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Mechanism and Function of Membrane Homeostasis of Sortase Modulated by an Evolutionarily Conserved Protein Involved in Pilus Assembly in Actinobacteria

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Mechanism and Function of Membrane Homeostasis of Sortase Modulated by an Evolutionarily Conserved Protein Involved in Pilus Assembly in Actinobacteria

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular Biology

by

Nicholas Ramirez

2022

ABSTRACT OF THE DISSERTATION

Mechanism and Function of Membrane Homeostasis of Sortase Modulated by an Evolutionarily Conserved Protein Involved in Pilus Assembly in Actinobacteria

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Doctor of Philosophy in Molecular Biology

University of California, Los Angeles, 2022

Professor Hung Ton-That, Chair

Bacteria utilize proteins at their surface for a multitude of processes including adhesion, biofilm formation, motility, and virulence. Thus, understanding the biogenesis and surface display of these factors is instrumental in our understanding of bacterial pathogenesis and virulence mechanisms. Within this dissertation we describe the identification and characterization of a newly identified peptide which is functionally conserved amongst Actinobacteria and serves to modulate anchoring of proteins to the cell wall through modulation of membrane homeostasis of the housekeeping sortase. In the oral cavity associated bacterial species, *Actinomyces oris*, we identified a small peptide consisting of 52 amino acids which is

encoded directly downstream of the gene encoding the housekeeping sortase SrtA. Henceforth we refer to this peptide as SafA for Sortase Associated Factor A.

Firstly, through bioinformatic analysis we found that nearly all Actinobacteria encode a SafA homolog immediately downstream of their respective housekeeping sortase genes, with the exception of *Bifidobacterium dentium* in which the genome does not contain a separate SafA reading frame, but rather the C-terminus of the housekeeping sortase harbors a domain homologous to SafA in *A. oris*. In *A. oris* we found that deletion of *safA* results in phenotypes consistent with deletion of the housekeeping sortase itself, which include the formation of abnormally long pili as detected by electron microscopy and the failure of *A. oris* to interact with another oral bacterial species *Streptococcus oralis*. Cellular fractionation and immunoblotting revealed that in the absence of SafA, SrtA is cleaved and released into the extracellular milieu. While software predictions did not identify a signal peptide sequence in SrtA, manual amino acid sequence, sequence analysis did in fact reveal that SrtA contains a tripartite domain consistent with a type I signal peptide sequence and a predicted cleavage site between A56 and S57. Edman degradation amino acid sequencing confirmed this cleavage site and mutational analysis revealed that the signal peptidase LepB2 is responsible for this observed cleavage of SrtA.

To elucidate how SafA protects SrtA from cleavage we utilized a Bacterial Adenylate Cyclase Two-Hybrid system which demonstrated that SafA and SrtA directly interact. Furthermore, we identified a three amino acid domain in SafA consisting of FPW residues which is essential for mediating this interaction. Finally, we found that ectopic expression of SafA from *A. oris*, *Corynebacterium diphtheriae*, and *Corynebacterium matruchotii* rescued the aforementioned functional defects of the *safA* mutant of *A. oris*, thus supporting the conclusion that SafA is both functionally and evolutionarily conserved.

The findings described herein demonstrate a new paradigm for the modulation surface protein display in Actinobacteria. The conservation of SafA across Actinobacteria coupled with the essential role for sortases in mediating anchoring of pili and key virulence factors provides a unique target and opportunity to inhibit the virulence of Actinobacteria species.

The dissertation of Nicholas Ramirez is approved.

Megan McEvoy

Peter Bradley

Renate Lux

Robert Clubb

Hung Ton-That, Committee Chair

University of California, Los Angeles

2022

This dissertation is dedicated to the memory of our dear friend, colleague, and laboratory member Dr. Matthew James Paul Scheible. Whose impact on each of our lives and the scientific community will not be forgotten.

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Acknowledgements

Firstly, I would like to thank my mentor and committee chair Professor Hung Ton-That. All of my previous undergraduate research was in environmental microbiology, as I started graduate studies I wanted to study pathogenic mechanisms in bacteria. The overall aim of the Ton-That laboratory was a perfect fit, and Dr. Ton-That has been a great mentor along these past years, as I have found he quickly was able to match the style of mentorship which I needed to succeed. I additionally would like to thank all of the present and past members of the Ton-That laboratory as each of them has had a direct impact on these findings, either by aiding in experimental design, conducting experiments, or in feedback during presentation and manuscript preparation. Additionally, I would like to thank all of co-authors who contributed to the main findings of this dissertation as presented in chapter 4, which is displayed as a manuscript which is currently in press by Proceedings of the National Academy of Sciences of the United States of America. Firstly, I would like to thank Dr. Chenggang Wu who was instrumental in the conception and gathering of the preliminary data that led to the development of the SafA manuscript and who serves as co-first author for the manuscript describing these findings. I would also like to thank Dr. Julie Chang who was instrumental in my initial training in the Ton-That laboratory and who contributed multiple cloned constructs as well as electron microscopy images used in this manuscript. I would also like to thank Dr. Sara Siegel, who initially characterized the signal peptidase LepB2 as part of her graduate work as a member of the Ton-That laboratory. I would like to extend thanks to Dr. Asis Das for critical review and contributions to manuscript preparation. Lastly, I would like to thank Dr. Hung Ton-That for serving as principal investigator and corresponding author for these described findings.

I would like to thank all members of my doctoral committee, as each member had a very distinct role in my development as a scientist and a researcher. Dr. Renate Lux and Dr. Robert Clubb have been a great source of information and a constant support system as each of our meetings and conversations always led to an interesting suggestion, question, or hypothesis that continuously helped to drive my research forward and continue to spark interest in this project. I would also like to extend sincere thanks to Dr. Peter Bradley, who in addition to serving on my doctoral committee, hosted me as a rotation student during my first quarter as a graduate student. As a student coming into UCLA with very little molecular biology experience, the time I spent in the Bradley lab was invaluable as Dr. Bradley was very present during my initial training which helped me to quickly refine my skills in molecular cloning while at the same time allowing me to gain exposure to novel methods such as genetic manipulation using CRISPR. I would also like thank Dr. Megan McEvoy, who has been a source of inspiration throughout my time at UCLA. Her ability to maintain a laboratory while also working so closely with underrepresented minority students through the MARC program, from which I am an alumnus is truly inspirational. I am also extremely grateful that she agreed to host me as a rotation student during my first year at UCLA. During my rotation, Dr. McEvoy gave me the freedom to explore areas which I found compelling while adapting my skills to fit the research the lab was conducting at the time, which ultimately led to my contributions being recognized with co-authorship on a manuscript describing our findings.

This work would not have been possible without the unconditional love and support of my family. My parents Juan and Sylvia Ramirez continue to sacrifice and work extremely hard to provide ample opportunities for each of their children to succeed and I am beyond thankful for all of the opportunities that I have been granted due to their hard work and determination. I have

always been extremely close to my mother, and through my undergraduate and graduate school career she has always been someone I can talk to at anytime of day or night, without fear of judgement. She always makes me feel validated and loved through all the trials that graduate school has brought on, and without this constant support I don't know that I could have completed these studies. I have learned lessons too numerous to count from my father, however of all things he has taught me, a strong work ethic is probably one of the most important. I strive to demonstrate the same level of strength, dedication, and perseverance that he does on a daily basis. I honestly don't know where I would be without the constant support of my parents and I am beyond thankful for their constant love, support, and guidance. Additionally, as the youngest of three children, I felt that I have always had another set of role models that I could look up to, and I am forever grateful for the support, love, and affirmation I have received from each of my older siblings Vibiana and Manuel Ramirez.

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Chapter 1 Introduction

1.1 Objectives

Bacteria utilize a variety of surface proteins, including the fiber like appendages known as fimbriae or pili for a multitude of functions including cell adhesion, motility, biofilm formation, and horizontal gene transfer (1-3). As Gram-positive bacteria have a thick cell wall at their surface, the mechanism in which proteins are localized to the surface had long remained elusive. Ultimately, it was revealed that all peptides destined for the cell wall in *Staphylococcus aureus* contain a cell wall sorting signal, consisting of a conserved C-terminal LPXTG motif followed by a hydrophobic domain and a positively charged tail (4, 5). Although the archetype housekeeping sortase SrtA was first identified in *S. aureus,* sortases have since been identified in many pathogenic Gram-positive organisms including *Corynebacterium diphtheriae*, and Group B *Streptococci*, the causative agents of diphtheria and neonatal meningitis respectively (6). Since these initial reports, sortases have been grouped into six distinct classes based upon structure and substrate preference (7). In addition to having the distinct role of anchoring peptides to the cell wall, in Gram-positive bacteria, pili are assembled by the pilus-specific sortase. Although the precise mechanism in which this assembly occurs varies slightly between species, generally this assembly process is conducted by the pilus-specific sortase which recognizes and hydrolyzes the cell wall sorting signal of a pilin monomer, then catalyzes a lysine transpeptidase reaction between another pilin monomer and the threonine residue of the previously recognized pilin (3, 8, 9). This reaction occurs repeatedly resulting in the formation of covalent linkages between pilin monomers to form a polymer. Ultimately, these pili are anchored to the cell wall by way of the housekeeping sortase which catalyzes a transpeptidation reaction between the terminal pilin present at the pilus base and peptidoglycan cross bridges. Although the general pilus assembly

mechanism remains conserved across Gram-positive bacteria, there are key differences in pilus composition and anchoring. Specifically, some organisms such as *Corynebacterium diphtheriae*, *Enterococcus faecalis*, or Group B *Streptococcus* species assemble heterotrimeric pilus polymers, or pili composed of three individual pilus subunits (6). In *C. diphtheriae* for example, these subunits are SpaC, SpaA, and SpaB which serve as the pilus tip, shaft, and base respectively (8, 10, 11). Previous reports have demonstrated that in *C. diphtheriae* the base pilin plays a crucial role in signaling pilus anchoring to the cell wall, thus allowing pili to be consistently assembled at an optimal length (11). In contrast to the heterotrimeric pili described here other species such as *Bacillus cereus*, commonly a causative agent of foodborne illnesses, assemble heterodimeric pili which are composed of only two subunits which serve as a tip adhesin and shaft (6, 12). Although previous studies have established a relatively clear model for both the assembly of heterodimeric and heterotrimer pili, one aspect that has remained elusive is what signals pilus anchoring in organisms that assemble heterodimeric pili. We have previously theorized that this process is regulated by the relative abundance and availability of pilin monomers and housekeeping sortase at the pilusosome, or that this process is regulated by some other yet to be identified factor(s) which may modulate protein anchoring (3).

