1 h, and this was followed by washing in PBS with 2% BSA. For double-labeling experiments, the same procedure was performed with another primary antibody and goat anti-rabbit IgG conjugated with gold particles that were of different sizes. The grids were washed five times with water before they were stained with 1% uranyl acetate. Samples were analyzed using a Jeol 100CX electron microscope.

Sequence analysis. BLAST searches were used to obtain homologous sequences of fimbria-associated proteins (1). The protein sequences for major fimbrial subunits and the sortases of *A. naeslundii* MG-1, *Corynebacterium diphtheriae* NCTC13129, *Corynebacterium efficiens* YS-314, *Corynebacterium jeikeium* K411, *Streptococcus agalactiae* 2603V/R, and *Streptococcus pyogenes* MGAS10270 were aligned with ClustalX (35). An alignment was produced for each protein family, and phylogenetic trees were reconstructed with the neighbor-joining algorithm (30) using the program PAUP 4.0 10 β .

RESULTS

Fimbrial gene clusters of *A. naeslundii* MG-1. To examine *A. naeslundii* MG-1 for the presence of fimbriae, cells were negatively stained with uranyl acetate and viewed by transmission electron microscopy. Typical images consistently showed that many long (length, 1 to 2 μ m) and thin filamentous structures were distributed over the entire bacterial surface (Fig. 1A).

FIG. 1. Fimbriae and fimbrial gene clusters of *A. naeslundii*. (A) *A. naeslundii* strain MG-1 cell negatively stained with uranyl acetate and viewed by transmission electron microscopy. Bar = 0.2 μ m. (B) Diagrams of two gene clusters identified in the chromosome of *A. naeslundii* MG-1, each of which contains one putative sortase gene (*srtC1* or *srtC2*) and two fimbria-associated genes (*fimP* and *fimQ* or *fimA* and *fimB*). A gene for a putative housekeeping sortase (*srtA*) is located elsewhere in the MG-1 chromosome. Similarity between *fim* gene products is indicated by different colors (black, white, and gray).

To determine whether sorting signals of precursor proteins and sortase enzymes play a role in the assembly of *Actinomyces* fimbriae, we searched the unfinished genome sequence of *A. naeslundii* MG-1 (The Institute of Genomic Research [TIGR]; <http://www.tigr.org/>) using *A. naeslundii* *orf365* and LPXTG sorting signals as queries in BLAST searches (1). We found two chromosomal gene clusters that contained a total of four predicted surface protein genes, each encoding an N-terminal signal peptide and a C-terminal sorting signal (*fimA*, *fimB*, *fimP*, and *fimQ*), as well as two putative sortase genes (*srtC1* and *srtC2*) (Fig. 1B). We found a third sortase gene, potentially encoding a housekeeping sortase (*srtA*), elsewhere on the chromosome. We used the designation Fim for *Actinomyces* fimbria-associated surface proteins based on the original description (41), and we designated the sortases SrtC1 and SrtC2 based on their possible involvement in the assembly of type 1 and type 2 fimbriae, respectively, following the convention of using SrtC for fimbria-specific sortases (12).

FimA and FimP exhibit significant homology (36 and 32% identity, respectively) to the major pilin subunits SpaH and SpaD of *C. diphtheriae* (15, 33) (Fig. 2A and B). This suggests that FimA and FimP may be the major fimbrial proteins. In fact, sequence alignment of several major fimbrial proteins revealed that FimA and FimP also contain the pilin motif, the E box, and the sorting signal with the LPXTG motif in addition

FIG. 2. Analysis of fimbrial shaft proteins and the corresponding sortases. (A) ClustalX (35) was used to align the protein sequences for major fimbrial subunits and the predicted sortases of *S. agalactiae* 2603V/R, *S. pyogenes* MGAS10270, *C. efficiens* YS-314, *C. diphtheriae* NCTC13129, *A. naeslundii* MG-1, and *C. jeikeium* K411. (B and C) Phylogenetic trees of the major fimbrial proteins (B) and sortases (C) reconstructed with the neighbor-joining algorithm (30), using the program PAUP 4.0 10β. Locus tags are color coded to indicate substrate-sortase specificity.

