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Pilin and Sortase Residues Critical for Endocarditis- and Biofilm-Associated Pilus Biogenesis in *Enterococcus faecalis*

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Enterococci commonly cause hospital-acquired infections, such as infective endocarditis and catheter-associated urinary tract infections. In animal models of these infections, a long hairlike extracellular protein fiber known as the endocarditis- and biofilm-associated (Ebp) pilus is an important virulence factor for *Enterococcus faecalis*. For Ebp and other sortase-assembled pili, the pilus-associated sortases are essential for fiber formation as they create covalent isopeptide bonds between the sortase recognition motif and the pilin-like motif of the pilus subunits. However, the molecular requirements governing the incorporation of the three pilus subunits (EbpA, EbpB, and EbpC) have not been investigated in *E. faecalis*. Here, we show that a Lys residue within the pilin-like motif of the EbpC subunit was necessary for EbpC polymerization. However, incorporation of EbpA into the pilus fiber only required its sortase recognition motif (LPXTG), while incorporation of EbpB only required its pilin-like motif. Only the sortase recognition motif would be required for incorporation of the pilus tip subunit, while incorporation of the base subunit would only require the pilin recognition motif. Thus, these data support a model with EbpA at the tip and EbpB at the base of an EbpC polymer. In addition, the housekeeping sortase, SrtA, was found to process EbpB and its predicted catalytic Cys residue was required for efficient cell wall anchoring of mature Ebp pili. Thus, we have defined molecular interactions involved in fiber polymerization, minor subunit organization, and pilus subcellular compartmentalization in the *E. faecalis* Ebp pilus system. These studies advance our understanding of unique molecular mechanisms of sortase-assembled pilus biogenesis.

The burden of human disease caused by *Enterococcus faecalis* and *Enterococcus faecium* has grown over recent decades (1). Enterococci are currently leading causes of hospital-acquired infections (HAIs), including bloodstream infections, wound infections, and catheter-associated urinary tract infections (CAUTIs) (2). Rising rates of antimicrobial resistance, an increase in invasive medical interventions, and a proliferation of indwelling medical devices have contributed to the changing epidemiology of these formerly rare enterococcal infections (1). Adhesion to and biofilm formation on damaged tissue and abiotic surfaces, such as central venous and urinary catheters, are critical components of enterococcal pathogenesis that complicate successful treatment. Additional therapeutic and prophylactic strategies are needed to combat these infections.

Relatively little is understood about the molecular mechanisms governing enterococcal pathogenesis. However, cell surface proteins have been shown to play significant roles in *E. faecalis* virulence in a variety of disease models and assays of biofilm formation (1, 3–6). Among these, the sortase-assembled endocarditis- and biofilm-associated pilus (Ebp pilus) is important for *in vitro* biofilm formation and virulence in animal models of ascending urinary tract infection (UTI) in *E. faecalis* (7) and *E. faecium* (8) and for infective endocarditis in *E. faecalis* (9). Furthermore, we recently demonstrated that the EbpA minor subunit of these pili mediates *E. faecalis* colonization of bladders and intrabladder implants in a mouse model of CAUTI (10). Additionally, sera from hospitalized patients with enterococcal infections react with pili on the surface of *E. faecalis* cells and recombinant Ebp structural proteins (9, 11), further supporting an *in vivo* role for Ebp pili in nosocomial enterococcal infection.

Sortase-assembled pili like the Ebp pili are found among diverse Gram-positive genera (12–15). The unique biochemistry of

the sortase transpeptidase enzymes results in two distinguishing features of these pili: their covalent linkage to the peptidoglycan cell wall (16) and the interpilin isopeptide bonds that connect their structural subunits (17). These pili comprise one to three structural subunits. One major pilin, EbpC in the case of *E. faecalis* Ebp pili, forms the fiber backbone. The remaining minor or ancillary structural subunits, EbpA and EbpB in Ebp pili, occur at specific locations within the pilus fiber, often the tip or base (9). Each pilus subunit includes a cell wall sorting signal (CWSS) at its C terminus made up of a sortase recognition motif (Leu-Pro-Xaa-Thr-Gly or LPXTG-like peptide sequence), a hydrophobic transmembrane domain, and a positively charged C-terminal tail (12). Pilus-associated sortase enzymes are necessary for polymerization of the major pilin and for incorporation of minor pilins into the fiber. Although some pilus systems utilize multiple pilus-associated sortases with redundant and unique functions, only SrtC is

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necessary for assembly of the *E. faecalis* Ebp pilus and incorporation of both the EbpA and EbpB minor subunits (18). Anchoring of pili to the cell wall is often, although not always, achieved by the housekeeping sortase, a conserved, membrane-associated enzyme found in nearly all Gram-positive organisms (19). Like Ebp pili, the *E. faecalis* housekeeping sortase, SrtA (20), is also important for biofilm formation and in animal models of ascending UTI and CAUTI (21–23). However, it is not known whether SrtA participates in the biogenesis of Ebp pili.

Sortase transpeptidation is thought to occur in two steps on the bacterial membrane. First, the enzyme cleaves its substrate between the Thr and Gly residues of the LPXTG-like motif, leading to the formation of a sortase-substrate intermediate with a thioester bond between the side-chain sulfur of the sortase catalytic Cys residue and the carboxyl group of the substrate Thr residue. Next, this thioacyl intermediate is resolved upon nucleophilic attack by an acceptor molecule (6, 24, 25). Thus, sortases demonstrate two molecular specificities: (i) for cleavable substrates, partially determined by the substrates' LPXTG-like motifs and (ii) for acceptor nucleophiles, the basis of which is not fully understood. The housekeeping sortase accepts the cross-bridge peptide of the lipid II cell wall precursor as a nucleophile (26), leading to cell wall anchoring of its substrates. Studies of the *Corynebacterium diphtheriae* pilus islands first suggested that pilus-associated sortases accept conserved Lys residues within pilus structural subunits as nucleophiles, resulting in intermolecular isopeptide bonds formed between pilus subunits. In *C. diphtheriae*, the Lys residue in the pilin motif (WXXXVXVYPK peptide sequence) of the major pilin, SpaA, is necessary for its polymerization into fibers (12, 27). Canonical pilin motifs are present in N-terminal domains of many sortase-assembled pilus major subunits (6). Similarly, a conserved Lys residue within the minor anchor pilin SpaB was necessary for its incorporation into pilus fibers (14). Studies in *Actinomyces naeslundii* (28), *Streptococcus pyogenes* (group A streptococci [GAS]) (29), *Streptococcus agalactiae* (group B streptococci [GBS]) (30–32), and *Streptococcus pneumoniae* (the pneumococcus) (33–35) demonstrated that many molecular aspects of sortase-assembled pilus biogenesis are conserved but also revealed unique variations among different systems.

Pilus biogenesis and sortase activity are attractive targets for the development of Gram-positive antivirulence therapies (36). However, a unified understanding of the mechanisms governing these processes is required for rational drug design. The roles of conserved elements of pilus assembly and sortase catalysis, such as sortase recognition motifs, pilin-like motifs, and sortase catalytic residues, have not been directly tested in Ebp pilus biogenesis in *E. faecalis*. Here, we used a panel of strains expressing mutant pilin and sortase alleles to define molecular interactions important for pilus fiber polymerization, minor pilus subunit incorporation, and pilus anchoring to the cell wall. Our results reveal both universal and unique aspects of pilus biogenesis and sortase activity in *E. faecalis*.

MATERIALS AND METHODS

Bacterial growth conditions. All bacterial strains used in this study are listed in Table S1 in the supplemental material. *Escherichia coli* TOP10 was used for propagation of expression plasmids and grown at 37°C with agitation in Luria-Bertani (LB) broth or agar. For *E. coli* strains carrying plasmids, kanamycin (Kan) was added to growth media at 50 µg ml⁻¹ or ampicillin (Amp) at 25 µg ml⁻¹. *E. faecalis* strains were grown statically at

37°C in Bacto brain heart infusion (BHI) broth or agar supplemented with 25 µg ml⁻¹ of rifampin (Rif) for OG1RF-derived strains or 500 µg ml⁻¹ streptomycin (Str) for OG1X unless otherwise noted. Expression plasmids were maintained in *E. faecalis* by supplementation of growth media with 500 µg ml⁻¹ Kan. All antibiotics were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Media were purchased from BD (Becton, Dickinson and Company, Franklin Lakes, NJ).

General cloning techniques. The Ebp pilin amino acid sequence is based on the *E. faecalis* OG1RF sequence for the *ebpABC-srtC* locus (37), obtained as described previously (10). The SrtA and SecA sequences used in this study are derived from the *E. faecalis* V583 genome (38). The OG1RF and V583 SrtA amino acid sequences are 100% identical. The Wizard genome DNA purification kit (Promega Corp., Madison, WI) was used for isolation of bacterial genomic DNA (gDNA), and the Hurricane Maxi Prep kit (Gerard Biotech LLC, Oxford, OH) was used to prepare *E. faecalis* expression plasmid DNA. All plasmids used in the study are listed in Table S2 in the supplemental material. T4 DNA ligase and restriction endonucleases were purchased from New England BioLabs (Ipswich, MA). PCR was performed with Phusion DNA polymerase from Finnzymes (Thermo Fisher Scientific, Inc., Rockford, IL). The primers used in this study are listed in Table S3.