To this aim, the oral-cavity associated bacterium *Actinomyces oris* (one of the earliest colonizing bacterial species in the oral microbiome) has served as a fruitful model for elucidating the mechanism of heterodimeric sortase-mediated pilus assembly and cell wall peptide anchoring. In an attempt at gaining a deeper understanding of *A. oris* peptide anchoring and surface morphogenesis, previous studies by our laboratory have revealed a novel paradigm in which the housekeeping sortase SrtA of *A. oris* may have an unprecedented role in modulating pilus polymer length (13). Given that it is well established that functionally related genes tend to

associate within similar gene loci in bacteria, we probed the *srtA* gene locus in an attempt at identifying additional factors that may modulate SrtA activity. *A. oris* genome analysis revealed the presence of a small open reading frame of unknown function which encodes a 52 amino acid protein located immediately downstream of *srtA*. We named this peptide sortase associated factor A, or SafA. Comparative genomics revealed that *safA* can be identified immediately downstream of the housekeeping sortase genes across virtually all Actinobacteria. Of note, the housekeeping sortase SrtE in the Actinobacteria species *Bifidobacterium dentium* lacks a SafA reading frame, however the C-terminus of SrtE contains a domain homologous to SafA, thus indicating that SafA co-evolved with housekeeping sortases and SafA may modulate sortase activity through direct interactions.

Within this dissertation we aimed to identify and functionally characterize SafA in the oral cavity associated Actinobacteria species *A. oris*. To accomplish this, we used a combinatorial approach consisting of bioinformatic analysis, biochemical assays, and molecular biology approaches to identify and elucidate the mechanism of SafA-mediated modulation of surface morphogenesis in *A. oris*.

1.2 Dissertation Overview

This dissertation is separated into five chapters. Chapter 1 provides a brief overview of the previously established knowledge in the field of sortase mediated peptide anchoring and a brief overview of preliminary studies that led to the conceptualization of this work. Chapter 2 describes the overall functions and mechanisms of assembly of the main fimbriae types in both Gram-negative and Gram-positive bacteria. Chapter 2 as presented is a direct reprint of a previously published manuscript from eLS, formerly the Encyclopedia of Life Sciences reprinted with permission from John Wiley & Sons, Ltd. Chapter 3 provides a summary of the recent advancements in elucidating mechanisms of pilus assembly and peptide anchoring in *Actinomyces oris*, as well as a brief summary of the biotechnical applications of sortases as used for sortase-mediated peptide ligation also known as "sortagging". Chapter 3 is presented as a reprint of a previously published manuscript from Trends in Microbiology with permission from Elsevier Ltd. Chapter 4 serves as the main body of this dissertation in which we describe the identification and functional characterization of SafA in *A. oris*. Additionally in this chapter we present a novel paradigm in sortase activity modulation in which we demonstrate that the housekeeping sortase membrane localization is modulated by a signal peptidase. These findings as presented are a reprint of an upcoming manuscript which has been accepted for publication by Proceedings of the National Academy of Sciences of the United States of America and are presented in this dissertation with the permission of the National Academy of Science. In chapter 5 we present the conclusions drawn from this work as well as a description of the impact that these findings will have on bacterial pathogenesis and cell surface peptide anchoring. Additionally, in this chapter we suggest future experiments which may serve to elucidate the

precise regulation of and role of signal peptidase-mediated cleavage of the housekeeping sortase and additional experiments to determine if SafA has a role in mediating bacterial virulence.

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Bacterial Pili and Fimbriae

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Based in part on the previous versions of this eLS article 'Bacterial Pili and Fimbriae' (2001, 2010).

Advanced article

Article Contents

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Bacterial proteinaceous filaments termed pili or fimbriae are nonflagellar, hair-like structures protruding from the cell surface that are critical for bacterial virulence and fitness. Present in both Gram-negative and Gram-positive bacteria, pili are involved in many processes such as conjugation, adherence, twitching motility, biofilm formation and immunomodulation. Considerably diverse and complex, Gram-negative pili are formed by non-covalent polymerisation of various pilin subunits; many of these pili require chaperones and usher proteins for their assembly. In contrast, fewer pilus systems have been described for the Gram-positive counterparts; notably well studied are the heterotrimeric or -dimeric pili that are covalently assembled by a transpeptidase enzyme called sortase. Furthermore, type IV pili have been identified in several Gram-positive bacteria, especially in clostridia.

Introduction

In the early 1950s, nonflagellar appendages were first observed on the surface of Gram-negative bacteria by electron microscopy. Duguid named these Gram-negative proteinaceous organelles fimbriae (Latin word for threads; singular, fimbria) whereas Brinton called them pili (Latin word for hair; singular, pilus) (Telford *et al.*, 2006). At present, researchers use both terms interchangeably. *Corynebacterium renale* is the first Gram-positive bacterium shown to contain pili (Telford *et al.*, 2006).

The structures and pathways of pilus assembly in Gram-positive and Gram-negative bacteria are distinct. In Gram-negative bacteria, pili, several micrometres in length and 6–8 nm in width, are embedded into the outer membrane lipid bilayer and consist of homopolymers of the shaft protein. Additional proteins can also be polymerised into these noncovalent polymers and often function as adhesins. Chaperones and usher proteins are an integral part in some of the assembly systems. Many types of pili have been discovered, whose names were based initially on morphology or serology. For example, Briton classified six types of pili as types I–V and F based on distinct morphology; however, Duguid and colleagues divided different fimbriae into seven types, 1–6 and F, based on fimbrial thickness and haemagglutination activity. Later pili were named after their functions (e.g. CFA for colonisation factor antigens), their contribution to certain diseases (e.g. P or Pap fimbriae for pyelonephritis-associated pili), their receptors (e.g. S fimbriae bind to sialylgalactoside, and Dr. adhesins recognise Dr. blood group antigen), their expression patterns (e.g. TCP for toxin-coregulated pili) or their characteristic appearance (e.g. BFP for bundle-forming pilus). Notably, sex pili or F pili of *Escherichia coli* were named for their involvement in bacterial conjugation, a process that requires intimate cell-to-cell contact to allow DNA (deoxyribonucleic acid) transfer from donor to recipient cells. F pili likely consist of only mature F pilin, whose precursor is encoded by *traA* gene. Processing of the 12.8-kDa prepilin TraA requires gene products of *traQ* and *traX*. About a dozen or so *tra* genes may be required for F pilus biogenesis. The type IV pilus got its name from group IV pili that share morphological similarities and phenotypes with nonflagellar structures of *Acinetobacter*, *Moraxella* and *Pseudomonas* species involving twitching motility and natural genetic transformation. Nonetheless, not until the 1990s did molecular views of pilus biogenesis in Gram-negative bacteria begin to emerge. Pili were then classified into groups based on their assembly mechanisms. In the following section, we will mainly discuss the assembly mechanisms of pili assembled through the chaperone/usher pathway, type IV pili (T4P), type V pili and their functions in bacterial pathogenesis. **See also**: **Bacterial Cell Wall**

Pili were also found on the surface of Gram-positive bacteria such as *C. renale*, *Corynebacterium diphtheriae* and *Actinomyces naeslundii* and their mechanism of assembly is quite distinctive (Mandlik *et al.*, 2008b). Unlike Gram-negative bacteria, Gram-positive microbes do not possess an outer membrane and thereby use their cell wall peptidoglycan as a scaffold for the covalent attachment of many surface proteins. Cell wall anchoring of these surface proteins requires a transpeptidase enzyme called sortase. The archetype of cell wall anchored proteins is protein A of *Staphylococcus aureus* (Mandlik *et al.*,

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2008b). Protein A, as well as other surface proteins, harbours an *N*-terminal leader peptide and a *C*-terminal cell wall sorting signal (CWSS), which consists of the LPXTG motif, a hydrophobic domain and a positively charged tail (Mandlik *et al.*, 2008b). In *S. aureus*, sortase SrtA cleaves the LPXTG motif between threonine and glycine, forming an acyl–enzyme intermediate with the cleaved substrate via the thioester bond between the threonine residue of the LPXTG motif and the catalytic cysteine residue of SrtA. Resolution of this intermediate by the amino group of the stem peptide within the lipid II precursor leads to cell wall anchoring of the substrate (Mandlik *et al.*, 2008b). A notion that sortase also catalyses pilus assembly in Gram-positive bacteria came from an observation that fimbrial subunits of *A. naeslundii* contains the CWSS and their encoding genes are associated with a sortase gene as part of a fimbrial locus (Mandlik *et al.*, 2008b). This seminal hypothesis was examined in *C. diphtheriae*, the causative agent of diphtheria, as the experimental system (Telford *et al.*, 2006). Since then, sortase-mediated pilus assembly has been described in many other Gram-positive bacteria including streptococci, enterococci, *Bacillus cereus* and *A. naeslundii* (Mandlik *et al.*, 2008a,2008b; Telford *et al.*, 2006). In a later section, we will discuss the assembly mechanism of the prototype SpaA in *C. diphtheriae* and the roles of Gram-positive pili in many aspects of bacterial pathogenesis. It is noteworthy that type IV-like pili have been reported in some Gram-positive bacteria like *Clostridium perfringens* (Melville and Craig, 2013). In addition, related to the T4P and widespread in Gram-negative bacteria, the *tad* loci encoding fimbrial low-molecular weight protein (Flp) pili have been revealed in several actinobacteria (e.g. *Corynebacterium glutamicum* and *Mycobacterium* spp.) (Tomich *et al.*, 2007). Because little is known about their mechanisms of assembly, they will not be discussed further. **See also**: **Gram-type Positive Bacteria**

Pili of Gram-negative Pathogens: Assembly and Functions

Since their description in the early 1950s, pili of many Gram-negative pathogens have been widely studied and classified into two major pathways based on their mechanisms of assembly: pili of the chaperone/usher pathway and T4P (**Table 1**). Recently described is the pili assembled by the nucleation/precipitation pathway or curli as well as the newly described type V pili of *Porphyromonas gingivalis*. The requirement of the general secretion system (Sec) for pilins and pilus machinery to cross the inner membrane is common to these pathways.

Pili assembled by the chaperone/usher pathway

A wide range of Gram-negative pathogens assemble pili by the chaperone/usher pathway. For example, many *E. coli* strains produce type 1 and S pili; strains of uropathogenic *Escherichia coli* (UPEC) make P, Dr. and Afa pili; *Haemophilus influenza* and *Proteus mirabilis* contain Hif and PMF pili, respectively. In addition, *Salmonella* and *Yersinia* spp. express many types of pili assembled by the same mechanism (Thanassi and Hultgren, 2000).

The chaperone/usher pathway has been characterised by Hultgren's laboratories through extensive studies on P fimbria, which is considered the first and best characterised pilus structure. Expressed by UPEC that colonise the urinary tract causing infection of kidneys, P pili are produced by the *pap* gene cluster of 11 genes, which encodes regulatory proteins (PapI/B), rod terminator (PapH), outer membrane usher (PapC), periplasmic chaperone (PapD), major pilin subunit (PapA) and tip fibrillum components. The tip fibrillum is composed of a tip adhesin PapG, which is joined by an adapter PapF followed by the major tip component PapE and another pilin PapK. PapK serves as an adapter that links the tip fibrillum to the pilus rod comprised of PapA and anchored to the outer membrane by PapH. The tip adhesin PapG binds to the Galα(1–4)Gal moieties of the globoseries of glycolipids present on the surfaces of kidney cells and erythrocytes (Sauer *et al.*, 2000).

