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A conserved signal-peptidase antagonist modulates membrane homeostasis of actinobacterial sortase critical for surface morphogenesis

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Most Actinobacteria encode a small transmembrane protein, whose gene lies immediately downstream of the housekeeping sortase coding for a transpeptidase that anchors many extracellular proteins to the Gram-positive bacterial cell wall. Here, we uncover the hitherto unknown function of this class of conserved proteins, which we name SafA, as a topological modulator of sortase in the oral Actinobacterium *Actinomyces oris*. Genetic deletion of *safA* induces cleavage and excretion of the otherwise predominantly membrane-bound SrtA in wild-type cells. Strikingly, the *safA* mutant, although viable, exhibits severe abnormalities in cell morphology, pilus assembly, surface protein localization, and polymicrobial interactions—the phenotypes that are mirrored by *srtA* depletion. The pleiotropic defect of the *safA* mutant is rescued by ectopic expression of *safA* from not only *A. oris*, but also *Corynebacterium diphtheriae* or *Corynebacterium matruchotii*. Importantly, the SrtA N terminus harbors a tripartite-domain feature typical of a bacterial signal peptide, including a cleavage motif AXA, mutations in which prevent SrtA cleavage mediated by the signal peptidase LepB2. Bacterial two-hybrid analysis demonstrates that SafA and SrtA directly interact. This interaction involves a conserved motif FPW within the exoplasmic face of SafA, since mutations of this motif abrogate SafA-SrtA interaction and induce SrtA cleavage and excretion as observed in the *safA* mutant. Evidently, SafA is a membrane-imbedded antagonist of signal peptidase that safeguards and maintains membrane homeostasis of the housekeeping sortase SrtA, a central player of cell surface assembly.

actinobacteria | pilus assembly | signal peptidase | sortase | antagonism

Significance

Cell wall anchoring of surface proteins in Gram-positive bacteria requires a sortase enzyme. Here, we unveiled the hitherto unknown function of an evolutionarily conserved small transmembrane protein, named SafA, genetically linked to the housekeeping sortase in Actinobacteria. We show that *Actinomyces oris* SafA interacts with the housekeeping sortase SrtA via the conserved FPW motif and prevents SrtA cleavage by the signal peptidase LepB2, hence maintaining membrane homeostasis of SrtA. This function is conserved as ectopic expression of SafA from *Corynebacterium diphtheriae* and *Corynebacterium matruchotii* in the *A. oris safA* mutant rescues its defects in cell morphology, pilus assembly, surface protein localization, and polymicrobial interactions. Thus, SafA represents an archetypal antagonist of signal peptidase that modulates surface assembly in Actinobacteria.

Most Gram-positive bacteria, with a notable exception of the *Mycobacterium* species, encode a housekeeping transpeptidase enzyme called sortase that catalyzes cell wall anchoring of surface proteins and pili (1–3). First discovered in *Staphylococcus aureus* with the prototype SrtA (4), the large sortase family members are divided into six classes (i.e., SrtA–SrtF) based on protein sequence homology and substrate preference (1, 5, 6). Sortases of class A and class E are considered housekeeping sortase enzymes that perform cell wall anchoring of surface proteins, whereas class C sortases are "polymerases" that covalently link pilin substrates into pilus polymers of various size, which are then anchored to bacterial peptidoglycan by the housekeeping sortase (1, 7–9). While sortases are critically important for bacterial virulence, their genes are dispensable for cell viability and fitness, with the exception of the housekeeping sortase of *Actinomyces oris* (8, 10–13).

A. oris, an oral colonizer that interacts with a wide range of microbes and plays an important role in oral biofilm development (14), expresses a housekeeping class E sortase, SrtA, and two class C sortases, SrtC1 and SrtC2 (2). SrtC1 and SrtC2 are specifically required for assembling the type 1 and type 2 heterodimeric fimbriae (or pili), respectively (15, 16), with the latter essential for polymicrobial interactions (or coaggregation) and biofilm formation (16–18). Coaggregation involves the adhesin CafA located at the tip of type 2 fimbriae (18), and biofilm formation requires FimA making up the type 2 fimbrial shaft; thus, a mutant strain lacking *fimA* is defective in biofilm formation (16, 17) and coaggregation (16). The housekeeping sortase SrtA is mainly responsible for covalent attachment of both fimbrial types to the cell wall (19), as well as many surface proteins such as AcaC (or GspA) and AcaB (18), although SrtC2 is able to mediate cell wall anchoring of fimbriae when *srtA* is genetically inactivated (20).

In contrast to many other sortases studied to date, *A. oris srtA* is an essential gene since *srtA* deletion is lethal to cells, with conditionally *srtA*-depleted cells exhibiting cell morphology and coaggregation defects and abnormal elongation of pili (19). The genetic basis of this lethality was determined by a Tn5 transposon screen in the absence of *srtA*, which generated many suppressor mutations mapped to 7 genes including *gspA*

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and *lepB2* (19, 21). *gspA* encodes a cell wall anchored glycoprotein, GspA, and in the absence of *srtA*, GspA glycopolymers are accumulated in the cytoplasmic membrane, resulting in a membrane toxicity phenomenon we referred to as lethal glyco-stress (19). LepB2 is one of two signal peptidases in *A. oris* (21). Critically, a nonpolar, in-frame deletion mutant lacking both *lepB2* and *srtA* is viable yet defective in producing cell wall anchored GspA polymers (19, 21). We hypothesized that LepB2 might be responsible for membrane processing of factors linked to GspA glycosylation (21). It is still unclear, however, why the housekeeping sortase SrtA is uniquely essential in *Actinomyces*.

The analysis of many bacterial genomes sequenced to date has enabled identification of *srtA* homologs and numerous sortase-associated factors (5, 22), among which is a previously neglected small transmembrane protein, hereafter called SafA (saf for sortase-associated factor), encoded by a gene immediately downstream of the housekeeping sortase *srtA* in *A. oris* (19). This transmembrane protein is highly conserved in Actinobacteria (see *SI Appendix*, Fig. S1*A*)—Gram-positive bacteria with high G+C content in their genomes—and it is absent from Firmicutes—Gram-positive bacteria with low G+C content in their genomes. The conserved linkage and gene arrangement consisting of a housekeeping sortase followed immediately by *safA* (see Fig. 1*A*) suggested to us that they are functionally related. Here, we employed a combination of biochemical and genetic approaches to demonstrate that indeed SafA is a signal-peptidase antagonist