to several conserved motifs that have yet to be characterized (Fig. 2A). Similarly, the fact that FimB is homologous (36% identity) to the SpaG tip protein of *C. diphtheriae* suggests that FimB may be a minor fimbrial component. The SpaHIG pilus is assembled by two sortases (SrtD and SrtE) encoded by genes in the same pilus gene cluster that contains the genes encoding SpaH, SpaG, and SpaI (33), whereas formation of the SpaDEF pilus requires sortases SrtB and SrtC encoded by genes in the pilus gene cluster producing SpaD, SpaE, and SpaF (15). Consistent with the currently established specificity of sortases for pilins encoded by genes in the cognate pilus gene cluster, SrtC1 and SrtC2 are predicted to be homologous to SrtB and SrtD, respectively (Fig. 2C).

Surface localization of predicted fimbrial proteins in *A. naeslundii* strain MG-1. To determine whether the predicted fimbrial gene clusters are functional and to study fimbrial structures, we raised specific antisera against the predicted *A. naeslundii* fimbrial proteins by immunizing rabbits with recombinant proteins isolated from *E. coli* (see Materials and Methods). Using the antibody raised against purified FimP (anti-FimP), as well as gold-labeled IgG, we examined *A. naeslundii* strain MG-1 using electron microscopy and observed immuno-gold labeling along bacterial fimbrial fibers (Fig. 3A). There was no labeling of these structures on bacteria treated with control rabbit sera (data not shown). The data demonstrated that FimP is located all along the fimbrial shaft and hence is a major subunit of the fimbriae, which is consistent with its homology to other well-characterized major pilins, such as SpaD of corynebacteria.

By contrast, when we stained MG-1 with antibody against

FimQ (anti-FimQ) and gold-labeled IgG, we observed gold particles both on the bacterial cell surface and at the distal ends of fimbriae, but not along the fimbrial shaft (Fig. 3B and C), as observed for labeling with anti-FimP (Fig. 3A). Importantly, FimP/FimQ double staining not only confirmed these results but also demonstrated that FimP and FimQ are present in the same fimbrial structure (Fig. 3D and E). We concluded that FimP is a component of the fimbrial rod, whereas FimQ is located at or near the fimbrial tip, as well as on the bacterial surface.

To determine whether FimA and FimB form a comparable structure, we stained wild-type strain MG-1 with specific antibody raised against purified FimA (anti-FimA) or FimB (anti-FimB), as well as gold particles conjugated with IgG. We observed FimA staining all along the fimbrial shaft (Fig. 4A). In contrast, FimB staining was observed on the cell surface and at a distance, but never along the fimbrial shaft (Fig. 4B and C), as observed for labeling with anti-FimA (Fig. 4A). FimA/FimB double staining showed that FimA and FimB were present in the same fimbrial structure (Fig. 4D and E). Together, the data demonstrated that FimA is the major fimbrial subunit and that FimB is located peripherally in the fimbrial tip region.

FimP and FimQ are components of type 1 fimbriae and FimA and FimB constitute type 2 fimbriae. The results described above did not show that FimP and FimQ form a fimbrial structure that is distinct from the structure consisting of FimA and FimB. To address this question, we analyzed spontaneous mutants of *A. naeslundii* T14V that express either type 1 or type 2 fimbriae (4) by electron microscopy. Strain 5519,

FIG. 3. Fimbrial structures consisting of FimP and FimQ. *A. naeslundii* strain MG-1 was immobilized on carbon grids and stained with a specific antiserum. Single-label experiments were performed by staining cells with anti-FimP (A), anti-FimQ (B), and IgG-conjugated 12-nm gold particles. For double labeling (D and E), cells were first reacted with anti-FimQ, followed by IgG-conjugated 18-nm gold particles (open arrowheads), and then were reacted with anti-FimP, followed by IgG-conjugated 12-nm gold particles (solid arrowheads). The area in the box in panel B is enlarged in panel C. Fimbriae were viewed by transmission electron microscopy. Bars = 0.2 μ m.

which is known to express only type 1 fimbriae ($1^+ 2^-$) (4), had FimP/FimQ-labeled fimbriae, like strain MG-1 (compare Fig. 5A and B and Fig. 3A to C). Double labeling confirmed that FimP and FimQ are in the same fimbrial structure (Fig. 5C and