Newly synthesised pilin subunits, including PapG, PapF, PapE, PapK, PapA and PapH, are translocated across the inner membrane through the general Sec-dependent pathway involved in the type II secretion system. Efficient release of pilin subunits from the inner membrane requires the periplasmic chaperone protein PapD, which then escorts them to the assembly site in the outer membrane usher PapC. In addition to PapD, the periplasmic disulfide isomerase DsbA is also required for correct folding of pilin subunits and chaperones (**Figure 1**). Periplasmic chaperone–pilin subunit complexes are then targeted to the outer membrane usher protein PapC, where the pilin subunits are dissociated from chaperones, translocated across the outer membrane and assembled into pilus structures. Pilus assembly occurs in an orderly fashion beginning with PapG, followed by the tip fibrillum and then the pilus rod. It is thought that the differential affinities between PapC and various PapD–pilin subunit complexes may be crucial factors in determining their final position in the pilus (Thanassi *et al.*, 1998). In the periplasm, the chaperone interacts with pilin subunits by a mechanism termed donor strand complementation (DSC), whereby the chaperone inserts parallel its G1 β-strand into the subunit groove, resulting in efficient folding of the subunits and preventing them from aggregation. Although pilus subunit–subunit interactions are considered to occur by a mechanism similar to DSC termed donor strand exchange, in the latter each subunit donates a strand in an antiparallel manner to complete the immunoglobulin fold, imparting a more stable interaction (Sauer *et al.*, 2000). **See also**: **Chaperones, Chaperonins and Heat-Shock Proteins**

Pilus adhesins and tissue tropism

Colonisation of a host by a microbe is the first step of infection that often requires specific adherence to host receptors. As described previously, pathogenic bacteria produce many adhesins presented at the tip of pilus structures that mediate attachment to the host surface to overcome cleansing mechanisms such as sneezing, coughing and constant fluid flow in gastric or urinary tracts (Craig *et al.*, 2019; Telford *et al.*, 2006). Clearly, pili are major virulence determinants and pilus

Table 1 Bacterial pili/fimbriae

Chaperone/usher pathway assembled pili of Gram-negative bacteria

Organism	Pilus type	Major pilin	Chaperone/ usher	Functions	Diseases
E. coli	Type 1	FimA	FimC/D	Host-tissue adhesion, coaggregation, immunomodulation, biosensor and biofilm formation	Urinary tract infections (UTIs), cystitis, sepsis and meningitis
	S	SfaA	SfaE/F		
	P (UPEC)	PapA	PapC/D		
	Dr (UPEC)	DraA	DraB/C		
	Afa (UPEC)	AfaA	AfaB/C		
H. influenza	Hif	HifA	HifB/C	Host-tissue adhesion, colonisation and biofilm formation	Bronchitis, otitis, meningitis and septicemia
P. mirabilis	PMF	PmFA	PmfC/D	Host-tissue adhesion and colonisation	UTIs
Y. pestis	F1	Caf1	Caf1M/A	Antiphagocytic and adhesion	Plaque

Type IV pili of Gram-negative bacteria

Organism	Pilus type	Major pilin	Putative adhesin	Functions	Diseases
(a) Type IVa pili					
N. meningitidis	MCP	PilE	PilC	Host-tissue adhesion, invasion and twitching motility	Meningitis and sepsis
N. gonorrhoeae	GCP	PilE	PilC	Host-tissue adhesion, invasion and twitching motility	Gonorrhoea and pelvic inflammatory disease (PID)
P. aeruginosa	Pa pilus	PilA	PilA?	Attachment, twitching motility, colonisation and biofilm formation	UTIs, respiratory system infections and bacteremia
(b) Type IVb pili					
EPEC	BFP	BfpA		Host-tissue adhesion, colonisation, biofilm formation and dispersal	Diarrhoea
V. cholerae	TCP	TcpA		Cell adherence, autoaggregation, colonisation, virulence and CTXΦ phage receptor	Cholera
ETEC	CFA/III	CofA		Intestinal colonisation and adhesion	Traveller's diarrhoea
A. actinomycetemcomitans	Tad	Flp		Adhesion, aggregation and biofilm formation	Periodontal disease
P. aeruginosa	Tad	Flp		Biotic and abiotic adhesion and biofilm formation	UTIs, respiratory system infections and bacteremia

Type V pili of Gram-negative bacteria

Organism	Pilus type	Major pilin	Putative adhesin	Functions	Diseases
P. gingivalis	FimA	FimA	BovFim1C?	Biofilm formation, aggregation, epithelial binding	Gingivitis, periodontitis
	Mfa1	Mfa1	Mfa4?		

Pili of Gram-positive bacteria

Organism	Major pilin	Minor pilin	Pilus-specific sortases	Functions	Diseases
C. diphtheriae	SpaA	SpaB,C	SrtA	Lung, laryngeal and pharynx epithelial cells attachment	Diphtheria
	SpaD	SpaE, F	SrtB, C		
	SpaH	SpaI, G	SrtD, E		
GBS (2602 V/R)	GBS80 (PI1)	GBS52, 104	SrtC1,2	Host-cell adhesion, translocation antiphagocytosis	Neonatal sepsis, meningitis and pneumonia
	GBS59 (PI2)	GBS67, 150	SrtC3,4		
GAS (M1)	Spy0128	Cpa, Spy0130	SrtC1	Epithelial cell attachment	Necrotising fasciitis, toxic shock and strep throat
S. pneumoniae	RrgB	RrgA, C	SrtC1,2,3	Adhesion and immunodulation	Pneumonia, bacteremia, otitis media and meningitis
E. faecalis	EbpC	EbpA, B	SrtC	Adhesion and biofilm formation	UTIs, endocarditis and bacteremia
A. oris	FimP (type 1)	FimQ	SrtC1	Tooth colonisation	Dental caries and periodonitis
	FimA (type 2)	FimB	SrtC2	Bacteria and host cell adhesion	
B. cereus	BcpA	BcpB	SrtD	Host-tissue adhesion and biofilm formation	Food-borne disease

Proft and Baker (2009). Reproduced with permission of Springer Nature.

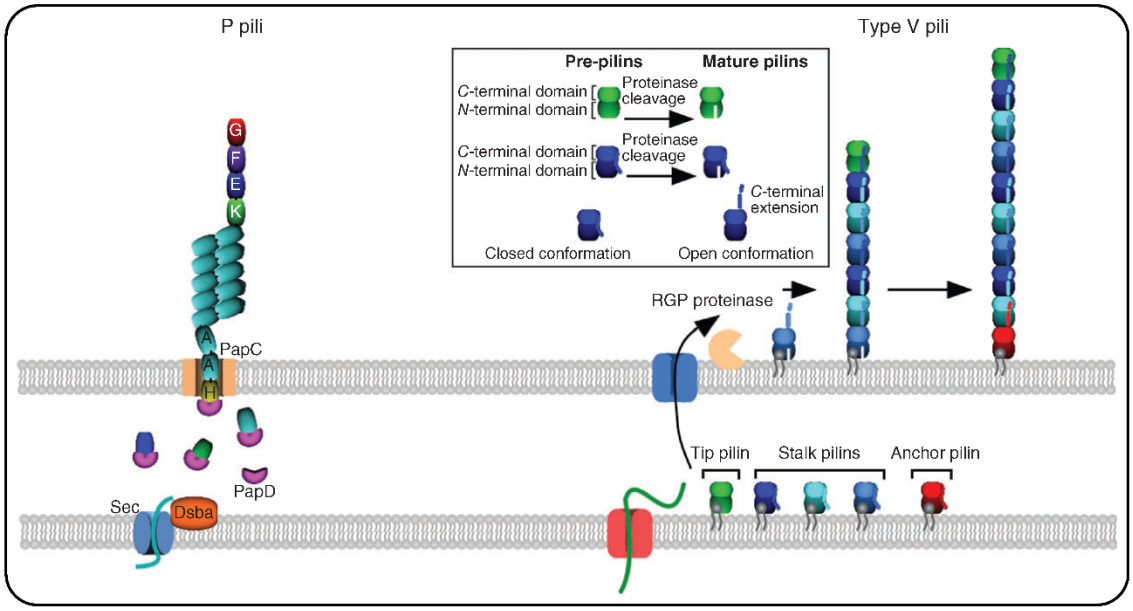

Figure 1 Assembly mechanisms of P pili assembled by the chaperone/usher pathway and Type V pili. P subunits (PapA, E, F, G, H, K) are translocated across the cytoplasmic membrane by the Sec machinery and they interact sequentially with the periplasmic disulfide isomerase DsbA and the chaperone PapD. DsbA mediates disulfide bond formation in the subunits and PapD, and it is required for the correct folding of PapD. PapD is needed for the release of subunits from the cytoplasmic membrane and for the proper folding of the subunits via donor strain complementation. In the absence of PapD, subunits enter into nonproductive aggregations that are sensed by the Cpx and σE signal transduction pathways (not shown). Chaperone–subunit complexes are targeted to PapC in the outer membrane, where subunit–subunit interactions lead to the formation and translocation of a linear pilus fibre across the outer membrane through the usher channel. Once on the cell surface, the pilus rod can twist into its final helical conformation, which may facilitate secretion of the pilus. Type V pilus assembly in *P. gingivalis* begins with secretion of prepilins into the periplasm followed by secretion through the outer membrane where an arginine or lysine-specific proteinase cleaves one β sheet from the *N*-terminal domain. Assembly of the pilus occurs through interactions between the channel created from proteinase cleavage and a flexible C-terminal extension of another pilin. Type V pili are anchored directly to the outer membrane through lipidation of the *N*-terminus of the anchor pilin. Adapted from Li and Thanassi (2009), Thanassi and Hultgren (2000).

expression is frequently associated with diseases; for instance, in EPEC (enteropathogenic *Escherichia coli*) expression of P pili associated with pyelonephritis or type 1 pili with cystitis. As aforementioned, P pili contain an adhesin PapG at the pilus tip that binds specifically to Galα(1–4)Gal moieties of glycolipids present on uroepithelial cells and erythrocytes. Similar to P pili, type 1 pili contain FimH as the adhesive tip pilin. Unlike PapG, FimH mediates bacterial binding to mannose-oligosaccharides, thus conferring tissue tropism for this pathogen.

Curli fibres

Curli are short amyloid fibres produced by Gram-negative bacteria, such as *E. coli* and *Salmonella enterica*, which mediate host-cell adhesion, aggregation and biofilm formation (Barnhart and Chapman, 2006). In *E. coli*, seven curli genes are organised into two tightly regulated operons, and their expression is influenced by a variety of environmental factors including osmotic stress, nutrient deprivation, and temperature through two-component regulatory systems (Barnhart and Chapman, 2006). Curli precursors (CsgA, CsgB, CsgE, CsgF, CsgG) are synthesised in the cytoplasm and secreted into the periplasm through the SecYEG translocon (Barnhart and Chapman, 2006). CsgA and CsgB are two subunit proteins that are translocated across the outer membrane through the channel formed by the lipoprotein CsgG. CsgE binds the subunits and targets them to the secretion channel, while CsgC prevents premature polymerisation of subunits in the periplasm (Evans *et al.*, 2015; Nenninger *et al.*, 2011). Of note, expression of the *csgBAC* genes is regulated by the transcriptional activator CsgD (Barnhart and Chapman, 2006). Once secreted, the nucleator CsgB mediates assembly of CsgA into amyloid fibres in a CsgF-dependent manner. While the exact role of CsgF is not clear, it is essential for the formation of curli fibres, possibly acting as a coupling factor that coordinates the nucleator CsgB for CsgA secretion and surface polymerisation of curli (Bhoite *et al.*, 2019).