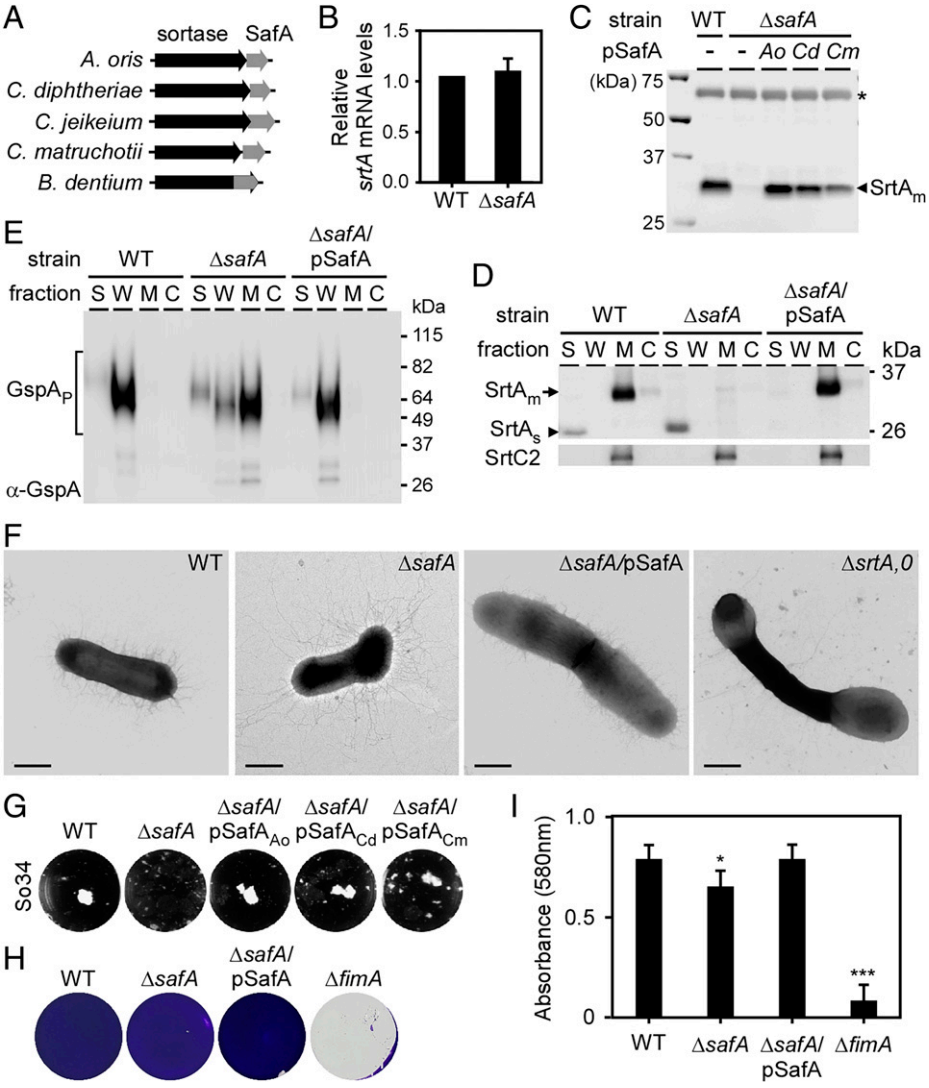

Fig. 1. A conserved membrane protein, SafA, is required for membrane localization of the housekeeping sortase SrtA, cell morphology, surface assembly, and biofilm formation. (*A*) Presented are genetic loci coding for the housekeeping sortase (black) and a conserved membrane protein, SafA (gray), found in Actinobacterial species; note that the *B. dentium* sortase harbors a SafA domain at its C terminus. (*B*) Relative expression of *srtA* in the Δ*safA* mutant, as compared to the parent strain, was determined by qRT-PCR. Results are presented as average of three independent experiments with error bars representing SD. 16S rRNA was used as reference. (*C*) Cells of the *A. oris* parent strain (WT), Δ*safA* mutant, and Δ*safA* mutant harboring a plasmid expressing *safA* from *A. oris* (*Ao*), *C. diphtheriae* (*Cd*), or *C. matruchotii* (*Cm*) were grown to midlog phase and normalized prior to isolation of membrane fractions. Membrane protein samples were analyzed by immunoblotting with antisera raised against SrtA (α-SrtA). Shown are molecular mass markers (kDa) and a nonspecific band (asterisk) serving as loading control. (*D*, *E*) Equivalent cells of indicated strains grown to midlog phase were subjected to cell fractionation. Protein samples collected from culture supernatant (S) cell wall (W), membrane (M) and cytoplasmic (C) fractions were immunoblotted with antisera raised against SrtA, SrtC2 (*D*), or GspA (*E*), with SrtC2 used as membrane control. Membrane-bound SrtA ($SrtA_m$), secreted SrtA ($SrtA_s$), and GspA polymers (P) are indicated. (*F*) Midlog phase cells of indicated strains and a conditional *srtA* deletion mutant (Δ*srtA*, 0) were immobilized on carbon-coated nickel grids and stained with 1% uranyl acetate prior to viewing with an electron microscope. (Scale bar: 0.5 μm.) (*G*) Equal cell numbers of indicated *A. oris* strains and *S. oralis* So34 were mixed in coaggregation buffer (20 mM Tris, 150 mM NaCl, 100 mM $CaCl_2$) prior to imaging. (*H*, *I*) Indicated *A. oris* strains were analyzed for their ability to form monospecies biofilms, which were stained by crystal violet and quantified by measuring absorbance at 580 nm. Results in (*I*) are average of three independent experiments performed in triplicate. Statistical significance was determined by *t* test using GraphPad Prism; $*P < 0.05$; $***P < 0.001$.

that interacts with SrtA and prevents SrtA cleavage by the signal peptidase LepB2, hence maintaining membrane homeostasis of the housekeeping sortase. Remarkably, SafA homologs are not only highly conserved, they are functionally interchangeable, leading us to propose that the mechanism of signal-peptidase antagonism by SafA is conserved in Actinobacteria. Thus, our study presents a paradigm for future investigations in other bacteria of this phylum, many of which are human commensals and pathogens.

Results

An Evolutionarily Conserved Membrane Protein Is Required for Membrane Localization of the Housekeeping Sortase SrtA in *A. oris*. To date, no *trans*-acting factors directly affecting sortase-catalyzed surface assembly have been identified. Considering that functionally related genes tend to cluster together within bacterial genomes, we began to probe the function of *safA*, coding for a small transmembrane protein of 52 amino acids, located immediately downstream of the gene for the housekeeping sortase SrtA (Fig. 1*A*). As stated above, the *srtA-safA* locus appears to be a common feature in Actinobacteria, as *safA* homologs are found in close proximity with the housekeeping sortase gene in many Actinobacterial species including *C. diphtheriae*, *Corynebacterium jeikeium*, and *Corynebacterium matruchotii* (Fig. 1*A* and *SI Appendix*, Fig. S1*A*). Interestingly, in *Bifidobacterium dentium*, a SafA-like domain is fused to the C terminus of the housekeeping sortase (Fig. 1*A* and *SI Appendix*, Fig. S1*B*), further supporting the idea of coevolution and functional relationship between SrtA and SafA.

To elucidate the function of SafA in *A. oris*, we first generated a nonpolar, in-frame deletion mutant of *safA*, using a previously described method of plasmid mediated allelic exchange we developed for *A. oris* (23). To examine whether deletion of *safA* affects *srtA* expression, we isolated mRNA from the parent (WT) and *safA* deletion mutant (Δ*safA*) strains and determined the *srtA* expression level by quantitative reverse transcription PCR (qRT-PCR). As shown in Fig. 1*B*, no significant difference in the expression level of *srtA* was observed between both strains. Next, to determine the expression level of the membrane-bound SrtA protein, protein samples isolated from the membrane of *A. oris* strains were analyzed by immunoblotting with antibodies against SrtA (α-SrtA). Surprisingly, the SrtA level in the Δ*safA* mutant was drastically reduced compared to the WT strain, and this defect was recused by a plasmid expressing *safA* from *A. oris* (Ao) (Fig. 1*C*, second, third, and fourth lanes). Remarkably, ectopic expression of *safA* from *C. diphtheriae* (Cd), or *C. matruchotii* (Cm) in the Δ*safA* mutant also enhanced the level of membrane-bound SrtA ($SrtA_m$) (Fig. 1*C*, last 2 lanes), demonstrating the functional conservation of SafA in Actinobacteria.