D). Importantly, immunostaining with anti-FimA or anti-FimB did not result in detection of any labeled structures (Fig. 5E and F). This demonstrated that the fimbrial structures formed by FimP and FimQ are specific and that their assembly is

FIG. 4. Fimbrial structures containing FimA and FimB. *A. naeslundii* strain MG-1 was analyzed as described in the legend to Fig. 3, except that the cells were stained with anti-FimA (A), anti-FimB (B), and IgG-conjugated 12-nm gold particles. For double labeling (D and E), cells were stained with anti-FimB, followed by IgG-conjugated 18-nm gold particles (open arrowheads), and then were stained with anti-FimA, followed by IgG-conjugated 12-nm gold particles (solid arrowheads). The area in the box in panel B is enlarged in panel C. Bars = 0.2 μ m.

FIG. 5. Components of type 1 fimbriae on the surface of *A. naeslundii* strain 5519 ($1^+ 2^-$). Bacteria were stained with anti-FimP (A), anti-FimQ (B), anti-FimA (E), or anti-FimB (F) and IgG-conjugated 12-nm gold particles. For double labeling (C), cells were stained exactly as described in the legend to Fig. 3 (open arrowheads, FimQ; solid arrowheads, FimP). The area in the box in panel C is enlarged in panel D. Bars = 0.2 μ m.

independent of the type 2 fimbriae consisting of FimA and FimB.

Conversely, strain 5951, which expresses only type 2 fimbriae ($1^- 2^+$), produced labeled fimbriae when anti-FimA and anti-FimB were used. Consistent with the results described above (Fig. 4), the FimA label was distributed along the fimbrial rod and the FimB label was found at the distal ends of the fimbriae, as well as on the bacterial surface (Fig. 6A and B). Double staining confirmed that FimA and FimB are in the same fimbrial structure in this strain (Fig. 6C and D). In contrast, immunostaining with anti-FimQ or anti-FimP did not result in detection of any labeled structures (Fig. 6E and F). Together, our data show that FimA and FimB constitute the type 2 fimbrial structures, which are distinct from the type 1 fimbriae, which consist of FimP and FimQ. Additional electron microscopy and biochemical analysis established that the two distinct types of fimbriae consisting of FimA and FimB and of FimP

FIG. 6. Components of type 1 fimbriae on the surface of *A. naeslundii* strain 5951 ($1^- 2^+$). Bacteria were stained with anti-FimA (A), anti-FimB (B), anti-FimP (E), or anti-FimQ (F) and IgG-conjugated 12-nm gold particles. For double labeling (C), cells were stained exactly as described in the legend to Fig. 4 (open arrowheads, FimB; solid arrowheads, FimA). The area in the box in panel C is enlarged in panel D. Bars = 0.2 μ m.

and FimQ are also produced by the T14V strain of *A. naeslundii* (data not shown).

Sortase SrtC2 is required for assembly of type 2 fimbriae.

The presence of a sortase gene in each fimbrial gene cluster suggests that a specific sortase might be required for the formation of the cognate type of fimbriae (Fig. 1B). To examine this, we generated an in-frame deletion of the type 2 fimbrial sortase *srtC2* gene in parental strain MG-1.

To generate the desired deletion of the *srtC2* gene, we used a kanamycin-resistant derivative of plasmid pUC19 (40), which cannot replicate in *A. naeslundii*. Briefly, a gene deletion cassette was constructed by crossover PCR (39) and cloned into pUC19-kan (see Materials and Methods). The deletion construct was then introduced into *A. naeslundii* MG-1 by electroporation, and kanamycin-resistant colonies resulting from chromosomal integration of the plasmid were selected. Kanamycin-resistant colonies were then serially passaged in the absence of kanamycin to allow growth of rare progeny in which

FIG. 7. Assembly of type 2 fimbriae requires the SrtC2 sortase. Isogenic derivatives of *A. naeslundii* MG-1 with the *srtC2* gene deleted ($\Delta srtC2$) (A to D) or this strain expressing *srtC2* from plasmid pSrtC2 (E and F) were stained with a specific antiserum against FimA (A and E), FimB (B and F), FimP (C), or FimQ (D) and IgG-conjugated 12-nm gold particles. Bars = 0.2 μ m.

the plasmid had been excised from the chromosome via a second recombination event. Kanamycin-sensitive colonies were then identified by replica plating and were subsequently screened for the expected deletion mutation by PCR analysis and Western blotting. While we obtained the desired $\Delta srtC2$ mutant by this procedure, so far we have not generated mutants with deletions in other fimbrial genes.