Type IV pili: assembly and function

T4P have been identified as key host colonisation factors in a wide range of pathogenic bacteria, including *Pseudomonas aeruginosa*, *Dichelobacter nodosus*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Vibrio cholerae* and enterotoxigenic *Escherichia coli* (ETEC) (Craig *et al.*, 2019). In addition to the adhesive

properties common to most pilus structures, in some species, T4P are associated with a form of movement on solid surfaces, termed twitching motility (Mattick, 2002). The physical basis of twitching motility has been proposed to rely on the ability of T4P to undergo reversible extension and retraction. As a common feature for surface structures of pathogenic bacteria, some T4P undergo phase variation that may allow for the successful escape from the host immune system (see later discussion).

T4P are related through their similarities in many respects, including the primary amino acid (aa) sequence of the structural subunits, the proteolytic processing and *N*-methylation of the prepilins, the conserved assembly machinery and, to a lesser extent, the mechanisms of transcriptional regulation (Craig *et al.*, 2019). T4P (5–7 nm in diameter and several micrometres in length) are mostly composed of a single structural subunit (pilin) of 15–20 kDa, like PilA in *P. aeruginosa*, PilE in *Neisseria* spp., bundlin in EPEC or TcpA in *V. cholerae*, although tip adhesins were identified, for example PilC of *N. gonorrhoeae*. Remarkably, the *C*-terminal two-thirds of many pilins, referred to as the variable domain, provides the basis for antigenic variation of T4P from different species, and in the case of *N. gonorrhoeae*, it is also the source of antigenic variation occurring within the same strain by gene conversion (see later discussion).

T4Ps of different species share several distinctive features, including a short, positively charged leader peptide ending with a glycine residue in the precursor (prepilin), *N*-methylation of the first aa residue in the mature protein (pilin), and a highly conserved hydrophobic *N*-terminal region referred to as the constant domain (Craig *et al.*, 2019). Although pilins of *P. aeruginosa* and *N. gonorrhoeae* belong to a subgroup type IVa, the pilins of the bundle-forming pili (BFP) of EPEC and the toxin-coregulated pilus of *V. cholerae*, BfpA and TcpA, respectively, are placed in a subgroup type IVb due to minor differences in those features. Specifically, type IVa pilins of approximately 150–160 aa in length contain a leader peptide sequence less than 10 aa, whereas the type IVb pilins (180–200 aa) consist of a longer leader peptide sequence (15–30 aa).

Given the similarities in the structural subunits, T4P are speculated to be assembled through common machinery that involves 12 or more proteins. Indeed, this is supported by the fact that *P. aeruginosa* is capable of producing T4P of *D. nodosus*, *Mycobacterium bovis* and *N. gonorrhoeae* from cloned fimbrial subunits. The assembly of T4P involves many proteins such as prepilin peptidases, nucleotide-binding proteins and outer membrane proteins as well as the pilin subunit. In *P. aeruginosa*, PilA is synthesised as a precursor with a leader peptide. During translocation by the Sec machinery, the prepilin leader peptide is cleaved and the resulting amino-group is *N*-methylated that utilises *S*-adenosylmethionine as a cofactor. Both activities require a leader peptidase PilD (also PilD in *N. gonorrhoeae*). The pilus is thought to grow from the inner membrane 'subassembly' to the outer membrane secretin (PilQ) and onto the bacterial surface. Pilus polymerisation requires cytoplasmic soluble ATPase PilB (PilF in *N. gonorrhoeae*), PilM, PilN, PilO, PilP, and pseudopilins FimT, FimU, PilE, PilX, PilV and PilW. PilD, PilF and PilM-P form the assembly core and they are essential for the polymerisation of the pilin subunit PilA. In the absence of any of pilus homeostasis effectors PilC, PilH-L, PilG, PilW and TfpZ/Y, ATPase PilT mediates the disassembly of the pili, leading to retraction. Thus, ATPases PilB and PilT constitute T4P motors that control pilus polymerisation and depolymerisation, respectively (**Figure 2**).

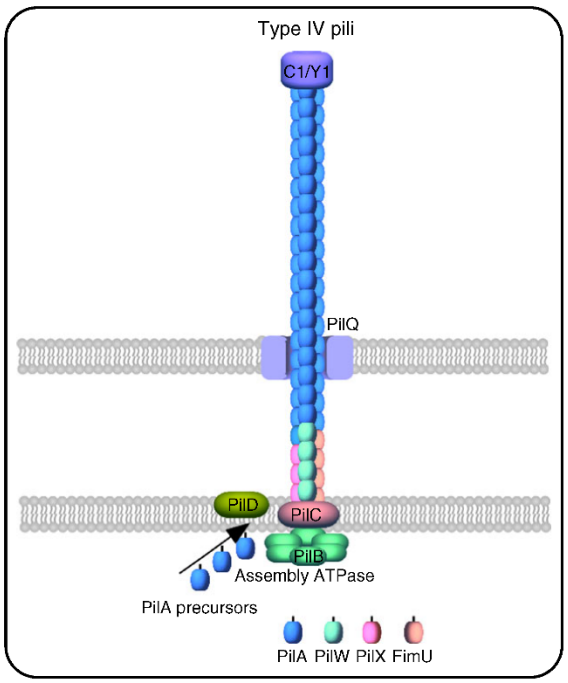

Figure 2 Assembly of type IV pili in Gram-negative bacteria. For the assembly and retraction of type IV pili, prepilin leader sequences are cleaved and *N*-methylated by prepilin peptidase PilD. PilA is assembled on a base of PilE, V, W, X and FimU by the cytoplasmic membrane protein PilC and the NTP-binding protein PilB. The pilus grows through the outer membrane pore composed of multimeric PilQ, which is stabilised by the lipoprotein PilP. Pili are retracted by ATPase PilT that is aided by PilU. Mattick (2002). Reproduced with permission of Annual Review of Microbiology.

Like the type IVa pili of *P. aeruginosa* and *N. gonorrhoeae*, the type IVb pili have been intensively studied in EPEC and *V. cholerae*. Among those, the BFP of EPEC is the prototype with all components for the pilus machinery identified (Milgotina and Donnenberg, 2009). A cluster of 14 genes sufficient for BFP biogenesis is located on a ~90-kb enteropathogenic *Escherichia coli* adherence factor (EAF) plasmid of EPEC strains, unlike most of type IVa pilus systems whose gene clusters are on chromosome. Bundlin, the only structural component known thus far, is synthesised as a prepilin as the *bfpA* gene product. For proper folding of BfpA, the periplasmic disulfide isomerase DsbA is required, as is the case for PapA aforementioned (Thanassi *et al.*, 1998). Prebundlin is cleaved into its mature form by the prepilin peptidase BfpP, encoded by *bfpP*, which also uses pilin-like proteins BfpI, BfpJ and BfpK. Proteins BfpB, BfpG and BfpU are part of the outer membrane translocation subassembly, whereby

BfpB appears to form pores from ring-shaped dodecamer complexes with a 7-nm inner diameter. The so-called inner membrane subassembly consisted of four proteins, BfpC–F with BfpD an ATPase that has Walker box A, among other Walker boxes, required for BFP biogenesis. BfpF is another ATPase proposed to be involved in pilus retraction. *bfpL* is the last gene in the bfp operon that is required for pilus biogenesis and stability of three pilin-like proteins BfpI–K.

There are currently two models of type IV pilus assembly and extrusion. In the 'rotational model' of pilus assembly, pilins dock at the base of the growing pilus through electrostatic interactions. Adenosine triphosphate (ATP) hydrolysis by the assembly ATPase PilB hexamer causes waves of conformational changes to the ATPase which results in rotation of the platform protein PilC. The rotation of the platform protein forces the extrusion of the pilus. Repeating cycles of this process results in the extrusion of the pilus from the bacterial membrane (Craig *et al.*, 2019).

The precise role of the platform protein in pili assembly and extrusion is not fully understood, and there is no evidence that the platform protein rotates during pilus assembly. Therefore, Craig *et al.* (2019) propose a 'compression model' of pilus assembly and extrusion. In this model, the platform protein PilC is in an open conformation which allows a pilin to localise between the base of the growing pilus and the platform protein. Docking of the pilus creates strain on the platform protein and forces it into a closed conformation. ATP hydrolysis by PilB compresses the platform protein further which results in extrusion of the pilus to alleviate the strain placed on the platform protein, and thus allowing it to readopt an open conformation and accept another pilin.

T4P and twitching mobility

T4P display a wide range of functions for bacterial virulence and fitness, including adherence, multicellularity and DNA uptake. For example, in *N. gonorrhoeae* production of T4P is a prerequisite for natural competence for DNA uptake and transformation, and, however, DNA transformation-mediated horizontal gene exchange is crucial for the antigenic variation of the major pilin PilE. Nonetheless, T4P are well known for their twitching motility that is the consequence of force generated from pilus retraction. Elegant studies of T4P in *P. aeruginosa* and *N. gonorrhoeae* have established this important principle that serves as a paradigm for many retractable pili in Gram-negative bacteria. Using laser tweezers, it was demonstrated that T4P of *N. gonorrhoeae* mediate crawling movement and formation of adherent microcolonies (Craig *et al.*, 2019). Nonpiliated mutants that lack the major pili subunit PilE or the polymerisation motor PilF were completely nonmotile, whereas *pilT* mutants unable to retract, otherwise piliated, failed to produce larger movements than 1 µm as observed for normal cells. The force generated from pilus pulling could exceed 80 pN (Craig *et al.*, 2019). In a separate study, using fluorescent microscopy Skerker and Berg observed extension and retraction of T4P in *P. aeruginosa*. When the distal end of a pilus was free, the pilus extended and retracted at rates of approximately 0.5 µm s^{-1}, about half of those of gonococcal T4P. When the pilus end adsorbed to the substratum, the pilus was retracted (Craig *et al.*, 2019). **See also**: **Bacterial Genetic Exchange**; **Homologous Genetic Recombination during Bacterial Conjugation**

How is pilus retraction related to colonisation and invasion of a pathogen? It has been postulated that T4P may provide initial attachment to host tissues, retract to bring the bacteria closer to host cell surfaces and trigger a signalling cascade leading to cytoskeletal reorganisation and invasion (Craig *et al.*, 2019). Several lines of evidence support this model. It has been demonstrated in *Neisseria* that pilus retraction is highly active during infection of human epithelial cells (Opitz *et al.*, 2009). Another study in *N. meningitidis* by Coureuil *et al.* (2009) demonstrates that adherence mediated by meningococcal T4P was found to recruit the Par complex of eukaryotic cells, which is consisted of Par3, Par6 and PKCξ and involved in the formation of positional landmarks on the plasma membrane, leading to the formation of ectopic early junction-like domains, consequently allowing the pathogen to cross the blood–brain barrier.