To investigate how the absence of SafA resulted in diminished membrane expression of SrtA without any change in *srtA* mRNA levels (Fig. 1*B*), we determined SrtA levels in subcellular compartments as well as the culture medium, using a previously described cell fractionation method (21). Proteins samples from equivalent amounts of the culture supernatant (S), cell wall (W), membrane (M), and cytoplasmic (C) fractions were analyzed by immunoblotting with polyclonal antibodies against SrtA (residues 52–253) (α-SrtA) (24). SrtA was detected mostly in the M fraction from the WT cells ($SrtA_m$), with a minor amount of a small fragment detected in the extracellular milieu (Fig. 1*D*, WT lanes). Intriguingly, the small SrtA species ($SrtA_s$) was the predominant form found in the supernatant of the Δ*safA* mutant, with only a miniscule amount of SrtA detected in the membrane fraction (Fig. 1*D*, Δ*safA* lanes). Ectopic expression of *A. oris safA* not only restored SrtA membrane localization but also prevented accumulation of $SrtA_s$ in the culture medium (Fig. 1*D*, last 4 lanes). The results suggest that SafA might block proteolytic cleavage and excretion of SrtA, thereby promoting the stable retention of SrtA on the cytoplasmic membrane.

Membrane-anchored SrtA normally catalyzes the anchoring of surface proteins on the cell wall. To determine if deletion of *safA* and the consequential mislocalization of SrtA results in altered cell wall anchoring of SrtA substrates, we extended our cellular fractionation experiment and immunoblotting to probe for the abundance and location of GspA—a highly expressed glycoprotein anchored to the cell wall by SrtA (19). In both wild-type (WT) and *safA* complementing strains, GspA was found in the cell wall fraction exclusively; by striking contrast, GspA was largely accumulated in the membrane compartment in the *safA* mutant with minor amounts also detected in the cell wall and the culture supernatant (Fig. 1*E*). This cell wall anchoring defect is similar to the phenotype we previously described with the genetic disruption or diminished expression of *srtA* (19). Since inactivation of *srtA* causes gross abnormalities in pilus assembly and cell morphology (stumpy and bent cells) (19), we sought to determine if *safA* deletion would produce similar phenotypes. Here, intact cells of various strains were analyzed by electron microscopy as previously reported (20). The results revealed that indeed unlike the WT strain, the *safA* mutant displayed an altered cell morphology and production of exceedingly long pili anchored to the cell wall by pilus-specific sortase SrtC2 (20)—both phenotypes similar to that of *srtA* disruption (Fig. 1*F*) that was due to toxic membrane accumulation of glycosylated GspA (19). Consistent with this, a mutant strain lacking both *safA* and *gspA* displayed the same phenotypes of cell morphology and pilus assembly as the WT strain (*SI Appendix*, Fig. S2). Importantly, ectopic expression of *safA* from *A. oris*, *C. diphtheriae*, and *C. matruchotii* successfully rescued the defects of the *safA* mutant (Fig. 1*F* and *SI Appendix*, Fig. S3).

As previously mentioned, the type 2 pili of *A. oris* are essential for mediating polymicrobial interactions or coaggregation in the oral cavity (2, 18). Because the long pili previously observed in the *srtA* mutant of *A. oris* are associated with a defective coaggregation phenotype (20), we subjected the *safA* mutant to a coaggregation assay as previously reported (18), whereby WT *A. oris* and *Streptococcus oralis* interact and form visible clumps of bacteria. As shown in Fig. 1*G*, the *safA* mutant was defective in forming aggregates with *S. oralis* (So34) compared to the WT. In further support of our hypothesis that SafA is both functionally and evolutionarily conserved, ectopic expression of *safA* from *A. oris*, *C. diphtheriae*, or *Corynebacterium matruchotii* rescued the coaggregation defect of the *safA* mutant (Fig. 1*G* and *SI Appendix*, Fig. S3). Since biofilm formation is mediated by the type 2 shaft FimA (16), and the *safA* deletion mutant still forms type 2 pili, albeit at a longer length than wild type, we sought to determine if loss of *safA* alters the ability of *A. oris* to form monospecies biofilm in vitro. The results show that relative to the WT strain, the *safA* mutant displayed a slight, albeit statistically significant decrease in its ability to form biofilms in vitro (Fig. 1 *H* and *I*); note that the observed defect of the *safA* mutant was not as drastic as what is observed in the *fimA* deletion mutant. Altogether, the results support that SafA is an evolutionarily conserved protein required for proper membrane localization of SrtA, hence bacterial coaggregation.

The Transmembrane SrtA Protein Contains a Noncanonical Signal Peptide Whose Cleavage Is Blocked by SafA. The results shown in Fig. 1*D* above suggest that SrtA might be subjected

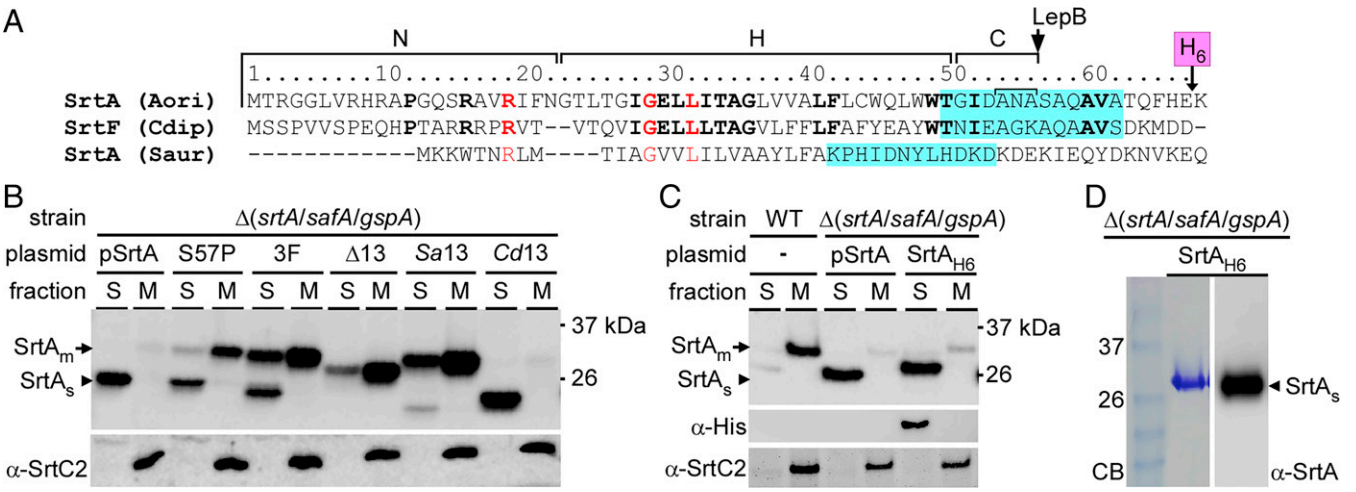

Fig. 2. The housekeeping sortase SrtA harbors a cleavable signal peptide sequence. (*A*) *A. oris* SrtA appears to contain a signal peptide sequence with a positively charged *N*-region, hydrophobic (H), and a C-region consisting of a conserved AXA motif (bracket) predicted to be cleaved by the signal peptides LepB. A recombinant SrtA protein was engineered with a 6-His tag (H_6) inserted after a Glu residue for protein purification. The housekeeping sortase of *C. diphtheriae* also contains a signal peptide sequence that is homologous to *A. oris* SrtA. (*B*) A triple mutant, Δ(*srtA-safA-gspA*), devoid of *srtA*, *safA*, and *gspA*, was transformed with a plasmid expressing wildtype SrtA (pSrtA) or its variants. Supernatant and membrane fractions of indicated strains were analyzed by immunoblotting with α-SrtA and α-SrtC2. A SrtA mutant strain with S57 mutated to P is indicated as S57P, whereas 3F indicates the AXA motif changed to FFF. Δ13 denotes a SrtA mutant, in which the 13-amino acid region, highlighted in cyan in (*A*), was deleted. *Sa*13 and *Cd*13 represent SrtA mutants that the highlighted 13-amino acid region of *A. oris* SrtA replaced by that of *S. aureus* or *C. diphtheriae*, respectively. (*C*) Similar to the experiment in B, protein samples of indicated strains were immunoblotted with specific antibodies. (*D*) Supernatants of the Δ(*srtA-safA-gspA*) mutant expressing His-tagged SrtA were subjected to affinity chromatography with nickel-Sepharose resins. Purified SrtA was analyzed by SDS-PAGE electrophoresis using Coomassie blue (CB) staining and immunoblotting with α-SrtA.