***E. faecalis* expression plasmids and strains.** To express mutant SrtA^{C200A} protein (SrtA with the C200→A mutation) in *E. faecalis*, we used splice by overlap extension PCR (SOE-PCR) (39) from *E. faecalis* gDNA to create an *srtA* open reading frame (ORF) encoding the mutant residue. This ORF was cloned downstream of the *rofA* promoter in the Gram-positive expression vector pAL1 (40). A DNA fragment upstream of and including the mutation was created by PCR using the forward primer EF3056i-f2 (encoding a BamHI restriction site) and the SOE-reverse primer EF3056 C200A-r. A DNA fragment downstream of and including the mutation was created using the SOE forward primer EF3056 C200A-f and the reverse primer EF3056-r3 (encoding a PstI restriction site). Upstream and downstream DNA fragments were spliced by PCR with EF3056i-f2 and EF3056-r3. The pAL1::SrtA^{C200A} plasmid was derived after digestion of the parent pAL1 plasmid and SOE-PCR products with EcoRI and PstI followed by ligation. All primer sequences are listed in Table S3 in the supplemental material.

We assessed pilus assembly in this study by providing the previously described EbpABC⁻ or EbpABC⁻ SrtC⁻ pilus knockout strains (10) with OG1RF or mutant alleles of the *ebp* locus in *trans*. The p-*ebpABC* and p-*ebpABCsrtC* expression plasmids (10) encode regions beginning 500 bp upstream of the *ebpA* translational start codon (as proposed by Nallapareddy et al. [9]) and ending with the *ebpC* or *srtC* stop codon, respectively. The plasmids p-*ebpABC*^{K186A}, p-*ebpABC*^{K179A}, and p-*ebpABC*^{K978A}, encoding putative pilin-like motif mutant alleles of EbpC, EbpB, and EbpA, respectively, were derived by site-directed mutagenesis of p-*ebpABC* with the Quikchange II kit (Stratagene) using the sense/antisense primer pairs HVN170/HVN171, HVN142/HVN143, and HVN134/HVN135, respectively.

To scramble the EbpB and EbpA LPXTG motifs on expression plasmids, we used SOE-PCR using OG1RF gDNA as a template with the SOE forward/SOE reverse primer pairs HVN220/HVN221 and HVN197/HVN198, respectively, encoding the mutant sequences. Forward and reverse primers internal to the *ebpABC srtC* locus and that included restriction sites present in the genome but unique within the locus were designed for these reactions: HVN218/HVN217 and HVN191/HVN192, respectively. The p-*ebpABC*^{NTPLE}*srtC* and p-*ebpABC*^{GTPLE} plasmids were derived by double digestion of the parent plasmids p-*ebpABCsrtC* and p-*ebpABC* and the respective SOE-PCR products with EcoRI/StuI or SbfI/AatII, respectively, followed by ligation.

To generate an expression plasmid lacking the *ebpA* coding sequence, we used SOE-PCR to generate a DNA fragment encoding the region 500 bp upstream of the *ebpA* translational start codon followed immediately by the *ebpB* start codon with the SOE forward/SOE reverse primers HVN149/HVN148 encoding the mutant sequence, OG1RF template

gDNA, and the forward/reverse primers HVN145/HVN192. Thus, to create *p-ebpBCsrtC*, the parent plasmid *p-ebpABCsrtC* and the SOE-PCR product were digested with XhoI and AatII followed by ligation. Plasmids incorporating the mutant allele (which lacked the *ebpA* coding sequence and the 3 *ebpA-ebpB* intergenic bp) were selected by PCR screening for lack of *ebpA*. All plasmids derived in this study were confirmed by sequencing.

Competent cells were prepared from the OG1RF-derived EbpABC⁻, EbpABC⁻ SrtC⁻, SrtA⁻, and SrtC⁻ SrtA⁻ strains and transformed by electroporation essentially as described elsewhere (41), except BYGT medium (1.9% [wt/vol] BHI, 0.5% yeast extract, 0.2% glucose, 100 mM Tris-Cl [pH 7.0]) was substituted for M17 medium. All of the resultant strains are listed in Table S1 in the supplemental material.

Bacterial cell fractionation. Bacteria were grown overnight, diluted 1:100 into TSBG (BBL Trypticase soy broth supplemented with 0.25% glucose), and grown statically at 37°C for 8 h. Equal numbers of cells (as determined by optical density at 600 nm [OD₆₀₀]) were harvested by centrifugation at 5,000 × *g* for 10 min and washed once in phosphate-buffered saline (PBS). Cell wall fractions, cell lysates, and culture medium fractions were prepared as described previously (10).

For isolation of bacterial cell membranes, cells harvested and washed as described above were stored at -80°C. Cell pellets were thawed on ice and digested in protoplast buffer (20% sucrose, 10 mM Tris-Cl, 50 mM sodium chloride, 1 mM EDTA [pH 8.0]) with 250 U ml⁻¹ mutanolysin and 10 mg ml⁻¹ lysozyme (Sigma-Aldrich Corp.) in 1/50 of the original culture volume for 2 h at 37°C. Protoplasts were harvested by centrifugation (~20,000 × *g*, 20 min, 4°C) and washed once in PBS. Washed protoplast pellets were resuspended in ~1/5 the original culture volume of a mixture of 20 mM Tris-HCl (pH 8.0), 150 mM sodium chloride, and 1 mM EDTA with Complete protease inhibitor cocktail (Roche), and cell membranes were disrupted by sonication. The resultant cell lysates were incubated with DNase I (5 μg ml⁻¹) and RNase A (10 μg ml⁻¹) for 15 min on ice. Lysates were then subjected to two successive centrifugations at 15,700 × *g* for 30 min each at 4°C to remove any unlysed cells or protoplasts. Membranes were then isolated by ultracentrifugation at 165,000 × *g* for 2 h at 4°C. Pellets containing bacterial membranes were resuspended in ~1/50 the original culture volume of 10 mM Tris-Cl-1 mM EDTA and are referred to as “membrane” fractions in the text. All bacterial cell fractions were stored at -20°C.

Preparation of protoplast lysates. Bacteria were grown, harvested, and washed as for bacterial cell fractionation above. Cell pellets were resuspended in half the original culture volume of protoplast buffer with lysozyme and mutanolysin as described above and incubated at 37°C with agitation. At 0, 1, and 2 h after addition of cell wall hydrolases, a sample from each culture was harvested by centrifugation at 20,000 × *g* for 5 min and washed in PBS.

Generation of anti-SrtA immune sera. A modified version of *E. faecalis* V583 SrtA was constructed in which internal amino acid residues 13 to 46, encompassing a transmembrane helix, were deleted (SrtA^{ΔTMH}). DNA encoding the modified version of the *srtA* gene (EF3056) was amplified from V583 gDNA using primers 5′ SrtANtermNheI and SrtACtermXhoI (listed in Table S3 in the supplemental material) and inserted between the NheI and XhoI restriction sites of the pET24c plasmid (Novagen). The resultant plasmid (pSJH-274) was transformed into *E. coli* BL21(DE3)/pLysS (Novagen) to create strain SJH-1399 for SrtA^{ΔTMH} overexpression. SJH-1399 cells were induced with isopropyl-D-1-thiogalactopyranoside (IPTG) at an OD₆₀₀ of 0.6 at 37°C. After centrifugation (4,000 × *g*, 10 min), cells were resuspended in lysis buffer (50 mM Tris-HCl, 2 mM EDTA) disrupted by two passages through a French press at room temperature, and centrifuged at 17,000 × *g* for 45 min at 4°C. The supernatant cell lysate was loaded onto a 100 ml DEAE-Sepharose column (GE Healthcare, Waukesha, WI). The column was washed with 2 column volumes of lysis buffer followed by a linear gradient of potassium chloride (0 to 1.0 M). The fractions containing SrtA^{ΔTMH} were pooled, and the concentration of ammonium sulfate in the pooled fractions was slowly adjusted to 1 M. The

pooled sample was applied to a 20-ml phenyl-Sepharose column (GE Healthcare) that was pre-equilibrated with buffer A (50 mM Tris-HCl, 2 mM EDTA, 1 M ammonium sulfate). The column was washed with 2 column volumes of buffer A followed by a linear gradient of lysis buffer in order to decrease the concentration of ammonium sulfate from 1.0 to 0 M. The fractions containing SrtA^{ΔTMH} were pooled, concentrated using an Amicon Ultra filter unit with a cutoff of 10 kDa (Millipore, Billerica, MA), and applied to a Superdex 75 column (isocratic gradient with 50 mM Tris-HCl, 2 mM EDTA). DEAE-Sepharose, phenyl-Sepharose, and Superdex 75 columns were connected to an ÄKTA fast protein liquid chromatograph (FPLC) (Amersham Biosciences Corp., Piscataway, NJ). SrtA^{ΔTMH} purity was verified by SDS-PAGE. The fractions containing SrtA^{ΔTMH} were finally pooled and concentrated to >1 mg/ml. Polyclonal antisera were raised against SrtA^{ΔTMH} in rabbits (Agro-Bio, La Ferté Saint-Aubin, France).