Type V pili: assembly and function

Type V pili are a recently described class of pili identified in the Gram-negative oral bacterium *P. gingivalis* (Xu *et al.*, 2016). Type V pili mediate *P. gingivalis* auto-aggregation and co-aggregation with *Streptococcus gordonii* (Park *et al.*, 2005). Type V pili are classified as major or minor pili, also referred to as FimA and Mfa1 pili, respectively. Major and minor pili are genetically encoded in different operons and differ in length with major pili ranging from 0.3 to 1.6 µm while minor pili range from 80 to 120 nm (Park *et al.*, 2005; Yoshimura et al., 1984, 2009). Unlike T4P, Type V pili are anchored directly to the outer membrane and do not rely on periplasmic or inner membrane anchoring proteins (Xu *et al.*, 2016).

Type V pili are composed of three distinct pilins: tip pilins (e.g. Mfa4), structural/stalk pilins (e.g. FimA4), and anchor pilins (e.g. Mfa2) (Xu *et al.*, 2016). Type V pilins consist of a *C*-terminal and *N*-terminal domain, each containing a β sheet consisting of seven β strands. Type V prepilins are expressed as lipoproteins and lipoprotein signal peptides mediates their transport to the periplasm (Xu *et al.*, 2016). Prepilins finish maturing after being shuttled to the outer membrane by an unknown protein(s). Pilus assembly occurs from the tip to the base, in which proteolytic cleavage of a conserved arginine/lysine residue in the *N*-terminal domain of a prepilin results in loss of one β strand from the *N*-terminus. Type V pilins contain a flexible appendage as part of the *C*-terminal domain which can exist in an extended 'open' conformation, or folded back into the pilin structure in 'closed' conformation (Xu *et al.*, 2016). The resulting gap in the *N*-terminal domain of the pilin is filled by the extended β sheet appendage from the *C*-terminal domain of another pilin (**Figure 1**). Type V pili subunits assemble in a right-hand twist conformation. The tip pilin is added through the same mechanism; however, the tip pilin does not contain the *C*-terminal extension, therefore, more pilins cannot be extended past the tip (Xu *et al.*, 2016). The pilus is anchored to the outer membrane through the addition of an anchor pilin which is structurally unique from the tip and stalk pili. Anchor pilins contain the extended β sheet appendage which mediates their attachment to the pilus; however, they do not contain the proteolytic cleavage site within the *N*-terminus; therefore,

additional pilins cannot be added to the anchor pilin (**Figure 1**). Xu *et al*. (2016) suggest that the *N*-terminus of anchor pilins is lipidated at a conserved cysteine residue, which mediates its attachment to the outer membrane.

Phase variation

Clearly, pilus adhesins are important virulence factors for pathogenic bacteria. However, because of their surface localisation, proper temporal and spatial expression of pilus structures is not only crucial for their function as adhesins but also very important for evading the host immune system or adapting efficiently to environmental changes. To achieve this, pathogens employ many pathogenic strategies, one of which is alternating their surface entities via phase variation and antigenic variation for example, reversible on and off switching of pilus genes leading to variation in P pilus expression in EPEC (Henderson *et al*., 1999). At 37 °C, P pilus expression is on; at temperatures below 26 °C, pilus expression is off. This has been proposed to occur by transcriptional regulation of pap pilus genes via DNA differential methylation. The intergenic region between *papI* and *papBA* of EPEC contains six binding sites for Lrp (leucine-responsive regulatory protein), two of which named GATC-I and GATC-II (site 5 and 2, respectively) are differentially methylated by Lrp and deoxyribonucleic acid adenine methylase (Dam). Lrp binding to sites 1–3 encompassing the GATC-II site, which is located within the promoter region of the *papBA* locus, results in the inhibition of *papBA* transcription. *papBA* transcription is activated when Lrp preferentially binds to sites 4–6, together with PapI binding to Lrp/DNA complex at the GATC-I site and methylation of the GATC-II site. Thus, in phase OFF, GATC-I is methylated and GATC-II unmethylated; inversely, in phase ON, GATC-I is unmethylated and GATC-II methylated. **See also**: **DNA Methylation**; **Site-specific Recombination**

Not all phase variation occurs at the transcriptional level. The phase-variable expression of *N. gonorrhoeae* type IV pilus adhesin, PilC, relies on slipped-strand mispairing, a process that occurs during DNA replication of regions containing highly repetitive DNA sequences (Seifert, 1996). The *pilC* gene contains a run of Gs near the beginning of the coding region. Frameshifts resulting from insertion or deletion of G residues by slipped-strand mispairing determine whether or not PilC will be expressed as an intact protein. The tRNA (transfer ribonucleic acid) for a rare leucine codon (UUG), encoded by *leuX*, appears to stimulate type 1 fimbrial expression, presumably by increasing the expression of FimB, which contains six leucine residues encoded by UUG (Newman *et al*., 1994). **See also**: **Gene Expression: Frameshifting**

In addition to phase variation, antigenic variation of fimbrial structural subunits is another common mechanism adopted by pathogenic bacteria to avoid attack by host immune system. One such example is the antigenic variation of the type IV pilin of *N. gonorrhoeae*, PilE, which results from the gene conversion between one of the several silent pilin genes (*pilS*) and the expressed gene (*pilE*) (Seifert, 1996). **See also**: **Antigenic Variation in Microbial Evasion of Immune Responses**

Pili of Gram-positive Pathogens: Assembly and Functions

Nonflagellar appendages named pili were first described on the surface of *C. renale*, an animal pathogen, in 1968 (Telford *et al*., 2006) or pili were then reported in several species of *Actinomyces* which produce two antigenic structures, that is type 1 and type 2 fimbriae (Mandlik *et al*., 2008b). Few years later, the major components of the type 1 and type 2 fimbriae, as FimP and FimA, respectively, were cloned. Pioneer work by Yeung and colleagues subsequently revealed the gene locus encoding each type of fimbriae; intriguingly, each locus contains genes encoding sortase and fimbrial subunits with the CWSS. This observation led to the discovery of sortase-mediated pilus assembly in *C. diphtheriae* (Mandlik *et al*., 2008b). With the advent of whole-genome sequencing coupled with genetic and biochemical methods, a similar assembly pathway has been subsequently described in *Streptococcus agalactiae* (group B *Streptococcus* or GBS), *Streptococcus pyogenus* (group A *Streptococcus* or GAS), *Streptococcus pneumoniae*, *Enterococcus* spp., *B. cereus* and *A. naeslundii* (**Table 1**; **Figure 3**).

Pilus assembly of the SpaA pili in *Corynebacterium diphtheriae*

By BLAST (basic local alignment search tool) homology searches in the genome sequence of *C. diphtheriae* NCTC13129 with *S. aureus* SrtA and the CWSS as queries, Ton-That and Schneewind revealed three pilus gene clusters with a total of nine surface protein genes (with *N*-terminal signal peptides and *C*-terminal sorting signals) named *spaA–I* and five sortase homologues named *srtA–E*. A sixth sortase gene (*srtF*) was found in a different chromosomal location (Telford *et al*., 2006). Rabbit-polyclonal antibodies raised against recombinant Spa proteins (Spa for sortase-mediated pilus assembly) were used to label pilus structures on the surface of *C. diphtheria* by immuno-electron microscopy. Three distinct pilus organelles were detected; each is built of a major pilus shaft, a tip pilin and another pilin, which forms the base and is also interspersed along the pilus shaft. The three pilus structures were named as the SpaA-, SpaD- and SpaH-type pili according to the major subunit that constitutes the pilus shaft. The assembly of each pilus requires sortase located at each locus; for example, sortase SrtA is specific for the SpaA-type pili, SrtB/SrtC for the SpaD pili and SrtD/SrtE for the SpaH pili. These sortases belong to sortase class C or pilus-specific sortases, whereas sortase SrtF is considered the housekeeping sortase (Mandlik *et al*., 2008b).

How does sortase catalyse the formation of covalently linked pilus polymers on the bacterial cell wall that remain intact from hash treatments such as hot sodium dodecyl sulfate (SDS) and formic acid? Genetic and biochemical evidence with the prototype SpaA pili has supported a biphasic model of pilus assembly catalysed by sortase, that is pilus polymerisation followed by cell wall anchoring of pilus polymers (**Figure 3**). Unlike the other two pilins SpaB and SpaC encoded by the SpaA gene cluster *spaA-srtA-spaB-spaC*, SpaA contains the pilin motif with a sequence of WxxxVxVYP*K*N, in addition to the CWSS, which

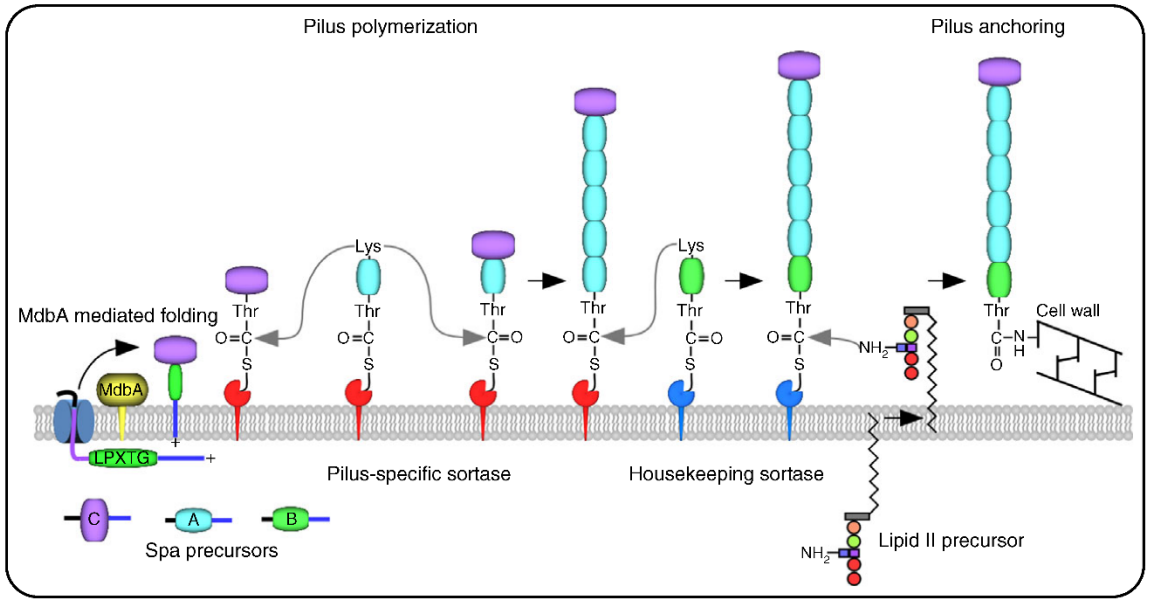

Figure 3 A biphasic model of sortase-mediated pilus assembly in Gram-positive bacteria with the prototype SpaA pili of *Corynebacterium diphtheriae*. Spa pilin precursors (SpaA, SpaB and SpaC) are translocated across the membrane by the Sec machinery with the removal of their leader peptide sequences. Folding of pilins is mediated by MdbA before the cleaved pilins are anchored to the cytoplasmic membrane by the sorting signal, which is compromised of an LPXTG sequence motif, followed by a hydrophobic domain and tail of positively charged residues (+). Sortases cleave the LPXTG motif between threonine and glycine, forming acyl–enzyme intermediates with the pilin substrates. Pilus polymerisation occurs by lysine-mediated transpeptidation reactions catalysed by pilus-specific sortase. This polymerisation is terminated when SpaB is attached to the pilus base by the housekeeping sortase. The housekeeping sortase catalyses cell wall anchoring of pilus polymers. Adapted from Mandlik et al. (2008a).