to proteolytic processing. This observation and the tangential connection between SrtA and the signal peptidase LepB2 mentioned earlier (19, 21) led us to examine whether SrtA harbors a signal sequence. Although the bioinformatics tool SignalP (https://www.cbs.dtu.dk/services/SignalP/) failed to identify a signal peptide sequence in SrtA, a close inspection of the protein sequence of SrtA revealed that in fact SrtA contains a positively charged domain (N), a hydrophobic domain (H), and a neutral polar domain (C) with a possible cleavage site between A^{56} and S^{57} after the cleavage motif AXA (Fig. 2*A*). All of these features are typical of a bacterial signal peptide sequence (25). We also found similar domains in the N-terminal sequence of the housekeeping sortase SrtF in *C. diphtheriae*, but not in *S. aureus* SrtA (Fig. 2*A*), which reportedly does not harbor a signal peptide (26).

To determine that *A. oris* SrtA contains a bona fide signal peptide, we generated various mutants within its predicted signal peptide and ectopically expressed these mutants in a mutant strain lacking both *srtA* and *safA* in the background of a genetic suppression Δ*gspA* that confers cell viability in the absence of *srtA* (19). This triple mutant Δ(*srtA*/*safA*/*gspA*) expressing ectopic SrtA mimicked the phenotype of Δ*safA* in that the small fragment of SrtA was released into the supernatant and the membrane-bound SrtA was only weakly detected in the membrane fraction by immunoblotting analysis (Fig. 2*B*, first 2 lanes). Since proline substitution of the residue in the +1 position relative to the cleavage site is known to inhibit the cleavage of substrate proteins by signal peptidases (27, 28), we generated a similar mutant, substituting S57 by P (S57P). Indeed, the S57P mutation greatly enhanced the membrane localization of matured SrtA in the absence of SafA (Fig. 2*B*, lanes S57P). Replacement of the potential cleavage site AXA motif with FFF residues also enhanced membrane retention (Fig. 2*B*, lanes 3F); it is noteworthy that in each case, a fraction of unprocessed SrtA was recovered from the culture supernatant implying the mutations might somehow perturb the membrane retention. Strikingly, deletion of a 13-amino acid region encompassing the AXA motif (Fig. 2*A*, highlighted in light blue) completely prevented SrtA cleavage and enhanced membrane localization of SrtA in the absence of SafA (Fig. 2*B*, lanes Δ13). A similar phenotype was observed when this region was replaced by 13 amino acids from *S. aureus* (Fig. 2*B*, lanes *Sa*13). Importantly, when the 13-amino acid region was replaced by a homologous region from the *C. diphtheriae* housekeeping sortase, this SrtA mutant was cleaved and released into the supernatant (Fig. 2*B*, lanes *Cd*13). This establishes that the proteolytic processing of the housekeeping sortase and its inhibition by SafA is a conserved phenomenon in Actinobacterial envelope morphogenesis.

Next, to map out the SrtA cleavage site(s), we engineered a recombinant SrtA protein with a 6-histidine tag inserted after E^{67} (Fig. 2*A*, H6), and this construct ($SrtA_{H6}$) was introduced in the same strain Δ(*srtA*/*safA*/*gspA*) (Fig. 2*C*). Compared to WT SrtA, $SrtA_{H6}$ was similarly processed (Fig. 2*C*). Using this H_6-engineered SrtA, we purified the cleaved SrtA fragment from the culture supernatant by affinity chromatography (Fig. 2*D*) and analyzed the cleaved sequence by N-terminal Edman degradation sequencing as previously described (21). The result (*SI Appendix*, Table S1) proved that the cleavage takes place between residues A^{56} and S^{57} as predicted (Fig. 2*A*).

The Signal Peptidase LepB2 in *Actinomyces oris* Cleaves SrtA's Signal Peptide. *A. oris* encodes two signal peptidases, LepB1 and LepB2, however, *lepB2* deletion suppresses the lethal phenotypes of *srtA* deletion, and LepB2 is required for pilus assembly (21). These results prompted us to determine whether SrtA is processed by the signal peptidase LepB2 or not. As shown in Fig. 3*A*, immunoblotting for SrtA in membrane and culture medium fractions demonstrate that while the Δ*lepB1* mutant did not change the membrane/culture medium distribution of SrtA as normally observed in the WT, the Δ*lepB2* mutant retained SrtA exclusively on the membrane without any SrtA cleavage or excretion into the medium. Further, in contrast to the Δ*safA* mutant, in which SrtA is largely cleaved and released into the medium (see Fig. 1*D*), the Δ*safA*/Δ*lepB2* double