Next, using the $\Delta srtC2$ mutant, we investigated the function of the SrtC2 sortase in fimbrial assembly by immunoelectron microscopy. Although the mutant produced many fibrils, none of the fibrils was labeled with anti-FimA or anti-FimB (Fig. 7A and B). The fact that these fibrils were labeled with both anti-FimP and anti-FimQ demonstrated that they represented the type 1 fimbriae (Fig. 7C and D). Expression of *srtC2* from a plasmid in the $\Delta srtC2$ mutant restored production of the type 2 fimbriae that were labeled with anti-FimA and anti-FimB (Fig. 7E and F).

To extend this observation, we performed a Western blot analysis with cell extracts prepared from wild-type strain MG-1

and mutant derivatives of this strain. Blotting with anti-FimA revealed FimA-containing material in lysozyme or formic acid extracts of wild-type bacteria but not in control cells boiled in SDS sample buffer (Fig. 8A). Heterogeneous high-molecular-weight bands of FimA (molecular mass, >200 kDa) were detected in the SDS-PAGE stack, while monomeric FimA (calculated molecular mass, 56 kDa) migrated at $\sim$ 64 kDa. Importantly, deletion of *srtC2* in strain AR1 eliminated synthesis of high-molecular-weight FimA but not synthesis of monomeric FimA (Fig. 8A). The SrtC2-encoding plasmid restored synthesis of high-molecular-weight FimA products in the $\Delta srtC2$ mutant (strain AR2) (Fig. 8A). Thus, the SrtC2 sortase is essential for the production of FimA polymers and for their covalent attachment to the cell wall.

Similarly, Western blotting with anti-FimB revealed both high-molecular-weight and monomeric FimB in lysozyme or formic acid extracts of wild-type bacteria but only monomeric FimB in extracts of the $\Delta srtC2$ mutant (Fig. 8B). Again, expression of *srtC2* from a plasmid restored the production of high-molecular-weight immunoreactive material in the deletion mutant (Fig. 8B). The monomeric FimB migrated as an 86-kDa protein, which was slightly less than the predicted molecular mass ($\sim$ 100 kDa), while polymeric FimB migrated in the same region as polymeric FimA (molecular mass, >200 kDa). By contrast, a blotting experiment with anti-FimP and anti-FimQ revealed polymeric forms of the type 1 fimbrial proteins in extracts of the $\Delta srtC2$ mutant with a mobility pattern that is similar to that of the wild type (Fig. 8C and D). Thus, neither the polymerization nor the cell wall linkage of type 1 fimbriae depends on SrtC2. Together, these results establish the specific role of SrtC2 in the assembly of type 2 fimbriae from FimA and FimB monomers.

DISCUSSION

Successful infection by bacterial pathogens requires that the infecting bacteria attach to and colonize specific host tissues. This step generally depends on specific interactions of fimbriae or pili displayed on bacterial cell surfaces with distinct host cell receptors, which govern the bacterial host range and sites of infection (27, 34). Previous work with *A. naeslundii* established that this basic principle applies to the colonization of the tooth surface by this bacterium, which expresses two distinct types of adhesive fimbriae (41). The type 1 fimbriae mediate bacterial binding to specific peptides in the salivary proline-rich proteins that coat the tooth enamel. The type 2 fimbriae, on the other hand, permit binding of *Actinomyces* to the host-like saccharide motifs present on the surface of streptococci, which are prominent members of the dental plaque biofilm community. The type 2 fimbriae also mediate the binding of *Actinomyces* to a wide range of host cells, including neutrophils (31), triggering inflammatory responses and progressive damage in the surrounding tissues (29). However, the precise mechanism by which these fimbriae are assembled and their molecular architecture are poorly understood.