Generation of anti-SecA immune sera. Full-length *E. faecalis* V583 *secA* (EF1763) was amplified from V583 genomic DNA using the primers rSecA-f and rSecA-r (listed in Table S3 in the supplemental material) and inserted between the NdeI and XhoI restriction sites of pET19b (Novagen) for inclusion of an N-terminal His tag. The resulting plasmid (pSJH-469) was used to transform *E. coli* BL21(DE3)/pLysS, creating strain SJH-1898. SJH-1898 cells were grown to an OD₆₀₀ of 0.6, SecA expression was induced with 1 mM IPTG, and cells were grown for another 4 h at 37°C. Cell lysates were prepared by sonication. SecA protein was purified by chromatography using a Talon cobalt affinity column (Clontech, Mountain View, CA) followed by a Source15Q column (GE Healthcare) connected to an ÄKTA FPLC (Amersham Biosciences Corp.). SecA purity was verified by SDS-PAGE and by N-terminal sequencing. Polyclonal antisera were raised against purified SecA in rabbits (New England Peptide, Gardner, MA). Western blots of SDS-PAGE of SecA purified from *E. coli* and OG1RF cell lysates probed with the immune sera revealed a single band ~100 kDa. No reactivity of preimmune sera was observed.

Western blots. Bacterial cell fractions derived as described above were diluted in β-mercaptoethanol-containing sample loading buffer and boiled for 10 to 15 min. SDS-PAGE was performed using NuPAGE Novex 3 to 8% Tris-acetate precast protein gels run in NuPAGE Tris-acetate SDS running buffer (Life Technologies Corp., Carlsbad, CA) to detect the pilus high-molecular-weight ladder (HMW) and pilin monomers and NuPAGE 4 to 12% Bis-Tris precast protein gels run in morpholinepropane-sulfonic acid (MOPS)-SDS running buffer (Life Technologies Corp.) to visualize EbpB, SrtA, and SecA monomers. Western blotting was performed as described previously (10) additionally using the anti-SecA and anti-SrtA immune sera derived here. White space in the figures separates distinct immunoblots or exposures, and lines on a blot show that the lanes were reordered or deleted using Adobe Photoshop CS2 (Adobe Systems, Inc., Mountain View, CA).

Negative-stain immunogold EM. Cells adsorbed to grids were stained with uranyl acetate and incubated with either rabbit (11) or mouse (10) anti-EbpC immune sera followed by the appropriate goat IgG conjugated to either 18-nm or 12-nm colloidal gold particles (Jackson ImmunoResearch Laboratories, West Grove, PA). Negative-stain immunogold electron microscopy (EM) was performed as described previously (10).

Deep-etch immunogold electron microscopy. Bacterial cells were grown on TSBG (BBL Trypticase soy broth with 0.25% glucose), and then the cells were centrifuged and washed twice with 1× PBS. Cells were deposited onto glass slides, fixed, and labeled as described previously (42) using rabbit anti-EbpA sera and 18-nm gold bead-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Samples were freeze-dried and imaged as described previously (42).

RESULTS

Deletion of *srtA* does not affect Ebp pilus polymerization or minor pilin incorporation. To determine the role of the *E. faecalis* housekeeping sortase SrtA in pilus biogenesis, we examined pilus

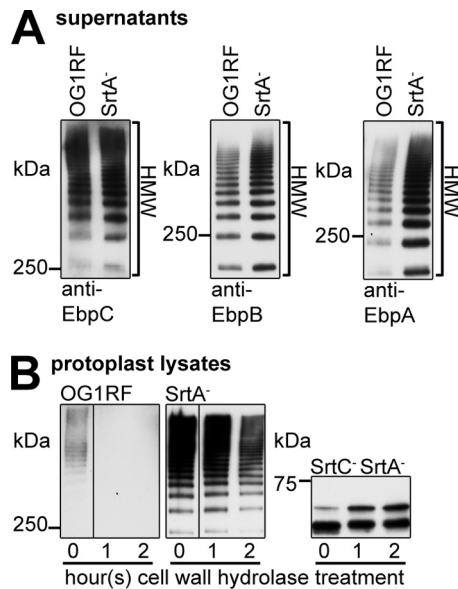


FIG 1 Pili expressed by the *SrtA*⁻ strain incorporate all structural subunits and remain associated with protoplasts after depletion of cell wall material by treatment with cell wall hydrolases. (A) Culture supernatants of OG1RF and the *SrtA*⁻ strain were assessed by Western blotting with the indicated antipilin immune sera. (B) OG1RF, *SrtA*⁻, or *SrtC*⁻ *SrtA*⁻ cells were treated with lysozyme and mutanolysin for the indicated time. Protoplasts were isolated by centrifugation, and protoplast-associated pili or EbpC monomers were detected on Western blots probed with anti-EbpC immune sera. HMW, high-molecular-weight ladders.

polymerization, minor pilin incorporation, and pilus subcellular compartmentalization in a markerless, chromosomal *srtA* deletion mutant in the OG1RF background (*SrtA*⁻ mutant) (22). A Western blot analysis of OG1RF and *SrtA*⁻ mutant culture supernatants using anti-EbpC (Fig. 1A, left), anti-EbpB (Fig. 1A, middle), and anti-EbpA (Fig. 1A, right) immune sera revealed similar high-molecular-weight ladders (HMWs) for both strains. These HMWs seen on Western blots indicate EbpC polymerization and EbpB and EbpA incorporation into pilus fibers since the intermolecular isopeptide bonds between pilus subunits do not dissociate upon boiling in SDS. Thus, *SrtA* is not necessary for assembly of Ebp pilus subunits, consistent with reports for all other sortase-assembled pilus systems (43).

Ebp pili are not incorporated into the cell wall in an *SrtA*⁻ strain. Pilus assembly by sortases takes place in the bacterial membrane, but mature pili are covalently attached to peptidoglycan cross-bridges in the cell wall, presumably as a result of processing by the housekeeping sortase. To determine whether *E. faecalis* *SrtA* affects subcellular compartmentalization of Ebp pili, we performed Western blot analysis of OG1RF and the *SrtA*⁻ strain using anti-EbpC sera on bacterial cell fractions where pili have been detected: (i) cell wall fractions to detect peptidoglycan-linked pili, (ii) culture supernatants, and (iii) protoplast lysates to detect membrane-associated pili. In parallel, we monitored apparent EbpC monomer subcellular compartmentalization in the *SrtC*⁻ *SrtA*⁻ strain that cannot polymerize pili as a control for cell fractionation techniques. As this strain lacks all predicted sortase coding regions, EbpC monomer should be retained in the cell membrane via the hydrophobic membrane-spanning domain of its CWSS that remains uncleaved in the absence of sortases.

Cell wall material was isolated by digestion of strains with cell wall hydrolases followed by centrifugation to remove protoplasts and undigested cells. Pilus HMWs were present in cell wall fractions of OG1RF and the *SrtA*⁻ strain (data not shown), consistent with previously published results for *E. faecalis* OG1X (40). However, EbpC monomer was also detected in cell wall fractions of the *SrtC*⁻ *SrtA*⁻ strain (data not shown), suggesting that some membrane-associated protein copurified with cell wall material for unknown reasons. Possibly, EbpC-containing sites of pilus assembly in the membrane of *E. faecalis* are intimately associated with the cell wall. Thus, we could not determine whether pili detected in cell wall fractions of the *SrtA*⁻ strain were anchored to the cell wall, potentially by another factor, such as *SrtC*, or membrane associated but copurifying with cell wall material.

Substrates of the housekeeping sortases, including the sortase-assembled pili of *C. diphtheriae* (44), are often found in culture supernatants and not in cell wall fractions in the absence of the enzyme for unknown reasons. Thus, we performed Western blot analysis of filter-sterilized culture supernatants. Although, we detected pilus HMWs in supernatants of the *SrtA*⁻ strain, similar amounts were also present in OG1RF supernatants (Fig. 1A). The mechanism leading to detection of pili in supernatants of these two strains is unknown but was not affected in the absence of *SrtA*.

To further investigate the effect of the *srtA* deletion on pilus subcellular compartmentalization, we next performed Western blotting of protoplast lysates, the pelleted cellular material remaining after progressive depletion of cell wall material by digestion with lysozyme and mutanolysin for up to 2 h. No pilus HMWs were observed associated with OG1RF protoplasts after only 1 h of cell wall hydrolase treatment (Fig. 1B, left), arguing that all of the pili were anchored to the cell wall and released by this treatment. As expected, hydrolase treatment did not affect the presence of EbpC monomers in *SrtC*⁻ *SrtA*⁻ protoplasts (Fig. 1B, right). For unknown reasons, pilin monomers were consistently detected as doublets on SDS-PAGE, as seen for EbpC in Fig. 1B (right panel). Similarly, pilus HMWs were still observed after 2 h of hydrolase treatment of the *SrtA*⁻ strain (Fig. 1B, middle), suggesting that in the absence of *SrtA*, pili were retained in the bacterial cell membrane where pilus assembly occurs. Notably, the *SrtA*⁻ strain consistently produced more pili than OG1RF (compare the 0-h lanes of OG1RF and the *SrtA*⁻ mutant in Fig. 1B). Although we cannot rule out the possibility that some pili in the *SrtA*⁻ mutant are linked to the cell wall, as discussed above, this increased pilus expression may contribute to copurification of membrane-associated pili with cell wall fractions in the *SrtA*⁻ strain.