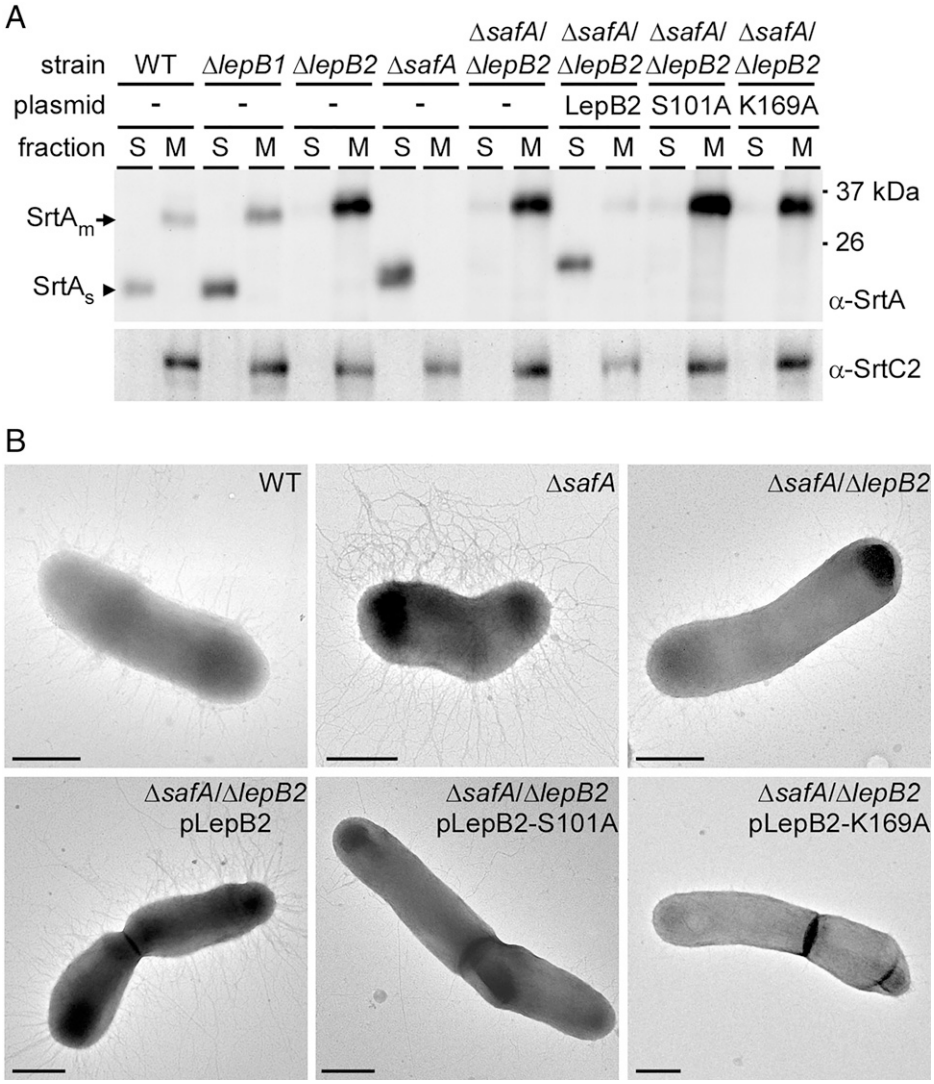

Fig. 3. SafA prevents SrtA from cleavage by the signal peptidase LepB2. (*A*) Indicated strains, including strains expressing wild type LepB2 or its catalytically inactive mutants (S101A and K169A), were analyzed by immunoblotting with α-SrtA and α-SrtC2. (*B*) Cells of indicated strains were analyzed by electron microscopy as described in Fig. 1*F*. (Scale bars: 0.5 μm).

mutant displayed mostly unprocessed SrtA on the membrane (Fig. 3*A*, lanes Δ*safA* and Δ*safA*/Δ*lepB2*). The same result was also observed in strain Δ*safA*/Δ*lepB2* expressing catalytically inactive LepB2 (i.e., S101A or K169A) (21), as opposed to the catalytically active counterpart (Fig. 3*A*, last six lanes). Clearly, LepB2 is the signal peptidase that processes and releases SrtA in the absence of SafA.

To further illuminate the impact of LepB2-mediated SrtA cleavage, we analyzed the aforementioned mutants by electron microscopy. Unlike the Δ*safA* mutant, which was stumpy and produced long pili, the Δ*safA*/Δ*lepB2* strain displayed the WT cell morphology, although it produced less pili (Fig. 3*B*). This is consistent with our previous report that establishes the role of LepB2 in pilus assembly, although deletion of *lepB2* alone does not affect cell morphology (21). Ectopic expression of LepB2 in this double mutant yielded the phenotypes of stumpy cells and long pili as observed in the Δ*safA* mutant (Fig. 3*B*). Furthermore, expression of the catalytically inactive LepB2 mutants, S101A or K169A, in Δ*safA*/Δ*lepB2* phenocopied this double mutant (Fig. 3*B*). Altogether, these results establish that SafA is necessary to prevent SrtA cleavage by the signal peptidase LepB2 so as to enable proper anchoring of surface proteins and assembly of pili.

SafA Directly Interacts with SrtA, Preventing SrtA from Cleavage by the Signal Peptidase LepB2. SafA is predicted to contain a transmembrane (TM) domain (residues 13–35), with its N terminus facing toward the cytoplasm and the C terminus toward the exoplasm (see TMHMM 2.0 Server, www.cbs.dtu.dk/services/TMHMM/) (Fig. 4*A*). To confirm this topological prediction, we generated two yellow fluorescent protein (YFP) fusion proteins with SafA, whereby YFP is attached to either the N or C terminus of SafA; a cytoplasmic YFP construct was used as control (Fig. 4*B*). Analysis of these fusion constructs demonstrated that they functionally complemented the Δ*safA* mutant and were able to restore membrane localization of SrtA (Fig. 4*C*). Next, fluorescence microscopy demonstrated that only the N-terminal YFP-SafA fusion protein was fluorescent with intensity similar to the cytoplasmic YFP control, whereas the C-terminal SafA-YFP construct displayed spotty YFP signal (*SI Appendix*, Fig. S4). Considering that the unfolded proteins are transported through the Sec translocon, we surmised that in the N-terminal YFP-SafA construct, YFP remained cytoplasmic, hence fluorescent.

To further confirm this point, we used the same set of strains in the fluorescence microscopic experiment for a proteolytic protection assay, whereby protoplasts of these strains obtained by