is recognised by pilus-specific sortase SrtA. SpaA also harbours intramolecular isopeptide bonds that confer mechanical stability (Kang *et al.*, 2009), which withholds unfolding forces up to 525 pN, making SpaA as one of the most mechanically stable proteins known (Echelman *et al.*, 2016). After their synthesis in the cytoplasm, the pilin precursors are transported across the membrane through the secretion machinery (Sec) where folding of the pilins is mediated by the thiol-disulfide-oxidoreductase MdbA, before the pilins are embedded into the membrane by the hydrophobic domain and charged tail (Reardon-Robinson *et al.*, 2015). Membrane-bound sortase cleaves the LPXTG motif of the CWSS between threonine and glycine and forms an acyl–enzyme intermediate with the pilin substrates. To ensure SpaC as the tip entity, the SpaA–SpaC isopeptide bond must be formed by the SrtA-catalysed transpeptidation reaction, in which a nucleophilic attack by the conserved lysine of the SpaA pilin motif resolves the Thr-Cys bond of the SpaC-SrtA acyl–enzyme intermediate (**Figure 3**). The pilus shaft is then extended by the cyclic addition of SpaA to the resultant SpaC–SpaA polymers formed in the preceding reactions. Pilus polymerisation is then switched to the cell wall anchoring phase when a SpaB is attached to the growing pilus base by a similar transpeptidation reaction involving lysine of SpaB (Mandlik *et al.*, 2008a). This cell wall anchoring step is catalysed by the housekeeping sortase SrtF (Mandlik *et al.*, 2008b). Recent studies, employing *in vitro* pilus polymerisation, confirm the role of SpaB as a stop signal for pilus polymerisation (Chang *et al.*, 2018). Thus, SpaB functions as a molecular switch that regulates pilus length and cell wall anchoring of pilus polymers.

Sortase-mediated pilus assembly in other Gram-positive bacteria

Like *C. diphtheriae*, pilus and sortase genes of many other Gram-positive bacteria are organised in clusters (pathogenicity islands or PIs) that are often flanked by transposase and inverted repeats, evidence of horizontal gene transfer. All share characteristic features of the sortase-dependent assembly pathway: pilus-specific sortase(s) and pilins harbouring the pilin motif and the CWSS. Unique to corynebacteria, streptococci and enterococci, these pathogens produce heterotrimeric pili composed of a major pilus shaft and two minor pilins (Mandlik *et al.*, 2008b; Telford *et al.*, 2006). In *S. agalactiae*, the major cause of neonatal sepsis and meningitis, genomic, genetic and biochemical analysis of several GBS clinical isolates identified three pilus variants: PI-1, PI-2a and PI-2b. All three pilus islands have the same basic organisation with three pilus gene encoding pilins containing the LPXTG motif along with two sortase genes (Telford *et al.*, 2006); however, the major difference between the pilus gene clusters is their gene sequence. Furthermore, genes encoding transcriptional regulators are present only in PI-1 and PI-2a loci but not in

the PI-2b locus, which also encodes a signal peptidase. Whether the transcriptional regulators and signal peptide are involved in pilus assembly in GBS remains to be investigated. By polymerase chain reaction (PCR) analysis, it was shown that at least 1 of 3 pilus variants was present in 289 strains; PI-1 and PI-2a loci are more predominant (72% and 73% of strains, respectively) than the PI-2b (27% of strains) (Margarit *et al.*, 2009). In *S. pyogenes*, heterotrimeric pili are encoded by nine highly variable pathogenicity islands known as the fibronectin-binding, collagen-binding T antigen (FCT) regions (Falugi *et al.*, 2008). Interestingly, the major pilin subunits are T antigens in the T-typing system of GAS described by Lancefield and colleagues more than 60 years ago that have been characterised as extremely resistant to trypsin (Lancefield and Dole, 1946). Of note, the major pilin Spy0128 of GAS strain SF370 constitutes the pilus shaft held together by intermolecular isopeptide bonds formed between the threonine residue of the LPXTG motif and the lysine residue that is not part of the canonical pilin motif (Kang *et al.*, 2007). Similar to *S. agalactiae*, *S. pyogenes* pilus gene clusters are present in all clinical isolates tested so far, but a degree of sequence variability of GAS pilus genes is significantly larger as compared to those of GBS, speculating a role of GAS pili in tissue tropism (discussed later). Using colony hybridisation and PCR amplification, it was shown that approximately 95% of *Enterococcus faecalis* clinical isolates carried the endocarditis- and biofilm-associated pilus (ebp) genes (Nallapareddy *et al.*, 2006), that is EbpA/EbpB/EbpC, whereas the *epb* locus was found in a small number of *E. faecium* clinical isolates (~33%) (Cobo Molinos *et al.*, 2008; Sillanpaa *et al.*, 2009). Unlike GBS, GAS or *E. faecalis* pilus loci, the pilus locus *rlrA* (designated PI-1) of *S. pneumoniae* was present in a smaller number of clinical isolates (~30%) (Aguiar *et al.*, 2008; Barocchi *et al.*, 2006), whereas the PI-2 locus distributes only in 16% of all strains investigated (Bagnoli *et al.*, 2008). Interestingly, pili of the *S. pneumoniae* PI-2 locus are consisted of two components, just like bacillus and actinomyces pili (Bagnoli *et al.*, 2008) (see later discussions).

Actinomyces oris (formerly *A. naeslundii*) and *B. cereus* were found to express heterodimeric pili (Budzik *et al.*, 2007; Mandlik *et al.*, 2008b). *Actinomyces* spp. are members of the pioneering colonising bacteria that attach to the tooth surface, forming a surface matrix for subsequence colonisation of the intermediate and late colonising oral bacteria. *A. oris*, a more predominant species of actinomyces in dental plaque, was shown to produce two functionally distinct types of fimbriae, named type 1 and type 2. Mishra *et al.* (2007) characterised two fimbrial gene clusters in *A. oris* strain MG1: the *fimQ-fimP-srtC1* encodes the type 1 fimbriae composed of FimP forming the shaft and FimQ as the tip and the *fimB-fimA-srtC2* for the type 2 fimbriae with the major fimbria FimA and tip fimbria FimB. Strikingly, a surface protein with a LPXTG motif, encoded by the gene *cafA* that is not genetically linked to the type 2 fimbrial cluster, is found at the tip of the FimA polymers. The CafA protein apparently forms a fimbrial structure that is distinct from the canonical type 2 fimbriae (Reardon-Robinson *et al.*, 2014). Similar to *Actinomyces*, *B. cereus*, a soil-dwelling pathogen, produces only type of pili that are made of the major pilin subunit BcpA and the tip pilin BcpB (Budzik *et al.*, 2007). With the apparent lack of a SpaB-like protein in both *Actinomyces* and *Bacillus* pilus systems, it is proposed that the housekeeping sortase mediates cell wall anchoring of pilus polymers via the major pilin subunit. Indeed, in *A. oris* the housekeeping sortase SrtA remarkably not only functions as the cell wall anchoring sortase but also acts as a molecular ruler that controls optimal pilus length required for polymicrobial interactions (Chang *et al.*, 2019).

Type IV pili: assembly and function in Gram-positive bacteria

T4P are not limited to Gram-negative bacteria, as they have also been identified in multiple genera of Gram-positive bacteria including *Clostridium, Heliobacterium* and *Streptococcus* where they have a role in gliding motility, host cell adhesion and biofilm formation (Melville and Craig, 2013; Piepenbrink and Sundberg, 2016). The Type IV pilus assembly mechanism and protein components are similar between Gram-negative and Gram-positive bacteria. In *C. perfringens,* PilA prepilins contain a leader peptide sequence and are secreted through the membrane by sec machinery, while PilD peptidase cleaves the prepilin into a mature pilin subunit. Homologs of assembly ATPase (PilB), retraction ATPase (PilT) and platform protein (PilC) mediated pilus polymerisation and de-polymerisation as described previously. PilM, PilN and PilO homologs in *C. perfringens* appear to form a complex similar to that seen in the *P. aeruginosa* pilus assembly core. In Gram-negative bacteria, the PilQ secretin appears to allow the pilus to grow through the outer membrane. In Gram-positive bacteria, it is unclear which protein(s) act as a channel to mediate pilus growth through the thick peptidoglycan cell wall. Melville and Craig (2013) speculate that PilM, PilN and PilO interact with an unidentified protein channel embedded within the cell wall to form a stable channel for the pilus to grow through.

Functions of pili

Because of their extended structures, pili are one of the first molecules that interact with host cells, initiating a pathogenic programme and triggering inflammatory responses from host. Although their mechanism of assembly has been discovered recently, functions of Gram-positive pili were examined much earlier, especially for several key concepts of bacterial pathogenesis such as tissue tropism and bacterial coaggregation, a prerequisite of biofilm biogenesis.

Adherence and tissue tropism

It is not surprising that all Gram-positive pili are involved in many aspects of bacterial pathogenesis including adherence and colonisation as the first step of infection. Nonetheless, *Actinomyces* fimbriae are an adhesive principle of tissue tropism and bacterial coaggregation in Gram-positive bacteria that were examined in the early 1980s. *Actinomyces* were shown not only to colonise the tooth surface but also coaggregate with oral streptococci that initiate subsequent colonisation of late oral bacteria. This remarkable ability is attributed to the two fimbriae; type 1 fimbriae mediate *Actinomcyes* binding to saliva-treated hydroxyapatite or salivary proline-rich proteins (PRPs) that coat the tooth enamel, and type

2 fimbriae interact with polysaccharide receptors or regulated secretory pathways (RSPs) on the surface of oral streptococci. The type 2 fimbriae also bind to carbohydrate-containing receptors on host epithelial cells, erythrocytes and polymorphonuclear leukocytes (Mandlik *et al.*, 2008b).