Pili produced by *SrtA* mutants accumulate in bacterial cell membranes. To further investigate the role of *SrtA* in Ebp pilus subcellular localization, we isolated bacterial cell membranes from OG1RF and the *SrtA*⁻, *SrtA*⁻/pAL1 (vector control strain), and *SrtA*⁻/pAL1::*SrtA* strains. The previously described pAL1::*SrtA* plasmid encodes an OG1 allele of *srtA* under the control of the GAS *rofA* promoter (40), which complements pilus production in OG1RF *SrtA*⁻ to levels similar to WT and does not affect the subcellular localization of the Ebp pilus, as judged by EM (data not shown). A vector control plasmid, pAL1, has the coding region for the GAS alkaline phosphatase *PhoZ* under the control of the *rofA* promoter (40). Western blots of membrane fractions probed with anti-EbpC sera revealed pilus HMWs only in membranes isolated from the *SrtA*⁻ and *SrtA*⁻/pAL1 strains but not in

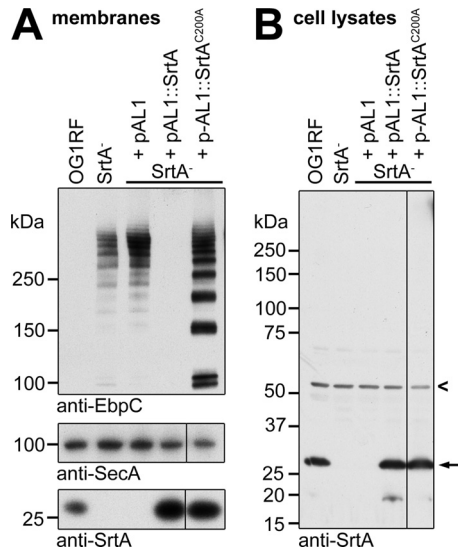


FIG 2 Pili accumulate in the cell membranes of strains lacking SrtA or strains with the predicted catalytic Cys 200 residue mutated to Ala. Western blots were performed with the noted antiserum on (A) membrane fractions and (B) cell lysates of OG1RF, the $SrtA^-$ strain, and the $SrtA^-$ strain carrying either p::AL1 (vector control), p::AL1-SrtA, or p::AL1-SrtA^{C200A}. The arrow and caret denote SrtA and a nonspecific band, respectively.

those from OG1RF or the $SrtA^-$ /pAL1::SrtA strain (Fig. 2A, top blot). Thus, lack of SrtA resulted in accumulation of pili in bacterial cell membranes, consistent with our above observation that the $SrtA^-$ strain pili were present in a bacterial cell compartment protected from treatment with cell wall hydrolases. Western blots performed on the same samples using antisera generated against the SecA membrane protein (Fig. 2A, middle blot) and SrtA (Fig. 2A, bottom blot) revealed that equal amounts of membrane material were isolated and analyzed for each strain and that SrtA was expressed in OG1RF and the $SrtA^-$ /pAL1::SrtA strain.

To determine whether lack of the SrtA protein or lack of its catalytic activity resulted in the observed membrane accumulation of pili in strains lacking SrtA, we mutated the predicted SrtA catalytic Cys 200 residue to Ala on the pAL1::SrtA expression plasmid, resulting in the plasmid pAL1::SrtA^{C200A}. The SrtA and SrtA^{C200A} alleles were expressed to similar levels from the pAL1 plasmid backbone in whole-cell lysates (Fig. 2B) and membrane fractions (Fig. 2A, bottom blot). Membranes isolated from the $SrtA^-$ strain carrying pAL1::SrtA^{C200A} showed the same pattern of accumulation of pilus HMWs in membranes as that seen in the $SrtA^-$ and $SrtA^-$ /pAL1 strains (Fig. 2A), suggesting it was the lack of SrtA's catalytic activity that led to accumulation of pili in bacterial membranes.

EbpB is the only pilin processed by SrtA in the absence of SrtC. The results described above suggest that SrtA is important for efficient sorting of pili from the membrane to the cell wall. To determine which pilin or pilins serve as SrtA-cleavable substrates to achieve cell wall anchoring, we used Western blot analyses to detect unprocessed pilin monomers that retain their CWSS in the absence of SrtA. We examined pilin migration on SDS-PAGE in two previously described mutants: an $SrtC^-$ mutant that cannot polymerize pilus fibers but expresses all pilin monomers (10) and the $SrtC^- SrtA^-$ mutant additionally lacking the *srtA* coding sequence. In Western blots of cell lysates, migration of EbpC and

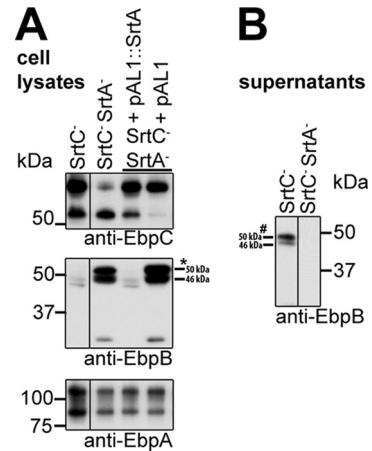


FIG 3 EbpB monomer doublets migrate at a higher molecular mass in the absence of SrtA. Western blots on SDS-PAGE of (A) cell lysates or (B) supernatants of the $SrtC^-$ and $SrtC^- SrtA^-$ strains and the $SrtC^- SrtA^-$ strain carrying either pAL1 (vector control) or pAL1::SrtA were probed with the indicated anti-pilin sera. The symbol # shows the EbpB monomer released mainly to culture supernatants of the $SrtC^-$ strain. The asterisk shows the larger EbpB monomer primarily associated with cells in strains lacking SrtA.

EbpA monomers did not differ between the two strains (Fig. 3A, top and bottom blots, respectively). However, the EbpB monomer migrated at a higher molecular mass (50 kDa) on SDS-PAGE in cell lysates of the $SrtC^- SrtA^-$ mutant (Fig. 3A, asterisk) than in lysates of the $SrtC^-$ mutant, consistent with the posttranslational cleavage of the EbpB CWSS (46 kDa) only in the presence of SrtA. This phenotype was complemented by transformation of the $SrtC^- SrtA^-$ mutant with pAL1::SrtA but not with pAL1. Furthermore, greater EbpB monomer signal was observed in cell lysates of strains lacking SrtA than in those of strains expressing SrtA (Fig. 3A, middle blot). EbpB monomers were primarily detected in culture supernatants of the $SrtC^-$ strain but not in those of the $SrtC^- SrtA^-$ mutant (Fig. 3B, #), suggesting that sorting of EbpB to the culture medium depended on its processing by SrtA. Taken together, these results suggest that EbpB, but not EbpC or EbpA, is an SrtA substrate.

EbpC's pilin-like motif Lys 186 is necessary for EbpC fiber polymerization. The next aspect of pilus biogenesis we examined was fiber polymerization. In *E. faecalis*, EbpC is the only structural subunit necessary (9) and sufficient (10) for Ebp pilus polymerization. To test whether the EbpC pilin-like motif (ELAVVHIYPK [conserved residues are underlined]) (9) was involved in pilus polymerization, we mutated the Lys 186 residue, predicted to participate in sortase formation of interpilin isopeptide bonds, to Ala on the expression plasmid p-*ebpABC*. This plasmid contains the OG1RF *ebp* locus beginning 500 bp upstream of *ebpA*'s translational start codon to include the native promoter and ending with the *ebpC* stop codon. We have previously shown that a chromosomal deletion mutant lacking all of the Ebp structural subunit coding regions complemented with p-*ebpABC* (EbpABC⁻/p-*ebpABC*) polymerizes pili similar to those of OG1RF (10). We examined pilus assembly in the EbpABC⁻ strain carrying p-*ebpABC* or the mutant plasmid (p-*ebpABC*^{K186A}) using negative-stain immunogold electron microscopy (EM) and Western blot analyses. Bacterial cells with pilus fibers decorated with gold beads were visible by EM from cultures of the EbpABC⁻/p-