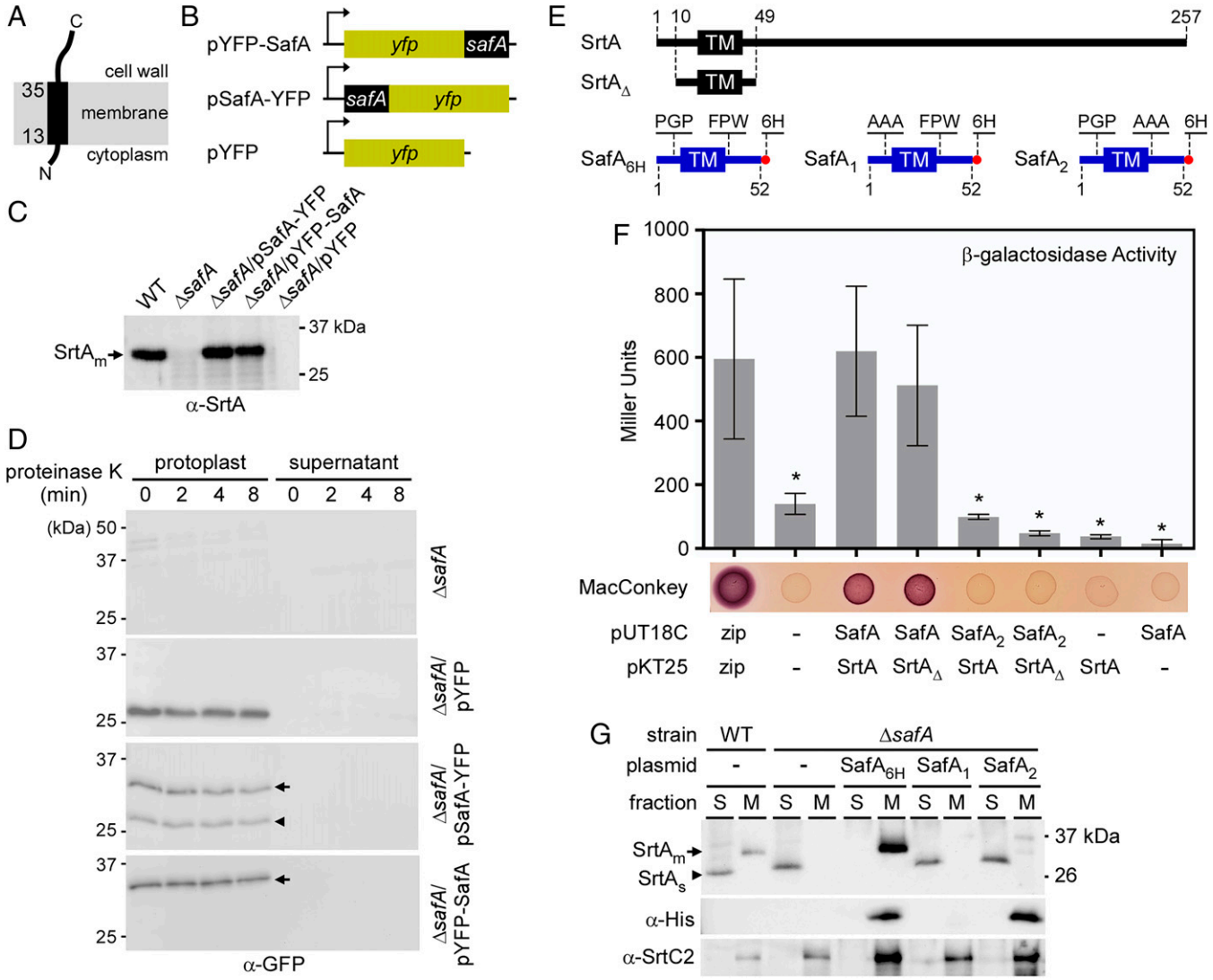

Fig. 4. Conserved residues within SafA are essential for interaction with SrtA. (*A*) Membrane topology of SafA is predicted by TMHMM (36), with the N terminus facing the cytoplasm and the C terminus toward the cell wall. (*B*) Shown are recombinant plasmids expressing yellow fluorescent proteins (YFPs) that were fused in frame to SafA at the N or C terminus. A cytoplasmic YFP was used as control. (*C*) Membrane fractions of the parent strain, Δ*safA*, or this mutant expressing various YFP constructs in (*A*) were analyzed by immunoblotting with α-SrtA. (*D*) Midlog phase cells of indicated strains were treated with cell wall hydrolase to remove peptidoglycan. Obtained protoplasts were then treated with proteinase K. At timed intervals, protein samples from protoplasts and supernatants were collected and analyzed by immunoblotting with α-GFP antibody. The cleaved and uncleaved SafA and YFP fusion proteins are marked by an arrowhead and arrows, respectively. (*E*) *Top*, a schematic diagram of full-length SrtA (257 amino acids) highlights a truncated region ($SrtA_{\Delta}$; residues 10–49) encompassing the predicted SrtA transmembrane (TM) domain. *Bottom*, shown are recombinant SafA constructs, with or without a 6-His tag (red). (*F*) Different SrtA and SafA constructs (without H6) were fused to the T18 or T25 fragment of adenylate cyclase, and the T18 and T25 construct pairs were coexpressed in *E. coli* BTH101 cells. SrtA-SafA protein interaction was determined by MacConkey agar plating or quantified by β-galactosidase activity. Constructs with leucine zipper proteins were used as positive control. (*G*) The parent strain, its isogenic Δ*safA* mutant, or this mutant expressing His-tagged SafA or His-tagged mutant SafA were analyzed by immunoblotting with specific antibodies as previously described in Fig. 3*A*.

digesting their cell wall by mutanolysin in an isotonic solution were subjected to proteinase K treatment; at timed intervals protein samples were obtained for immunoblotting with antibodies against a green fluorescent protein (GFP) that is cross-reactive with YFP. Consistent with the results in *SI Appendix*, Fig. S3 and Fig. 4*C*, the N-terminal YFP-SafA construct was protected from proteolytic cleavage, similar to that of the cytoplasmic YFP control, while the C-terminal SafA-YFP construct demonstrated exoplasmic exposure for proteolytic processing (Fig. 4*D*).

Since both SafA and SrtA are membrane localized, we hypothesized that they might interact. To examine this attractive possibility that also provides a mechanism for how SafA might protect SrtA from secretory processing, we utilized the bacterial adenylate cyclase-based two-hybrid (BACTH) assay (29, 30). We fused SrtA with the T25 subunit of adenylate cyclase from *Bortedella pertussis* and SafA with the T18 subunit; both constructs were expressed in an *E. coli* strain devoid of native adenylate cyclase. Evidence for SrtA-SafA interaction was determined by *E. coli* growth on MacConkey agar plates supplemented with maltose and further quantified by β-galactosidase activity. As shown in Fig. 4*F*, the full-length fusions of SrtA and SafA showed positive interaction, giving rise to strong signal similar to the positive control Zip proteins, whereas the construct pairs pUT18C/pKT25, lacking either SrtA or SafA, were negative, mirroring the negative control with empty vectors. Strikingly, the truncated SrtA construct ($SrtA_{\Delta}$), encompassing the SrtA TM domain (residues 10–49), was sufficient to interact with full-length SafA (Fig. 4*E* and *F*).

To probe this interaction further, we focused our attention to the conserved features of the SafA proteins from Actinobacteria. Sequence alignment analysis revealed several conserved motifs, such as PGP (residues 10–12) and FPW (residues 36–38), the latter of which is just outside of the TM domain facing the exoplasm

(*SI Appendix*, Fig. S1*B* and Fig. 4*E*). To determine if these conserved motifs are important for SafA functionality, we generated SafA mutants combined with a His-tag to monitor both SafA expression and membrane localization. The His-tagged constructs were introduced to the *A. oris* Δ*safA* mutant and analyzed by immunoblotting. Like the native SafA protein (Fig. 1), the recombinant WT His-tagged SafA was membrane embedded and enabled membrane localization of SrtA (Fig. 4*G*, lanes $SafA_{6H}$). In contrast, both SafA mutant constructs, with PGP or FPW replaced by AAA, failed to mediate SrtA membrane localization, nor protect SrtA from cleavage (Fig. 4*G*, lanes $SafA_1$ and $SafA_2$, respectively). Immunoblotting for the His-tag reveled that while the SafA mutant protein with PGP mutation ($SafA_1$) could not be detected in either membrane or medium, possibly due to protein instability, the other SafA protein with FPW mutation ($SafA_2$) was abundantly detected and membrane embedded (Fig. 4*G*, compare lanes $SafA_1$ with lanes $SafA_2$). It is important to note that $SafA_2$ was unable to interact with SrtA as determined by BACTH (Fig. 4*F*). We infer that in *A. oris*, the intramembranous SrtA and SafA interact with each other and that the exoplasmic minimotif FPW of SafA is essential for this interaction, as well as SafA's function as signal peptidase antagonist, allowing the protection of SrtA from proteolytic processing and proper membrane homeostasis that enables the physiological assembly of surface proteins on the Actinobacterial cell surface.

Discussion

Short open reading frames (ORFs) coding for small proteins in bacteria have been overlooked in traditional systematic genome annotations and comparative genomics (31). This is changing, however, with major recent advancements in computational genomic analysis tools, the available platforms, and greater opportunities for systematic experimentation technologies. Recently, small bacterial membrane proteins have emerged as key regulators that modulate many cellular processes, including transport, signal transduction, cell division, and membrane stability (32). We report here our studies of a single 52-amino acid transmembrane protein conserved in the Actinobacterium phylum that expands this emerging field. We show that this protein, SafA, modulates the membrane homeostasis of a key transpeptidase sortase enzyme in *A. oris*, SrtA, through a direct, protein-protein interaction to prevent the enzyme's proteolytic processing by a signal peptidase, and in turn facilitates the proper surface assembly of numerous bacterial adhesins that are variously involved in Actinobacterial commensalism or pathogenesis in humans and other organisms.