To obtain further insight into the molecular and biological properties of *A. naeslundii* fimbriae, we characterized these structures using strain MG-1, whose genomic sequence is being assembled at TIGR. Electron microscopy revealed that the type 1 fimbrial structures contain FimP along the fimbrial shaft

FIG. 8. Sortase SrtC2 is required for polymerization of type 2 fimbriae but not for polymerization of type 1 fimbriae. *A. naeslundii* strain MG-1 (wt), an isogenic $\Delta srtC2$ derivative of strain MG-1 (AR1), or strain AR1 expressing *srtC2* from plasmid pSrtC2 (AR2) were treated with lysozyme (L) or formic acid (F) or were not treated (–) prior to extraction with hot SDS sample buffer. Proteins were separated by SDS-PAGE and detected by immunoblotting with anti-FimA (A), anti-FimB (B), anti-FimP (C), or anti-FimQ (D). The positions of monomeric products (FimA_M, FimB_M, FimP_M, and FimQ_M) and high-molecular-weight products (FimA_{HMW}, FimB_{HMW}, FimP_{HMW}, and FimQ_{HMW}) of fimbrial assembly, the molecular weight markers, and the stacking gel portion of the SDS-PAGE gel (stack) are indicated.

and FimQ at the distal tip (Fig. 3 and 5). Similarly, the type 2 fimbriae contain FimA along the shaft and FimB in the tip region (Fig. 4 and 6). Thus, each type of fimbriae consists of a major subunit that forms the shaft and a minor subunit that may form the tip. Interestingly, in addition to their location at the fimbrial tips, the minor subunits are also present on the bacterial surface (Fig. 3 to 6). By performing a biochemical analysis of the two types of fimbriae, we demonstrated that the cognate major and minor subunits are assembled into high-molecular-weight polymers that are linked to the cell wall (Fig. 8). Importantly, by creating and characterizing an in-frame deletion mutant, we showed that the SrtC2 sortase is required only for the formation of type 2 fimbriae and not for the formation of type 1 fimbriae (Fig. 7). This is consistent with the role of pilus-specific sortases that catalyze the assembly of various pili in many other gram-positive pathogens (32, 34, 38). These findings provide the first comprehensive description of *A. naeslundii* fimbriae and thus set the stage for a wide range of future studies.

The display of surface proteins in gram-positive bacteria involves five major pathways (7). Only one of these pathways is involved in the covalent attachment of surface proteins to the cell wall, utilizing the enzyme sortase, a transpeptidase that cleaves the LPXTG motif of the substrate precursor protein (between T and G) and links the threonine residue of the

cleaved product to the amino group of the cell wall cross bridge (36). Many proteins encoded by gram-positive bacteria contain the C-terminal sorting signal with the LPXTG motif (25). The vast majority of these proteins are linked to the cell wall in the manner just described. However, what distinguishes these surface proteins from the proteins that form pili and fimbriae is that the latter proteins are covalently cross-linked to each other by specific sortases, thereby forming a thread-like polymer which is ultimately linked to the cell wall. According to the current model (21, 27, 34, 38), pilus or fimbrial precursor proteins containing the N-terminal secretion signal are synthesized in the cytoplasm and transported across the cytoplasmic membrane by the general secretion (Sec) machinery. Upon translocation to the exoplasm, the fimbrial precursor proteins are captured by a specific sortase and assembled into high-molecular-weight structures that are anchored on the bacterial cell wall.

This general model applies to the assembly of different fimbriae in *Actinomyces*. First, we found that components of each of the two types of fimbriae are cross-linked to the cell wall. Lysozyme, a cell wall hydrolase, detaches the fimbriae from bacteria, and the fimbriae that are released have a mixture of high-molecular-weight species that are various sizes (Fig. 8), a hallmark of gram-positive bacterial pili (32, 39) that was first reported for *A. naeslundii* (45). When we treated *Actinomyces*