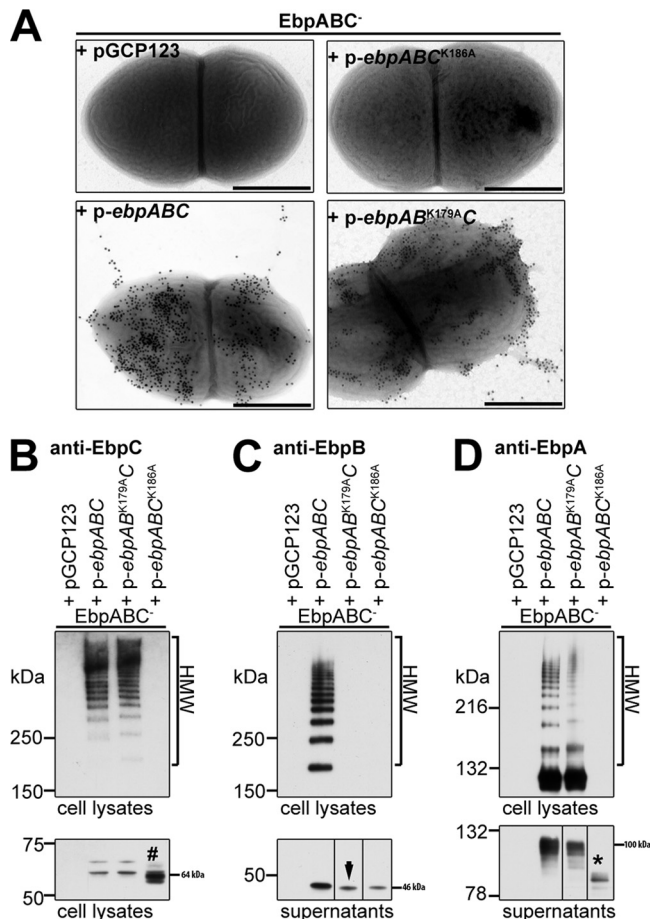


FIG 4 EbpC's pilin motif Lys 186 and EbpB's pilin motif Lys 197 are necessary for EbpC polymerization and EbpB incorporation into pilus fibers, respectively. (A) Negative-stain immunogold EM studies were carried out with anti-EbpC sera to visualize pilus fibers expressed by the EbpABC⁻ strain carrying either pGCP123 (vector control), p-*ebpABC*, p-*ebpAB*^{K179A}C, or p-*ebpABC*^{K186A}. Scale bars are 500 nm. (B to D) Western blots of the EbpABC⁻ strain carrying either pGCP123 (vector control), p-*ebpABC*, p-*ebpAB*^{K179A}C, or p-*ebpABC*^{K186A} were performed after SDS-PAGE of the indicated cell fractions with (B) anti-EbpC, (C) anti-EbpB, and (D) anti-EbpA immune sera. The top blots show pilus HMWs (brackets), while the bottom blots show pilin monomers (#, EbpC^{K186A}; arrow, EbpB^{K179A}; asterisk, EbpA degradation product).

ebpABC mutant, but not from cultures of the EbpABC⁻/pGCP123 strain (vector control strain) or EbpABC⁻/p-*ebpABC*^{K186A} strain (Fig. 4A), showing that the EbpC pilin-like motif Lys 186 was necessary for pilus fiber elaboration. Western blots of cell lysates of the EbpABC⁻/p-*ebpABC* strain probed with anti-EbpC immune sera revealed pilus HMWs (Fig. 4B, brackets), indicating EbpC polymerization. Likewise, HMWs were observed on Western blots of EbpABC⁻/p-*ebpABC* cell lysates developed with anti-EbpB and anti-EbpA sera (Fig. 4C and 4D, brackets), indicating incorporation of EbpB and EbpA into pilus fibers, respectively. In contrast, no HMWs were visible on Western blots of cell lysates or culture supernatants (data not shown) of EbpABC⁻/p-*ebpABC*^{K186A} cells probed with any antipilin sera (Fig. 4B to D, top blots), showing that the EbpC pilin-like motif Lys 186 is necessary for EbpC polymerization into fibers.

Mutation of EbpC Lys 186 affects EbpC and EbpA expression. Although not seen in cell lysates of strain OG1RF (data not

shown), we observed EbpC monomers (Fig. 4B and see Fig. 6B, bottom blots) and EbpA monomers (Fig. 6D, bottom blot) in cell lysates of the EbpABC⁻/p-*ebpABC* strain. We hypothesize that pilin production from the multicopy plasmid may saturate the capacity of SrtC produced from a single copy on the chromosome, leading to the presence of unprocessed pilin monomers in cell lysates. EbpB monomers were seen in culture supernatants (Fig. 4C, bottom blot), consistent with previously published results for OG1RF (10). Similarly, EbpB monomers were observed in the culture supernatants of the EbpABC⁻/p-*ebpABC*^{K186A} strain (Fig. 4C, bottom blot), showing that mutation of EbpC Lys 186 did not affect EbpB expression or processing. Although EbpA and EbpC^{K186A} monomers were expressed by EbpABC⁻/p-*ebpABC*^{K186A} cells, they migrated differently on reducing SDS-PAGE and localized differently than those from EbpABC⁻/p-*ebpABC* cells. The EbpC^{K186A} monomer shifted to a lower molecular mass (Fig. 4B, #). We hypothesize that the sortase recognition motif of the EbpC^{K186A} monomer (LPSTG) was cleaved by SrtC, leading to the observed downward shift on SDS-PAGE due to the loss of the CWSS. We failed to detect any EbpA monomer in cell lysates (data not shown), but a smaller EbpA species was observed in culture supernatants of the EbpABC⁻/p-*ebpABC*^{K186A} strain (Fig. 4D, asterisk). We have previously shown that the EbpA monomer is cell-associated in the absence of SrtC (10, 40), presumably tethered to the membrane via its uncleaved CWSS. Thus, we hypothesized that the EbpA species found in supernatants of the EbpABC⁻/p-*ebpABC*^{K186A} strain arose after EbpA processing by SrtC. Possibly, an EbpA-SrtC thioacyl intermediate could not be resolved in the absence of the EbpC Lys 186 nucleophile, leading to hydrolysis of the thioester bond and release of processed EbpA into the extracellular milieu, where it may have undergone proteolytic degradation, resulting in the observed EbpA species.

EbpB's pilin-like motif Lys 179 is necessary for its incorporation into pilus. We have previously shown that deletion of *ebpB* does not affect EbpC polymerization or incorporation of EbpA into pilus fibers (10), demonstrating the role of EbpB as an ancillary pilus subunit. Incorporation of minor subunits into sortase-assembled pilus fibers of other systems requires either a conserved Lys residue or LPXTG-like motif, depending on the pilin's location within the pilus fiber (6, 43, 45). EbpB encodes a pilin-like motif (SLTHIHLYPK) with a conserved Lys residue (9). We investigated the importance of EbpB's pilin-like motif in Ebp pilus assembly by mutating Lys 179 to Ala on the expression plasmid p-*ebpABC* and assessing pilus assembly by the mutant plasmid (p-*ebpAB*^{K179A}C) using the analyses described above. Gold bead-decorated fibers were observed on bacterial cells from cultures of EbpABC⁻/p-*ebpAB*^{K179A}C cells in immunogold EM studies using anti-EbpC antisera, similar to those from cultures of EbpABC⁻/p-*ebpABC* cells (Fig. 4A). Furthermore, HMWs were visible on Western blots of EbpABC⁻/p-*ebpAB*^{K179A}C cells probed with anti-EbpC and anti-EbpA sera (Fig. 4B and 4D, top blots), showing that EbpC polymerization and EbpA incorporation into pilus fibers were not affected by mutation of EbpB Lys 179. In contrast, no HMWs were visible when Western blots of cell lysates of the EbpABC⁻/p-*ebpAB*^{K179A}C strain were probed with anti-EbpB sera (Fig. 4C, top blot), although the EbpB^{K179A} monomer was visible in culture supernatants (Fig. 4C, bottom blot, arrow). Thus, mutation of the EbpB pilin-like motif Lys 179 did not affect

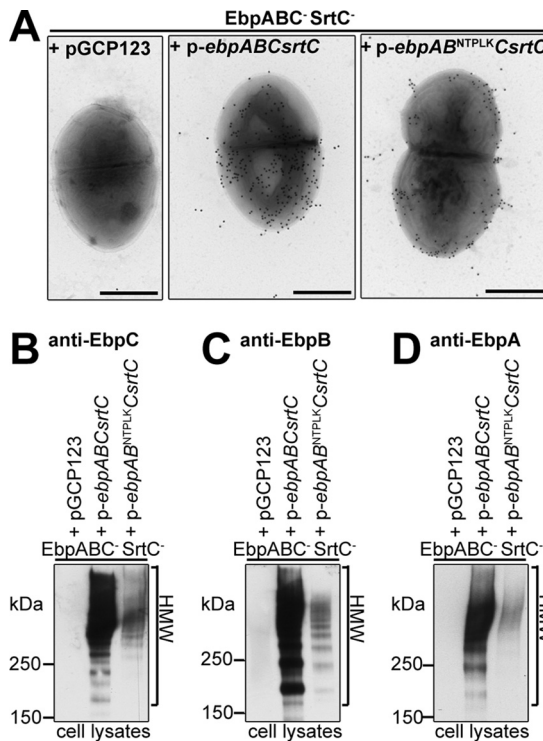


FIG 5 EbpB's sortase recognition motif is dispensable for EbpB incorporation into pilus fibers. (A) Negative-stain immunogold EM using anti-EbpC sera and (B to D) Western blot analyses of cell lysates were performed on the EbpABC⁻ SrtC⁻ strain carrying either pGCP123 (vector control), p-*ebpABCsrtC*, or p-*ebpAB^{NTPLK}CsrtC*. (A) Scale bars are 500 nm. Blots were probed with (B) anti-EbpC, (C) anti-EbpB, or (D) anti-EbpA immune sera. Pilus HMWs are indicated with brackets.

its expression or sorting to the culture medium but did prevent EbpB incorporation into pili.