Our study began with the realization that a small ORF located immediately downstream of the *A. oris* housekeeping sortase SrtA is conserved in both sequence and genetic linkage with the housekeeping sortase among many Actinobacterial species (Fig. 1*A*). We readily unveiled a functional connection between the two proteins—SrtA and SafA. While *safA* deletion did not affect *srtA* expression (Fig. 1*B*), this mutation induced processing of membrane-bound SrtA, resulting in excretion of a fraction of the processed sortase (Fig. 1*C* and *D*). Concomitantly, the mutation caused the hyper-accumulation of a SrtA substrate, GspA, known to cause toxicity and lethality of *A. oris* upon *srtA* inactivation (19). The physiological impact of SafA in preventing cleavage and release of SrtA was substantiated with complementation experiments, demonstrating that the defects in SrtA localization, cell morphology, and interbacterial coaggregation could all be rescued by the ectopic expression of SafA from not only *A. oris* but also other Actinobacteria including *C. diphtheriae* (Fig. 1). This lends strong support to our inference that the phenomenon our study uncovered is an evolutionarily conserved mechanism. Although SrtA's retention on the cytoplasmic membrane was grossly diminished in SafA's absence, it was not completely abolished (Fig. 1*D*). This result is significant because of our observation that while the deletion of *srtA* is lethal for *A. oris,* the deletion of *safA* is not. Thus, only a very small amount of membrane embedded SrtA enzyme suffices to allow bacterial survival.

Although SrtA takes part in the anchoring of pilus polymers to the cell wall, the housekeeping sortase is not essential for this process because the pilus-specific sortase SrtC2, which polymerizes pilins, can also catalyze the cell wall anchoring step (20). Nevertheless, the significant loss of membrane-embedded SrtA in the Δ*safA* mutant displays a pilus morphogenesis phenotype that mimics the pilus phenotype seen in the absence of SrtA (Fig. 1). Under each of these conditions, the defect in cell wall anchoring leads to the assembly of excessively long pilus polymers, so much that it hinders bacterial coaggregation (Fig. 1*G*). Because biofilm formation requires the fimbrial shaft FimA (16), it is expected that that *safA* mutant should form monospecies biofilms and indeed this was the case (Fig. 1*H* and *I*). It is interesting to note that subtle changes in the amount of the membrane-bound SrtA form can generate a differential impact on the various attributes of this enzyme critical for actinobacterial envelope morphogenesis, cell viability and cell–cell interaction.

A logical question that emerged from this initial analysis of the phenotypes of Δ*safA* mutant and its complementation by the conserved homologs is whether the proteolytic processing of sortase follows a basic biochemical pathway involved in the normal cell envelope morphogenesis and homeostasis. Based on conventional bioinformatics, we have long held the view that *A. oris* SrtA did not possess a signal sequence, though it seemed somewhat surprising because some sortases contain an N-terminal signal peptide sequence that is physiologically processed by signal peptidases (24, 33). Our compelling evidence that SrtA is cleaved in the *safA* mutant (Fig. 1), combined with the fact that the signal peptidase LepB2 is somehow linked to the lethality of *srtA* deletion (19), led us to reanalyze the sequence of the first 65 amino acids of SrtA manually, hence unveiling a typical tripartite domain of a signal peptide in this sequence (Fig. 2*A*). A combination of mutational, biochemical, and genetic analyses subsequently established that SrtA harbors a bona fide signal sequence and revealed the actual cleavage site (Fig. 2 and *SI Appendix*, Table S1), which is processed by LepB2, one of two signal peptidases that are encoded by the organism (Fig. 3).

The critical question of how SafA protects SrtA from cleavage by LepB2 signal peptidase was next addressed by first demonstrating that SafA is an integral membrane protein with a topology that places a conserved minimotif of SafA in the exoplasmic face of the membrane (Fig. 4). Subsequently, by a combination of bacterial two-hybrid experiments, alanine-substitution mutagenesis, and epitope tagging, we demonstrated conclusively that SafA and SrtA not only interact directly, but also that the exoplasmic motif FPW of SafA is critically involved in this interaction and the associated biochemical and cellular phenotypes (Fig. 4).

Together, these results lead us to propose a model for how SafA modulates SrtA function in envelope morphogenesis (Fig. 5). According to this model, SafA and SrtA are normally colocalized and embedded within the membrane via their respective transmembrane domains. This colocalization enables SafA's FPW motif to interact with the transmembrane domain of SrtA, to mask its cleavage site or cause steric hindrance, thereby preventing SrtA cleavage by LepB2 signal peptidase (Fig. 5*A*). In the absence of SafA, or when the FPW motif is mutated, the signal peptide of SrtA is unmasked, enabling LepB2 to process SrtA (Fig. 5*B*). As

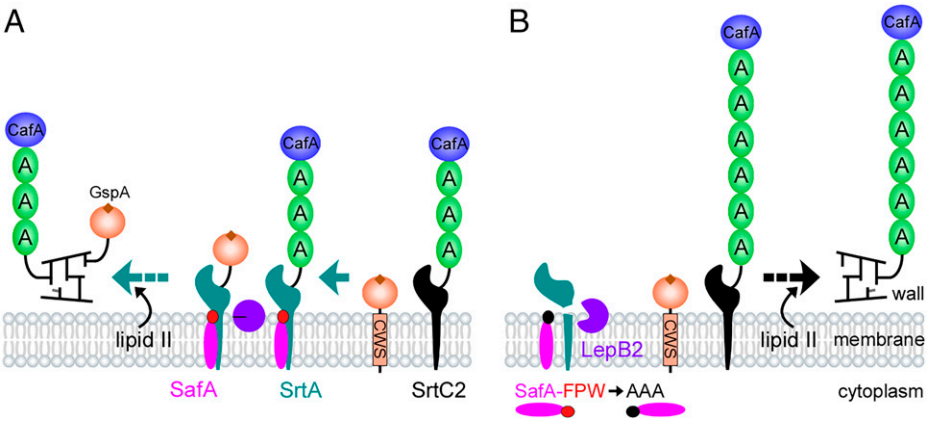

Fig. 5. A working model of SafA-mediated antagonism of signal peptidase. (*A*, *B*) See text for details.

the membrane is now depleted of SrtA, the pilus can continue to elongate until polymerization reaction switches to the cell wall anchoring step catalyzed by SrtC2 (20); furthermore, without sufficient membrane-bound SrtA, many other surface destined proteins including GspA are mislocalized (Fig. 5*B*). It is noteworthy that the molecular interaction between SafA and SrtA may be transient, or dynamic, in *A. oris* since we tried but failed to capture a SafA-SrtA complex by coimmunoprecipitation experiments, with or without the aid of crosslinking, after several attempts.

Notably, a small but appreciable fraction of SrtA is cleaved and secreted in the WT strain (Figs. 1 and 3), whereas the majority of SrtA is cleaved in the *safA* mutant (Fig. 1). This raises an intriguing question as to why some SrtA is still processed in the presence of SafA in the WT strain, and why some SrtA is retained in the membrane even in the absence of SafA in the *safA* mutant. Although it is possible that additional factor(s) might be involved in SrtA cleavage, we favor the possibility that it is the relative stoichiometry of LepB2, its substrate SrtA, and the antagonist SafA, and their distribution and colocalization on the membrane, that together dictate SrtA's membrane abundance, cleavage and excretion. As such, a small imbalance of these components may generate different outcomes. Future experiments will determine if this is the case, using a tightly controlled expression system and perhaps, an in vitro micelle system for transmembrane assembly and processing.