cells with formic acid (which dissociates noncovalent polymers [6]), the fimbrial antigens were not converted to the monomeric form (Fig. 8). This is consistent with the notion that fimbrial subunits are covalently cross-linked to a polymer. Second, we found that the formation of type 2 fimbriae requires a specific sortase, which is further evidence that there is covalent cross-linking of fimbrial subunits (Fig. 7 and 8). Third, the conserved sequence features of a protein that forms the pilus shaft, the pilin motif, and the sorting signal, as well as an E box (37), are present in both FimA and FimP, which make up the fimbrial shafts (Fig. 2, 3, and 4). Finally, *Actinomyces* FimA is polymerized into high-molecular-weight structures when it was expressed in *C. diphtheriae*, and the FimA polymerization in this heterologous system was found to be dependent on SrtD, a sortase responsible for assembly of the SpaHIG pili (37). Notably, the pilin motif of FimA (Fig. 2A) was critical for polymerization in corynebacteria; deletion of this motif or replacement by the motif of corynebacterial SpaA completely eliminated FimA polymerization (37).

A potentially unique feature of *A. naeslundii* fimbriae involves the apparent absence of a *spaB*-type gene in the fimbrial gene clusters. The SpaB-type proteins typically decorate the pilus shaft in corynebacteria and possibly in other gram-positive bacteria that have been studied (11, 24, 39). Yet the major fimbrial shaft proteins FimA and FimP contain the conserved E box, a motif shown to be essential for the linkage of SpaB to the major pilus shaft protein SpaA of *C. diphtheriae* (37). What then is the role of the E box in the FimA and FimP proteins? In the absence of a SpaB-like protein, why hasn't this motif degenerated over the course of evolution? It is tempting to speculate that the E box in FimA and FimP may link a SpaB-like protein, not yet identified, that is encoded elsewhere in the MG-1 genome. In further biochemical analyses of the two types of fimbriae of *A. naeslundii* MG-1 we will examine whether there are SpaB-like proteins in this organism and, if so, try to determine their role in adhesion.

The specific adhesive properties of type 1 and type 2 fimbriae were demonstrated previously by using the susceptibility of each type of fimbriae to inhibition by a fimbria-specific antibody (41). Thus, incubation of *A. naeslundii* T14V with Fab fragments of polyclonal antibodies raised against the type 1 fimbriae blocked bacterial adherence to saliva-treated hydroxylapatite (5). The Fab fragments of polyclonal antibodies raised against type 2 fimbriae, on the other hand, blocked *A. naeslundii* coaggregation with streptococci (28). Interestingly, similar experiments were performed with Fab fragments of monoclonal antibodies that were raised against the major fimbrial subunits FimA and FimP, which failed to block the corresponding adhesion reactions (2; J. Cisar, unpublished observation). Our finding that each of the two types of fimbriae contains minor tip proteins opens up the distinct possibility that FimB and FimQ may be the long-sought fimbrial adhesins. In this context it is important to note that recent studies with corynebacteria demonstrated that both the minor protein SpaB and the tip protein SpaC of the SpaABC pilus play critical roles in the specific corynebacterial adherence to pharyngeal epithelial cells, while the major subunit protein SpaA is entirely dispensable for adherence (20a). Work is under way to determine whether FimB and FimQ of *A. naeslundii* are in fact the fimbrial adhesins. The development of a more convenient

and versatile genetic system for manipulating the MG-1 strain should be invaluable for these studies, as well as for obtaining further insight into the underlying mechanisms of adhesion, colonization, and the initiation of inflammation in this prominent gram-positive oral species.

ACKNOWLEDGMENTS

We dedicate this paper to the memory of Maria Yeung in recognition of her many pioneering molecular studies of *Actinomyces* fimbriae.

We are grateful to Olaf Schneewind (University of Chicago) for his encouragement and generous help with reagents. We thank Kai P. Leung (Walter Reed Army Institute of Research) for providing the pJRD215 plasmid and Garry Myers (TIGR) for his help with the genome sequence of *A. naeslundii* MG-1. We are also indebted to Arlene Swierczynski for her help with immunoelectron microscopy and Anjali Mandlik, Andrew Gaspar, and Anu Swaminathan for helpful discussions.

This work was supported in part by the National Institute of Allergy and Infectious Diseases (NIH grant AI061381 to H.T.-T.) and by the Intramural Research Program of the National Institute of Dental and Craniofacial Research to J.O.C.

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