EbpB's sortase recognition motif is dispensable for its incorporation into pili. We determined the importance of the EbpB Leu-Pro-Lys-Thr-Asn predicted sortase recognition motif in pilus assembly by replacing the OG1RF sequence (LPKTN) with a scrambled sequence (NTPLK) on the expression plasmid p-*ebpABCsrtC*, which contains the OG1RF *ebp* locus beginning 500 bp upstream of *ebpA*'s translational start codon and ending with the *srtC* stop codon. Pilus assembly by the mutant plasmid (p-*ebpAB^{NTPLK}CsrtC*) was examined as described above. In immunogold EM studies, bacterial cells with gold bead-decorated fibers were observed in cultures of the EbpABC⁻ SrtC⁻/p-*ebpABCsrtC* and EbpABC⁻ SrtC⁻/p-*ebpAB^{NTPLK}CsrtC* strains but not in cultures of the EbpABC⁻ SrtC⁻/pGCP123 strain (vector control) (Fig. 5A), suggesting that scrambling the EbpB LPKTN motif did not affect pilus polymerization. Western blots of cell lysates of the EbpABC⁻ SrtC⁻ strain carrying either p-*ebpABCsrtC* or p-*ebpAB^{NTPLK}CsrtC* revealed pilus HMWs when developed with anti-EbpC (Fig. 5B), anti-EbpB (Fig. 5C), or anti-EbpA (Fig. 5D) sera. Thus, the EbpB LPKTN motif is dispensable for EbpC polymerization and EbpA and EbpB incorporation into pilus fibers. Interestingly, the pilus HMW signal on Western blots was fainter for the EbpABC⁻ SrtC⁻/p-*ebpAB^{NTPLK}CsrtC* strain, suggesting that EbpB's LPKTN motif may be important for efficiency of pilus assembly. We previously observed a similar decrease in piliation in

a mutant lacking EbpB (10). Thus, EbpB incorporation into pilus fibers relies on the Lys residue of its pilin-like motif but does not require its LPKTN sortase recognition motif, consistent with localization of EbpB at the pilus base.

EbpA's Lys 978 is dispensable for its incorporation into pilus fibers. We have previously shown that deletion of *ebpA* does not prevent EbpC polymerization or EbpB expression (10), confirming its role as a minor pilin. Although EbpA does not encode a canonical pilin motif, Nallapareddy et al. predicted a noncanonical pilin motif in EbpA that included Lys 978 (KYGEIH^{YAG}K) (9). We mutated this Lys to Ala on the expression plasmid p-*ebpABC* and assessed the ability of the EbpABC⁻ strain carrying either the parent p-*ebpABC* plasmid or the mutant p-*ebpA^{K978A}BC* plasmid to assemble pili. No difference was observed in the ability of either EbpA or EbpA^{K978A} to incorporate into pilus HMWs on Western blots (data not shown).

EbpA's sortase recognition motif is necessary for its incorporation into pili. To determine the role of EbpA's Leu-Pro-Glu-Thr-Gly predicted sortase recognition motif in pilus assembly, we replaced the OG1RF motif (LPETG) with a scrambled motif (GTPLE) on the p-*ebpABC* expression plasmid and assessed pilus assembly by the mutant plasmid (p-*ebpA^{GTPLE}BC*), as described above. Using anti-EbpC antisera, gold bead-decorated pilus fibers were observed in immunogold EM studies of bacterial cells from cultures of EbpABC⁻ cells carrying either p-*ebpABC* or p-*ebpA^{GTPLE}BC* but not cultures of EbpABC⁻/pGCP123 cells (Fig. 6A), showing that the EbpA LPETG sortase recognition motif was dispensable for pilus fiber elaboration. Western blots of cell lysates of the EbpABC⁻ strain carrying either p-*ebpABC* or p-*ebpA^{GTPLE}BC* developed with anti-EbpC or anti-EbpB sera revealed pilus HMWs (Fig. 6B and C, top blots), indicating no defect in EbpC polymerization or EbpB incorporation. However, when blots were developed with anti-EbpA sera, HMWs were observed only from cell lysates of the EbpABC⁻/p-*ebpABC* strain but not from those of the EbpABC⁻/p-*ebpA^{GTPLE}BC* strain (Fig. 6D, top blot), demonstrating that EbpA was not incorporated into pilus fibers in the latter strain. Similar EbpA low-molecular weight ladder (LMW) species were visible in cell lysates of both strains (Fig. 6D, open arrowheads), showing that EbpA expression was unaffected by the scrambled motif. Thus, in contrast to EbpB, EbpA incorporation into pilus fibers relies on its sortase recognition motif, consistent with its predicted localization to the pilus tip. We further confirmed that EbpA is located on the tip by deep-etch immunogold EM (Fig. 6E). If EbpA acted as a nucleophile in any sortase transpeptidation reaction, we would have expected to detect EbpA still incorporated into pilus HMWs in the EbpABC⁻/p-*ebpA^{GTPLE}BC* strain via this mechanism, just as we detected EbpB in pili of the EbpABC⁻ SrtC⁻/p-*ebpAB^{NTPLK}CsrtC* strain. Although EbpA contains Lys residues apart from Lys 978, the complete dissociation of EbpA with a scrambled sortase recognition motif from pili strongly suggests that none of these residues participates in sortase transpeptidation in *E. faecalis*.

EbpA protein is dispensable for normal pilus assembly. We previously showed that pili from the *ebpA* deletion mutant EbpA⁻ and EbpAB⁻ strains exhibited a perturbed morphology that manifested as compressed EbpC bands on SDS-PAGE and longer EbpC fibers visible in EM studies (10). We hypothesized that this altered pilus morphology resulted from *cis* effects of the *ebpA* deletion allele since expression of EbpA in *trans* did not restore normal pilus expression (10). Consistent with this hypoth-

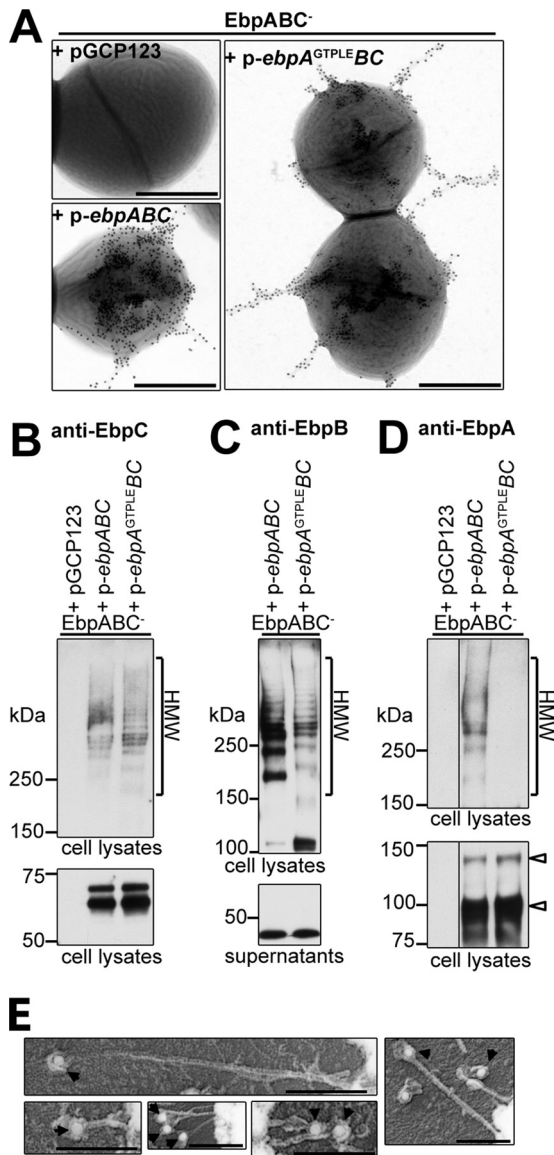


FIG 6 EbpA's sortase recognition motif is necessary for EbpA incorporation into pilus fibers and for EbpA's localization at the fiber. (A) Negative-stain immunogold EM studies were carried out with anti-EbpC sera to visualize pilus fibers expressed by the *EbpABC*⁻ strain carrying either pGCP123 (vector control), p-*ebpABC*, or p-*ebpA*^{GTPLE}BC. Scale bars are 500 nm. Western blots (B to D) of the *EbpABC*⁻ strain carrying either pGCP123 (vector control), p-*ebpABC*, or p-*ebpA*^{GTPLE}BC were performed after SDS-PAGE of the indicated cell fractions with (B) anti-EbpC, (C) anti-EbpB, and (D) anti-EbpA immune sera. The top blots show pilus HMWs (brackets), while the bottom blots show pilin monomers (arrowheads, EbpA^{GTPLE}). (E) Deep-etch immunogold EM was performed with anti-EbpA sera to determinate the localization of EbpA in the pilus fiber. Solid arrowheads indicate gold beads. Scale bars are 100 nm.