The fact that SafA homologs from the two Actinobacteria *C. diphtheriae* and *C. matruchotii* can rescue the *safA* mutant's defects in cell morphology, pilus assembly, and SrtA localization (Figs. 1 and 3) supports that the mechanism of SafA-mediated antagonism of signal peptidase is conserved in Actinobacteria. In this context, it is notable that in the genus *Bifidobacterium*, the class E sortases contain a SafA-like domain present as the C terminus of the sortase (*SI Appendix*, Fig. S1*B*), which further supports the idea of coevolutionary existence of class E sortases and SafA. Considering that *Bifidobacterium* is more ancient than other genera of the phylum Actinobacteria, including *Actinomyces*, *Corynebacterium*, and *Streptomyces* (34), we surmise that the SafA domain has further evolved to become a separate genetic entity. Since the presence of the antagonist in *cis* (as a linked domain of the protein) might lock the signal peptide of SrtA, the continued evolution that separated SafA from SrtA might provide organisms an opportunity for regulation of sortase via transient or stochastic inhibition of sortase cleavage by the signal peptidase. It remains to be determined whether the SafA-like domain of *Bifidobacterium* class E sortases functions similarly as *Actinomyces* and *Corynebacterium* SafA and whether or not the SafA-mediated antagonism of signal peptidase is limited to sortase. As such, the *A. oris* SafA system should serve as a prototypical antagonist of signal peptidase that would foster further investigations of this phenomenon in other important Actinobacteria. Last, whether the regulation of membrane localization of the housekeeping is novel to Actinobacteria or not remains an open question.

Materials and Methods

Bacterial Strains, Plasmids, and Media. Bacterial strains and plasmids used in this study are listed in *SI Appendix*, Table S2. *A. oris* strains were grown in heart infusion broth (HIB) or heart infusion agar (HIA) plates at 37 °C and in the presence of 5% CO_2. *S. oralis* was grown on HIA supplemented with a final concentration of 1% glucose and incubated at 37 °C in an anaerobic chamber. *E. coli* strains were grown on Luria-Bertani (LB) broth or agar in the presence or absence of 100 μg/mL ampicillin or 50 μg/mL kanamycin.

Generation of Strains and Plasmids. *A. oris* mutant strains and plasmids used in this study were constructed according to published protocols as described and listed in *SI Appendix* (16, 23).

Cellular Fractionation and Immunoblotting. Cell fractionation and immunoblotting analysis were conducted as previously described with some modification (19, 21). Briefly, 5 mL cultures of *A. oris* were grown in HI broth with shaking at 37 °C to midlog phase. Cells of different strains harvested by centrifugation were normalized to an OD_{600nm} of 1.0 and subjected to cell fractionation. Protein samples from culture supernatant (S), cell wall (W), membrane (M), and cytoplasmic (C) fractions were obtained by precipitation with 7.5% trichloroacetic acid. All samples were boiled in sodium dodecyl sulfate (SDS) containing 3 M urea prior to SDS-PAGE electrophoresis using 15% acrylamide gels and immunoblotting with antibodies against SrtA, SrtC2, or GspA (19, 24), as well as GFP (ABclonal) or poly-Histidine (Invitrogen).

Proteolytic Protection Assay. Cell wall digestion and protoplast isolation was conducted as previously described with some modification (19, 21). Briefly, 5 mL cultures of different *A. oris* strains grown to midlog phase at 37 °C were harvested by centrifugation and normalized to an OD_{600nm} of 4.0. Protoplasts were obtained by digestion with mutanolysin in SMM buffer (0.5M sucrose, 10 mM $MgCl_2$, and 10 mM maleic acid, pH 6.8). The protoplast suspension in SMM was treated with proteinase K (a final concentration of 5 μg/mL) for 2–8 min at 37 °C. Proteinase K digestion was quenched at time intervals by 0.2M PMSF, followed by centrifugation to separate supernatants from protoplasts. The treated protoplasts were subjected to repeated freeze–thaw cycles, and membrane fractions were obtained by centrifugation. Proteins samples from the membrane fractions and the supernatants were obtained by precipitation with 7.5% trichloroacetic acid. Samples were boiled in SDS sample buffer containing 3M urea prior to SDS-PAGE analysis with 15% acrylamide gels and immunoblotting with polyclonal anti-GFP (ABclonal).

Bacterial Coaggregation. Polymicrobial interactions were determined by previously published coaggregation assays (18, 20). Briefly, *A. oris* and *S. oralis* cells were grown in HIB and HIB supplemented with 1% glucose, respectively.

Bacterial cells were normalized by optical density, washed, resuspended in coaggregation buffer (20 mM Tris × HCl pH: 7.4, 150 mM NaCl, 1 mM $CaCl_2$) in a 1:1 ratio, and agitated by gentle rotational shaking. Coaggregation was recorded by a FluorChem Q (Protein Simple).

Biofilm Formation. *A. oris* biofilms were cultivated according to a previously published protocol with some modification (21). Overnight cultures of *A. oris* strains were used to inoculate fresh cultures (1:100 dilution) in HIB supplemented with 1% sucrose in 24-well plates, which were allowed to grow for 48 h at 37 °C in the presence of 5% CO_2. Biofilms were washed with phosphate buffered saline (PBS) three times prior to drying in a Savant speedvac (Thermo Scientific). Biofilms were stained with 1% crystal violet for 10 min, washed 3–5 times with water, de-stained, and dissolved in 30% acetic acid for 5 min, and quantified by measuring absorbance at 580 nm.

Bacterial Two-Hybrid. Cells of the *E. coli* adenylate cyclase deficient strain BTH101 were grown at 30 °C to midlog phase and washed three times in cold 10% glycerol to prepare for transformation. 200 ng of each plasmid construct (pUT18C and pKT25) were added to the 50 μL of electrocompetent cells. Transformations were conducted via electroporation in prechilled 1-mm gap cuvettes under the following conditions: 2.5 kV, 25 μF capacitance, 100 Ω resistance. Cells were allowed to recover for 2 h in LB at 30 °C prior to washing with sterile 0.9% saline and spreading onto MacConkey agar plates supplemented with 1% maltose, 50 μg/mL kanamycin, and 100 μg/mL ampicillin to select for cells containing both pUT18C and pKT25 plasmids.

For spot dilution and plating assays, cells of BTH101 strains containing both plasmid constructs were grown overnight in LB at 30 °C, washed twice and normalized to an OD_{600nm} of 0.1 in 0.9% saline. Aliquots (4 uL) of each cell suspension was spotted onto MacConkey agar plates supplemented with 1% wt/vol maltose, 50 μg/mL kanamycin, and 100 μg/mL ampicillin and incubated at 30 °C for up to 72 h prior to imaging.

To quantify BACTH interaction, a β-galactosidase assay was followed as previously described (29, 35). BTH101 cells grown overnight in LB supplemented with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG), 50 μg/mL kanamycin, and 100 μg/mL ampicillin were normalized by OD_{600} and harvested by centrifugation. Washed cells were resuspended in Z buffer (0.06M Na_2HPO_4, 0.04M NaH_2PO_4, 0.1M KCl, 1 mM $MgSO_4$, 0.05M β-mercaptoethanol, pH 7.0) and lysed by the addition of chloroform and SDS. Ortho-nitrophenyl-β-galactoside (ONPG) was added to cell lysate and incubated 35 min at 30 °C before quenching by the addition of Na_2CO_3. OD_{420nm} was recorded and Miller units calculated using the equation, Miller units = 1,000 * [(OD_{420nm}/(OD_{600nm} of culture * volume of culture in mL * reaction time in min)]. Experiments were performed in triplicate and statistical analysis was determined by *t* test using GraphPad Prism.

Electron Microscopy. Cell morphology and surface assembly were analyzed by electron microscopy according to published protocols with some modification (20). Briefly, cells of different *A. oris* strains were washed in 0.1 M NaCl, suspended in sterile water, immobilized on carbon coated nickel grids, and stained with 1% uranyl acetate prior to viewing under an electron microscope.

Data Availability. All study data are included in the article and *SI Appendix*.

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