This key concept of tissue tropism mediated by pili has been investigated in several other Gram-positive bacteria. As aforementioned, *C. diphtheriae* produces the SpaA-type, SpaD-type and SpaH-type pili. Although the SpaD and SpaH pili exhibit preferential binding to lung and laryngeal epithelial cells, the SpaA pili are essential for bacterial adherence to epithelial cells of pharynx, which is the major site of infection. This selective adherence requires two minor pilins SpaB and SpaC presented on the pili or as monomeric forms on the bacterial cell wall. The fact that minor pilins are critical for tissue tropism and that they are present both on the cell wall and pilus structures speculate a molecular model of pilus-mediated bacterial adherence during infection (Mandlik *et al.*, 2008b). Pilin adhesins displayed on the extended pili mediate bacterial distant contact with host cell, whereas cell wall-anchored pilins permit closer contact, thus forming an intimate zone of adhesion between the infecting bacterium and the host cells.

Similar to corynebacterial pili, GAS pili display tissue tropism as pili were shown to mediate attachment to human tonsil epithelial cells and skin keratinocytes, two major sites of infection, but not to liver or kidney cell lines (Abbot *et al.*, 2007). In GBS strain 2603 V/R, the minor pilin GBS52 of the PI-1 contributes to bacterial adherence to lung epithelial cells; consistently, GBS52-coated latex beads bind efficiently to the lung cells but not other tested epithelial cells (Krishnan *et al.*, 2007).

Bacterial coaggregation and biofilm

A classic example of bacterial coaggregation is the aforementioned fimbria-mediated coaggregation of *Actinomyces* with oral streptococci such as *S. gordonii*, *Streptococcus sanguinis* and *Streptococcus oralis*. A mutant strain lacking type 2 fimbriae fails to coaggregate with *S. sanguinis*. Reardon-Robinson *et al.* have identified CafA as a type 2 fimbriae adhesion, which mediates coaggregation. Coaggregation between these microbes is the key step leading to the formation of a more complex microbial community called biofilm or dental plaque. Using a flowcell model of *in vitro* biofilm conditioned with human saliva, Kolenbrander and colleagues showed that growth of *Fusobacterium nucleatum*, considered a bridging bacterium, was profoundly enhanced in a mixed-species biofilm of either fusobacteria with actinomyces alone or with actinomyces and *S. oralis* (Periasamy *et al.*, 2009).

The role of pili in biofilm formation has been demonstrated with *E. faecalis*, GAS and GBS pili. In *E. faecalis*, a mutant that lacks the Ebp pili displayed a major defect in biofilm formation as well as bacterial virulence as measured in a murine model of urinary tract infection (Nallapareddy *et al.*, 2006). In GAS, the pilus shaft is required for the formation of biofilm *in vitro*, whereas in GBS strain NEM316 both the pilus shaft and minor pilin contribute to this process (Manetti *et al.*, 2007).

Immunomodulation

On invading, pathogenic bacteria produce a wide variety of molecules called pathogen-associated molecular patterns (PAMPs) that activate immune cells. Lipopolysaccharide (LPS), secreted toxins and surface structures like flagella of Gram-negative microbes are typical PAMPs (Underhill, 2004). Do Gram-positive pili act to modulate the host immune response? Recent findings support the protective role of Gram-positive pili in bacterial survival within macrophages and neutrophils. When mice were challenged with *S. pneumoniae* strains that express pili or no pili, high levels of proinflammatory cytokines tumour necrosis factor-α (TNFα) and interleukin 6 (IL-6) were detected in the serum of animals infected with piliated pneumoniae only (Barocchi *et al.*, 2006). Although it is not clear how pneumococcal pili trigger inflammatory responses, the high level of cytokines generated may elicit tissue damage that allows for a more robust bacterial invasion. As aforementioned, the role of pili in mediating phagocytic survival is evident for *S. agalactiae*. A streptococcal strain that lacks the major pilin PilB is more susceptible to killing by macrophages and neutrophils *in vitro* as compared to the wild-type strain (Maisey *et al.*, 2007). This resistance to phagocytic killing may be due to the ability of pili to sequester the host cathelicidin antimicrobial peptides (Maisey *et al.*, 2007).

Concluding Remarks

With respect to morphology, assembly, genetics and regulation, bacterial pili/fimbriae are tremendously diverse; yet, they are commonly an adhesive principle of bacterial pathogenesis. Extending from the surface of cells, pili are able to overcome the forces of cell-to-cell repulsion caused by the negative charges on their respective surfaces. Pili allow cell-to-cell contact, whether it is interaction of a bacterium with another bacterium or a bacterium with its eukaryotic target cell. Specific adhesin–receptor interactions or nonspecific hydrophobic interactions can mediate these attachments. Bacteria have devised extraordinary strategies to assemble noncovalently or covalently these complex structures outside the cell, using mechanisms that are independent of the traditional cytoplasmic energy sources. Although some systems are well understood, it is clear that much work remains to elucidate mechanisms of assembly and function of many pilus systems. The past 10 years have marked an explosion in research studies of Gram-positive pili, many of which are being explored as vaccine candidates as therapeutic strategies to combat many deadly diseases caused by Gram-positive bacteria. The same principle has been employed and continues to be employed for Gram-negative counterparts.

List of Abbreviations

BLAST	basic local alignment search tool
BFP	bundle-forming pilus/bundle-forming pili
CFA	colonisation factor antigens
CWSS	cell wall sorting signal
DAM	DNA adenine methylase

DSC	donor strand complementation
EAF	EPEC adherence factor
EPEC	enteropathogenic *E. coli*
ETEC	enterotoxigenic *E. coli*
FCT	fibronectin-binding, collagen-binding T antigen
Flp	fimbrial low-molecular weight protein
GAS	group A streptococcus or *Streptococcus pyogene*
GBS	group B streptococcus or *Streptococcus agalactiae*
Lrp	leucine-responsive regulatory protein
PAMPs	pathogen-associated molecular patterns
Pap fimbriae	pyelonephritis-associated pili
PRP	proline-rich proteins
RSP	regulated secretory pathway
Sec	general secretion system
T4P	type IV pili
TCP	toxin-coregulated pili
UPEC	uropathogenic *E. coli*.

Glossary

Biofilm Dense mucous-like aggregation of a bacteria often bound to some solid surface, which may consist of multiple bacterial species.

Pilus Thin appendage on the surface of bacteria that can mediate binding with multiple substrates including other bacterial cells and host tissue cells.

Sec transolocon Complex of membrane associated proteins responsible for transporting peptides across the bacterial cell membrane.

Sortase A bacterial enzyme that cleaves a C-terminal sorting signal between threonine and glycine residues in the conserved LPXTG motif and catalyzes a transpeptidase reaction between the cleaved product and an aminoglycine substrate.

Twitching motility A form of bacterial motility across solid surfaces which is mediated by repeated cycles of extension and retraction of type IV pili.

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REVIEWS

Review

New Paradigms of Pilus Assembly Mechanisms in Gram-Positive Actinobacteria

Nicholas A. Ramirez,[1] Asis Das,[2] and Hung Ton-That[1,3,*]

Adhesive pili in Gram-positive bacteria represent a variety of extracellular multiprotein polymers that mediate bacterial colonization of specific host tissues and associated pathogenesis. Pili are assembled in two distinct but coupled steps, an orderly crosslinking of pilin monomers and subsequent anchoring of the polymer to peptidoglycan, catalyzed by two transpeptidase enzymes – the pilus-specific sortase and the housekeeping sortase. Here, we review this biphasic assembly mechanism based on studies of two prototypical models, the heterotrimeric pili in *Corynebacterium diphtheriae* and the heterodimeric pili in *Actinomyces oris*, highlighting some newly emerged basic paradigms. The disparate mechanisms of protein ligation mediated by the pilus-specific sortase and the spatial positioning of adhesive pili on the cell surface modulated by the housekeeping sortase are among the notable highlights.

Highlights

Covalently linked pili are assembled on the cell surface of many Gram-positive bacteria via a biphasic mechanism whereby pilus polymerization is catalyzed by the pilus-specific sortase followed by cell wall anchoring of pili by the housekeeping sortase.

Pilus-mediated adhesion depends on pilus length, which is modulated by the housekeeping sortase via unique structural features.

Some Gram-positive surface proteins with the LPXTG motif may hijack a pilus assembly machine via molecular mimicry to be displayed at the pilus tip.

Pilus-specific sortase enzymes provide a bioconjugation tool via the formation of an isopeptide bond that is mechanically stable and less susceptible to proteolytic cleavage.

Covalently-Linked Pili of Gram-Positive Bacteria

Fiber-like appendages called 'pili' or 'fimbriae' are microscopic structures present on the cell surface of both Gram-negative and Gram-positive bacteria. They are involved in a wide range of cellular activities, including adherence, motility, conjugation, and virulence [1–3]. Among these, the only pilus form known to date in which individual subunits are covalently bonded is the pili that are assembled by the action of sortase enzymes conserved in Gram-positive bacteria [4], but not in Gram-negative bacteria that produce pili in which the monomer subunits are joined via protein–protein interaction. The various sortases found thus far are broadly grouped into six classes (SrtA–SrtF), based on sequence alignment and substrate preference [5,6]; of these, only members of the class C and class A/E sortases are shown to catalyze the two distinct steps of Gram-positive pilus assembly: pilus polymerization and anchoring of pili to the cell wall, respectively (Box 1) [7].

Historically, the connection between sortase and pilus polymerization was somewhat serendipitous, based on two types of genetic observations. First, it was recognized that the protein sequences of different fimbrial subunits in the actinobacterium *Actinomyces naeslundii* harbor the same C-terminal cell wall sorting signal (CWSS) as the classically defined cell surface protein, protein A of *Staphylococcus aureus* [8,9] (Box 1). Second, many sets of genes coding for sortase enzymes and surface proteins with the LPXTG motif are clustered in the same operons in the actinobacterium *Corynebacterium diphtheriae* [10]. Indeed, immunoelectron microscopic analysis using antibodies against some of these surface proteins revealed the presence of distinct classes of pili on the surface of *C. diphtheriae* [10]. Since the first demonstration of the essential function of specific sortases in pilus assembly in *C. diphtheriae* [3,10,11], the past decade has seen extensive investigations of pilus assembly in many other Gram-positive bacteria, including *Bacillus cereus*, *Enterococcus faecalis*, and streptococci [12–18]. Because both *Actinomyces* and *C. diphtheriae* continue to serve as excellent models in the studies of the genetic, biochemical, and structural mechanisms of Gram-positive pilus assembly, we focus our present review on these two actinobacterial species only to highlight the recent advances in the field. For a more comprehensive

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Box 1. Overview of Classes A, C, and E Sortases

Staphylococcus aureus SrtA is the prototype class A sortase [61], which recognizes an LPXTG motif, preceding a hydrophobic domain and a positively charged tail that together constitute a 'cell wall sorting signal' located at the C-terminus of a sortase substrate [62]. As a transpeptidase, SrtA catalyzes cell wall anchoring of surface proteins harboring the tripartite sorting signal by first hydrolyzing the peptide bond between threonine and glycine and then covalently joining the cleaved threonine residue to the pentaglycine peptide of lipid II in the cell wall [63]. Structurally, the class A sortase harbors a unique β-barrel fold, with the catalytic site containing the sole cysteine residue [64], which is absolutely required for sortase activity [65].