esis, our results above showed that pili lacking the EbpA protein in the *EbpABC*⁻/p-*ebpA*^{GTPLE}BC strain exhibited the normal morphology. To confirm this, we created an expression plasmid with only the EbpB and EbpC structural subunit coding regions (p-*ebpBCsrtC*) and examined pilus assembly as described above. Using anti-EbpC antisera, bacterial cells with gold bead-decorated pilus fibers were observed in immunogold EM studies of the

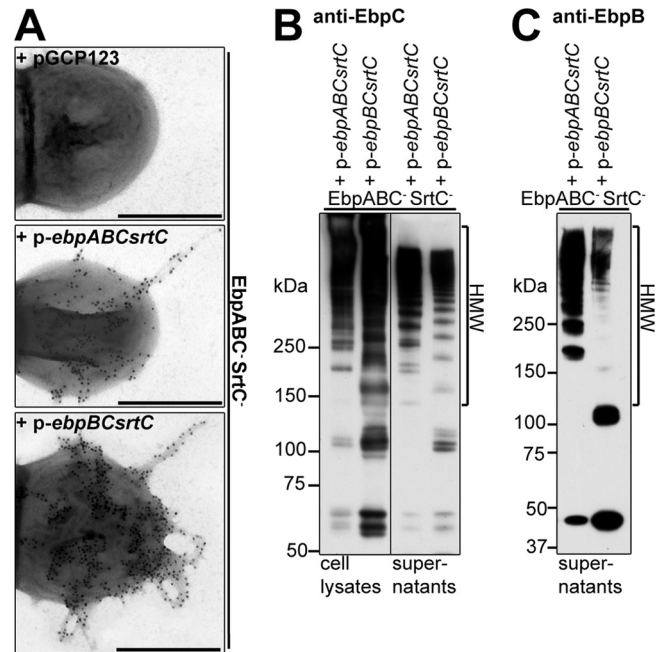


FIG 7 EbpA is not necessary for elaboration of EbpB⁻ and EbpC⁻-containing pili with normal morphology. (A) Negative-stain immunogold EM studies were carried out with anti-EbpC sera to visualize pilus fibers expressed by the *EbpABC*⁻ SrtC⁻ strain carrying either p-*ebpABCsrtC* or p-*ebpBCsrtC*. Scale bars are 500 nm. Western blots (B and C) of the *EbpABC*⁻ SrtC⁻ strain carrying either p-*ebpABCsrtC* or p-*ebpBCsrtC* were performed after SDS-PAGE of the indicated cell fractions with (B) anti-EbpC or (C) anti-EbpB immune sera. Pilus HMWs are indicated with brackets.

EbpABC⁻SrtC⁻ strain carrying either p-*ebpABCsrtC* or p-*ebpBCsrtC* (Fig. 7A). Furthermore, Western blots of cell lysates and culture supernatants of the *EbpABC*⁻ SrtC⁻ strain carrying either p-*ebpABCsrtC* or p-*ebpBCsrtC* developed with both anti-EbpC (Fig. 7B) and anti-EbpB (Fig. 7C) sera revealed pilus HMW in both strains. A prominent ~100-kDa band that reacted with both anti-EbpC and anti-EbpB sera was visible in the *EbpABC*⁻ SrtC⁻/p-*ebpBCsrtC* strain, possibly representing an EbpC-EbpB heterodimer. This and other differences in banding patterns between the two strains at low molecular masses are consistent with the loss of EbpA. Thus, the EbpA protein was dispensable for biogenesis of pili containing only EbpB and EbpC.

DISCUSSION

Sortase-assembled pili have been implicated as virulence factors in models of infections caused by many Gram-positive pathogens, including GAS (46, 47), GBS (48–50), and *S. pneumoniae* (51). Significant advances in the understanding of sortase-assembled pilus biogenesis have accompanied functional studies in these organisms and in the prototypical *C. diphtheriae* pilus systems. Here, we extend the understanding of pilus assembly to the nosocomial pathogen *E. faecalis* by investigating the roles of pilin-like motifs, minor pilin sortase recognition motifs, and the housekeeping sortase in biogenesis of the Ebp pilus, an important virulence factor in animal models of enterococcal infective endocarditis and ascending and catheter-associated UTIs (7, 9, 10).

Taken together, our results lead to a hypothetical model of Ebp pilus assembly in *E. faecalis* similar to that proposed for the biogenesis of SpaABC pili of *C. diphtheriae*. (i) Pilus assembly is ini-

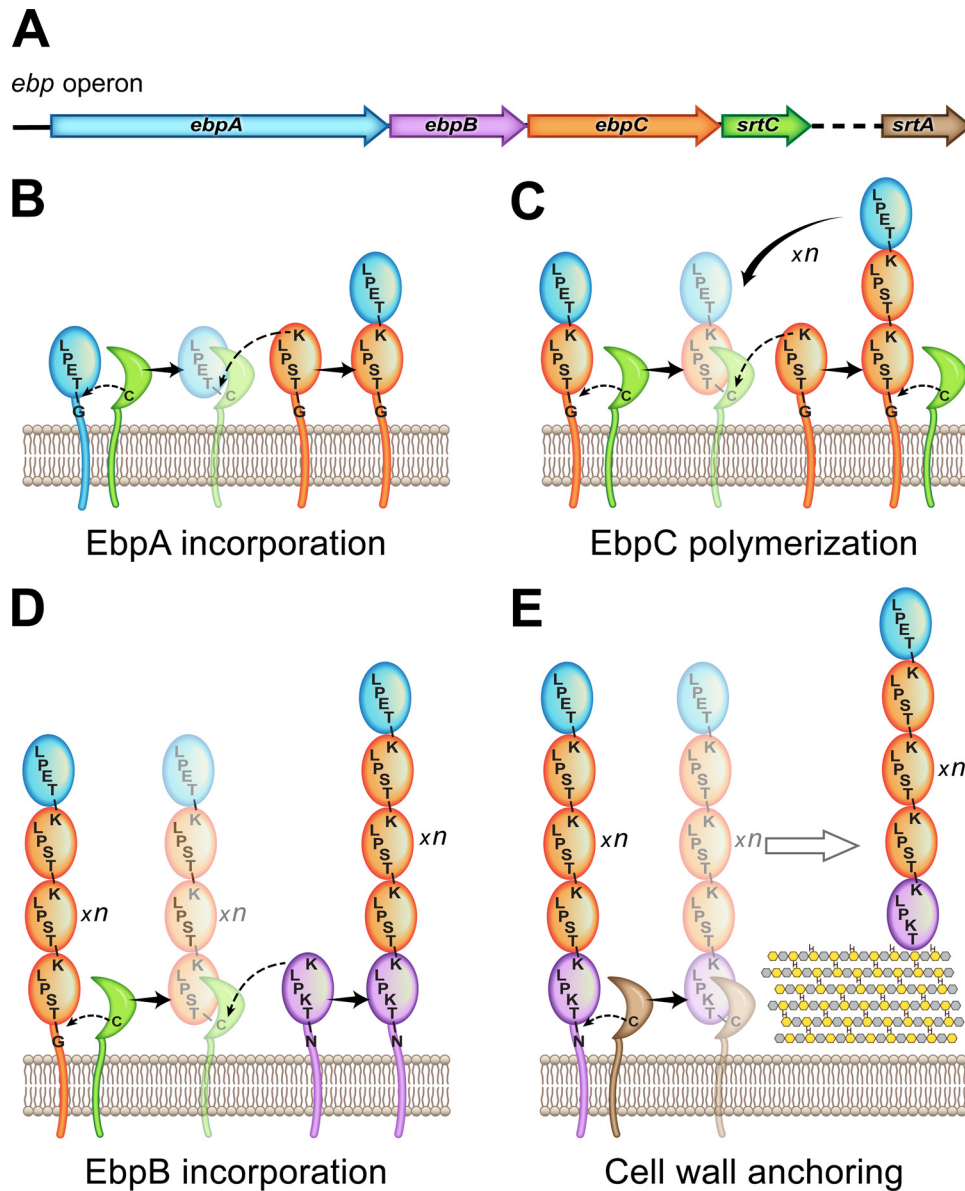


FIG 8 The *ebp* operon in *E. faecalis* OG1RF encodes the Ebp pilus structural subunits EbpA (blue), EbpB (purple), and EbpC (orange) and the pilus-associated sortase SrtC (green). The housekeeping sortase SrtA (brown) is encoded elsewhere in the genome (A). Sortase recognition motif amino acid sequences (LPETG for EbpA, LPKTN for EbpB, and LPSTG for EbpC), pilin motif Lys residues (K), and the sortase catalytic Cys residues (C) are shown by the one-letter code on their respective subunits. Predicted Srt subunit thioacyl intermediates are faded (A to E). EbpA incorporation relies on cleavage of its sortase recognition motif by SrtC and resolution of the resultant intermediate by the EbpC pilin motif Lys residue (B). EbpC polymerization requires SrtC transpeptidation between the sortase recognition motif of the most recently incorporated EbpC subunit of a growing fiber and the pilin motif Lys residue of an incoming EbpC subunit (C). EbpB is incorporated into a growing fiber via its pilin motif Lys residue (D). SrtA participates in anchoring fully polymerized pilus fibers to the cell wall, likely via transpeptidation of the EbpB sortase recognition motif with a cell wall precursor molecule.

tiated by SrtC cleavage of the EbpA LPETG sortase recognition motif. The pilin-like motif Lys 186 of EbpC acts as nucleophile to resolve the resultant EbpA-SrtC thioacyl intermediate, leading to incorporation of EbpA at the tips of pili (Fig. 8B). (ii) Pilus polymerization occurs as growing pilus fibers activated by SrtC cleavage of the most recently added subunit (EbpA-EbpC_n-SrtC thioacyl intermediates) are also resolved upon nucleophilic attack by EbpC Lys 186 (Fig. 8C). (iii) EbpB incorporation is achieved when a growing fiber activated by SrtC cleavage is instead resolved by the EbpB pilin-like motif Lys 179. Fully assembled pili compris-

ing all subunits are thus tethered to the bacterial cell membrane via the hydrophobic domain of the EbpB CWSS (Fig. 8D). SrtC processing of EbpB would permit continued fiber polymerization. Alternatively, these fibers may be activated by SrtA cleavage of the EbpB LPKTN sortase recognition motif. (iv) Sorting of pili to the cell wall then likely occurs when SrtA-activated pilus fibers (EbpA-EbpC_n-EbpB-SrtA thioacyl intermediates) are resolved by the lipid II cell wall precursor (Fig. 8E).

Evidence for the model includes our data showing that mutation of the EbpC pilin-like motif Lys 186 abrogates EbpC

polymerization, consistent with results from *C. diphtheriae*, *Bacillus*, and other sortase-assembled pilus systems. Strong evidence for the role of the major subunit pilin motif as an acceptor nucleophile for the pilus-associated sortase(s) comes from mass spectrometry studies by Budzik et al. in *Bacillus* that isolated and identified nonconsecutive peptides encompassing intermolecular isopeptide bonds (i) between two major pilins (17) and (ii) between a minor tip pilin and a major pilin (52).

Analysis of the EbpABC⁻/p-ebpABC^{K186A} strain expressing the nonpolymerizing EbpC^{K186A} protein led to insights into pilus assembly beyond identification of the SrtC acceptor nucleophile. Our results suggested that SrtC cleaved both the EbpA and EbpC LPXTG-like motifs in this strain. Although mutation of the EbpC Lys 186 prevented SrtC's acceptance of EbpC as a nucleophile, we expected to observe evidence of SrtC use of EbpB as a nucleophile, namely, EbpA-EbpB and EbpC-EbpB heterodimerization. We previously demonstrated that a putative SrtC-dependent EbpA-EbpB heterodimer forms in the absence of EbpC (10), and here we observed a probable EbpC-EbpB heterodimer formed in the absence of EbpA. However, no pilin heterodimers were observed in the EbpABC⁻/p-ebpABC^{K186A} strain, suggesting that mutation of the EbpC pilin-like motif somehow prevented nucleophilic attack by the EbpB pilin-like motif Lys 179. We hypothesize that local disruption of SrtC activity was caused by an accumulation of mutant EbpC^{K186A} protein. Indeed, we have previously shown that SrtC and thus pilus assembly occurs in distinct membrane domains on the bacterial cell and that EbpA and EbpC focally accumulate in the membrane in the absence of SrtC (40). We suspect that EbpC^{K186A} also accumulates within these membrane domains since pilus polymerization, and thus appropriate sorting of EbpC^{K186A}, was prevented. Here, EbpC^{K186A} may titrate SrtC enzymatic activity or sterically hinder access of SrtC to other pilins.

Our mutational analyses of the minor subunit pilin-like and LPXTG-like motifs also support the proposed model of Ebp pilus assembly. The EbpA LPETG motif was necessary for its incorporation into pili, while its pilin-like motif Lys 978 was dispensable, consistent with the predicted molecular requirements for a tip pilin. Additionally, deep-etch immunogold EM revealed that EbpA is indeed localized at the tip, consistent with our mutational analysis. Localization of a dedicated functional subunit to the cell-distal end of a pilus fiber is a common characteristic of bacterial pili, including the type 1 pili necessary for uropathogenic *E. coli* pathogenesis in UTI (53). We recently showed that a metal ion-dependent adhesion site (MIDAS) motif encoded within the predicted von Willebrand factor A (VWA) domain of EbpA is necessary for Ebp pilus function in a mouse model of enterococcal CAUTI.

Conversely, the EbpB LPKTN motif was dispensable for its incorporation into pili, while its pilin-like motif Lys 179 was necessary, consistent with the molecular requirements for a base pilin. Similarly, incorporation of the SpaB base pilin of *C. diphtheriae* depended on a conserved Lys residue, although not encoded within canonical pilin motif, but not the SpaB sortase recognition motif (14). We additionally demonstrated that EbpB, but not EbpA or EbpC, was processed by SrtA in the absence of SrtC, providing further evidence for terminal incorporation of EbpB since it is thought that cell wall anchoring of pili must occur via the cell-proximal pilus subunit. It is possible, however, that EbpB may also be cleaved by SrtC and thus incorporated within the pilus shaft in addition to its localization to the base. Recently, Linke et

al. crystallized the putative FctB anchor pilin from the GAS 90/306S strain and proposed that an enrichment of Pro residues just N terminal to the CWSS provides important rigidity for the cell wall anchoring pilus subunit (54). EbpB has no such clustering of Pro residues, suggesting this structural component of the base pilin is not universal among sortase-assembled pili.

The role of the housekeeping sortase in pilus biogenesis seems to vary among sortase-assembled pilus systems. In *S. pneumoniae*, deletion of the housekeeping sortase alone does not affect pilus fiber sorting to the cell wall, as determined by Western blots of cell wall fractions (55, 56), suggesting that pilus-associated sortases anchor fibers to the cell wall in the absence of the housekeeping sortase. In contrast, in *C. diphtheriae*, deletion of the housekeeping sortase (SrtF) leads to missorting of pilus fibers to the culture medium instead of the cell wall (44). In *E. faecalis*, we did not detect changes in the quantity of pili in cell wall or culture medium fractions in the absence of SrtA. Instead, we observed an accumulation of pili in the bacterial cell membrane, the site of pilus assembly, likely representing an intermediate stage of pilus biogenesis trapped in the absence of SrtA. Furthermore, we provide evidence that SrtA processes EbpB, which may represent the mechanism of pilus anchoring to the cell wall. Paradoxically, in our hands, pilus HMWs were detected by Western blotting in cell wall fractions of the SrtA⁻ strain, possibly due to the copurification of membrane-tethered pili with cell wall materials for unknown reasons. However, we cannot rule out the possibility that SrtC or some other factor anchors pili to the cell wall in the absence of SrtA, as seen for *S. pneumoniae* pili encoded by the *rlrA* island.

Interestingly, while in *C. diphtheriae* the phenotypes of mutants lacking SrtF and its substrate anchor pilin SpaB are similar, the phenotypes of the *E. faecalis* OG1RF SrtA⁻ and EbpB⁻ strains are discordant. In contrast to the SrtA⁻ strain, no accumulation of pili in bacterial cell membranes was observed in the EbpB⁻ strain (data not shown). It is possible that pili in the EbpB⁻ strain may be anchored to the cell wall by processing of EbpC by SrtA or SrtC. In that case, the signal for termination of pilus polymerization by SrtC is unclear. Alternatively, membrane-accumulated pili may simply be undetectable in the EbpB⁻ strain due to the reduction in piliation previously reported for this strain (10). In contrast, we show here that expression of pili in the SrtA⁻ strain was increased.

Detailed study of sortase-assembled pilus biogenesis began with pioneering work with the *C. diphtheriae* and *A. naeslundii* pilus islands and has now continued in the GAS, GBS, and *S. pneumoniae* pilus systems (6). Together, these studies show that some aspects of pilus biogenesis, such as a dedicated subunit and sortase(s) for fiber polymerization, are universal, while others, such as the importance of the housekeeping sortase in cell wall-anchoring, may vary among systems. In the rational development of small molecule therapeutics for Gram-positive infections that target sortase activity and sortase-assembled pilus assembly, it will be critical to understand how sortase activity and pilus biogenesis differ among bacterial species. We present here the first detailed study of the molecular determinants of pilus biogenesis in *E. faecalis*, an increasingly important human pathogen.

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