Class C sortases, or pilus-specific sortases, are found in bacterial species that produce covalently linked pili [5]. They are structurally similar to class A sortases with the eight-stranded β-barrel fold encapsulating the active site [66]. Unique to class C sortases is a flexible N-terminal hydrophobic 'lid' that covers the catalytic pocket and has been proposed to play a role in substrate recognition and sortase stability [6,67–70].

Present in various Gram-positive bacterial species and abundant in actinobacteria [5,71], class E sortase enzymes recognize a distinct LAXTG sorting motif [5]. Compared with sortases of classes A and C, structures of class E sortases have been less well studied, with only two available sortase structures from *Streptomyces coelicolor* and *A. oris* [60,72]. While both harbor the conserved eight-stranded β-barrel fold without the aforementioned lid, they contain a conserved tyrosine residue within the β3/β4 sheet that appears to be involved in the recognition of the LAXTG sorting motif [60,72].

description of pilus assembly in Gram-positive bacteria, we refer the reader to several excellent publications elsewhere [19–21].

Corynebacterium diphtheriae Pili Offer a New Paradigm for Protein Ligation

Assembly of the SpaA Pilus

The causative agent of human diphtheria *C. diphtheriae* is among the earliest bacterial species where pili were identified [22,23]. As in many Gram-positive bacteria [4], the *C. diphtheriae* genes coding for distinct pilin subunits and dedicated pilus-specific sortases, which are all class C sortases, are organized into three operons [10]. Together, they encode three distinct pilus types specified by their major subunits: SpaA-type, SpaD-type, and SpaH-type pili [10,24,25]. Pili constitute one of the major virulence factors in *C. diphtheriae*: A mutant devoid of all pilins is highly attenuated in virulence in a mouse model of diphtheritic toxemia [26]. Each type of *C. diphtheriae* pili is heterotrimeric, meaning that each pilus type is made of three distinct pilin subunits; however, it is important to note further that the various molecules of the same pilus type (SpaA, SpaD, or SpaH type) vary in their length. Of these, the most highly studied is the SpaA pilus, which is composed of the shaft pilin SpaA, the tip adhesin SpaC, and the base pilin SpaB anchored to the cell wall; assembly of this pilus requires the cognate sortase SrtA, a class C sortase [10] (Box 1). While all three pilins contain the CWSS, only SpaA has a recognizable pilin motif with a conserved lysine residue that serves as a nucleophile essential for sortase-mediated crosslinking of pilin monomers. In this reaction that can occur repeatedly, the pilus-specific sortase SrtA catalyzes hydrolysis of the LPXTG motif in a pilin subunit and links the cleaved threonine residue to the lysine residue within the pilin motif of another pilin adjoining on the bacterial membrane (Figure 1). Because SpaC resides at the pilus tip (the tip first rule), the first transpeptidation reaction must occur between SpaC and SpaA, linking the threonine residue of the SpaC LPXTG motif to the reactive lysine residue within the pilin motif of SpaA (Figure 1). Subsequently, pilus elongation ensues whereby SpaA pilins are added to the growing chain of pilus polymers. Ultimately, pilus polymerization is terminated with addition of SpaB (the base pilin), which is then anchored to peptidoglycan by the housekeeping sortase SrtF (class E sortase), whose gene is not genetically linked (or in proximity) to any of the three pilus gene clusters [27] (Figure 1). This biphasic mechanism of pilus assembly appears to be universally applicable to other Gram-positive bacterial pili studied to date [12,28–30].

Genetic and biochemical studies together have provided groundbreaking evidence to support the various steps of the aforementioned model (Figure 1). Importantly, X-ray crystallographic

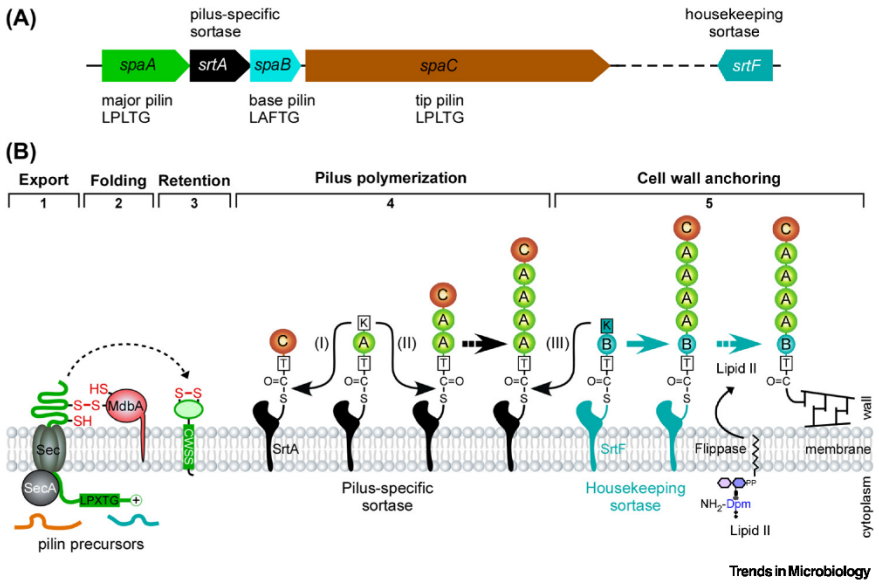

Figure 1. Assembly of the Heterotrimeric SpaA Pili in *Corynebacterium diphtheriae*. (A) Shown is the *spaA* pilus gene cluster in strain NCTC13129, encoding the pilus shaft SpaA, pilus base SpaB, and pilus tip SpaC and the pilus-specific sortase SrtA. The SpaABC pilins contain a cell wall sorting signal with the LPLTG or LAFTG motif. The housekeeping sortase gene *srtF* is located elsewhere in the bacterial chromosome. (B) A biphasic assembly mechanism of Gram-positive pili is depicted here with the SpaA-type pili. Pilin precursors are secreted in an unfolded state across the cytoplasmic membrane through the Sec translocon (Step 1). The membrane-bound thiol-disulfide oxidoreductase MdbA mediates disulfide bond formation and folding of pilin subunits (Step 2) prior to their insertion into the membrane (Step 3). The pilus-specific sortase SrtA catalyzed pilus polymerization into pilus fibers through successive lysine-transpeptidase reactions (Step 4). Incorporation of SpaB to the base of the pilus signals cell wall anchoring of the pilus by housekeeping sortase SrtF (Step 5). The order of the described transpeptidation reactions is marked by roman numerals (I–III). Reproduced, with permission, from [7].

studies of the SpaA pilin led to the discovery of an intramolecular disulfide bond and the role of a disulfide bridge-forming machine, termed 'MdbA,' which is critically involved in mediating the post-translocational folding of the SpaA precursor pilin prior to its polymerization by pilus-specific sortase SrtA in the exoplasmic environment [26]. As expected from the model, deletion of *srtA* completely abolishes SpaA pilus polymerization [10], as does the genetic replacement of the nucleophilic lysine residue of the pilin motif (lysine-to-alanine substitution) that prevents pilus crosslinking [11]. The complete loss of SpaA pilus assembly *in vivo* in the absence of SrtA also demonstrates the substrate specificity of the pilus-specific sortase enzyme SrtA, as other pilus-specific sortases expressed *in vivo* cannot substitute for SrtA function. When *srtF* or *spaB* is absent, however, abundant amounts of the generated pilus polymers are secreted into the culture medium, supporting the model that the pilus polymerization phase precedes the cell wall anchoring phase and that SpaB incorporation acts as a pilus termination switch in this biphasic mode of pilus assembly [27,31].

Recently, *in vitro* reconstitution of a Gram-positive pilus assembly system was described for the first time. The system uses recombinant sortase enzyme and pilin substrate proteins and has provided yet another foundational support for the biphasic model described above. This remarkable success in biochemical reconstitution was facilitated by structural genetic studies of the pilus-specific sortase SrtA, which uncovered novel structural features of the enzyme, on the one hand, and, on the other hand, also led to the engineering of a robust protein-polymerizing

machine. As outlined in Box 1, the pilus-specific sortase SrtA harbors a structural lid that appears to occlude the enzyme's catalytic pocket. Mutations of this lid could unmask the active site, thereby amplifying the net rounds of SpaA polymerization by this mutant enzyme (^{Cd}SrtA^{2M}) to a level that was never observed with the wild-type enzyme under comparable conditions [32].

The *in vitro* pilus assembly reaction contained the recombinant SrtA sortase truncated for its membrane localization domain and a SpaA protein devoid of its hydrophobic domain and charged tail [32], generating substantial amounts of pilus polymers within 24 h that were easily detected by SDS-PAGE and Coomassie staining. Importantly, electron microscopic sampling revealed SpaA polymers with many >1-μm-long fibers, while mass spectrometry authenticated the isopeptide linkage connecting individual subunits. Polymerization was abolished by a catalytic site mutation (C222A) and a pilin motif mutation (K190A); furthermore, the formation of an acyl-enzyme intermediate between SpaA and sortase, as the model predicted, was also observed. Remarkably, when SpaB or SrtF protein was added to the reaction, pilus polymerization was terminated, another prediction of the model [32]. The fact that SpaA polymers are formed without the presence of the tip pilin SpaC confirms the previous genetic observation that SpaC is dispensable for pilus assembly [10]. In essence, this test tube version of the reaction recapitulates much of the pilus assembly process that is observed in *C. diphtheriae* cells.

Protein Ligation with Pilus-Specific Sortase

The ability of a sortase to ligate proteins or peptides has significant implications in protein engineering, cell biology, and biomedicine. Indeed, prior to the work with a pilus-specific sortase described above, Mao and coworkers creatively used the most active recombinant sortase enzyme studied to date [i.e., *S. aureus* SrtA (^{Sa}SrtA)] in protein ligation [33]. In this study, the recombinant staphylococcal SrtA is capable of joining one substrate protein that is C-terminally tagged with the LPXTG peptide with another substrate N-terminally tagged with the G_n peptide (n = 1–5). Ploegh and colleagues further advanced this method for protein labeling in living cells [34]. Dubbed 'sortagging,' this method appears to be a promising new engineering tool, and it has been further optimized for site specificity and the ability to covalently link peptides to a variety of nonpeptide substrates, including folate, amino-terminated or glycine-tagged polyethylene glycol, and beads [33,35]. Sortagging was also used to create peptide nucleic acid cell-penetrating peptide conjugants, thus providing an exciting new tool for designing highly specific cell-permeable drug therapies [36]. Importantly, the high affinity of sortase for the LPXTG motif has yielded yet another protein capture method in which LPXTG motif–containing peptides can be efficiently and specifically captured from complex cell lysates [35,37]. The ability of sortase to mediate the stable anchoring of proteins to surfaces for microarray-based protein activity assays has also been explored, as staphylococcal SrtA has been used to covalently attach LPXTG-containing proteins to planar surfaces, such as glass coverslips, following treatment of the surface with aminosilane and oligoglycine [37]. Sortase protein–labeling technology is not limited to protein extract applications, as staphylococcal SrtA was used to efficiently ligate a modified pentaglycine probe to the surface of the transmembrane protein CD40L in live HEK293T cells [34]. Finally, Tanaka and colleagues successfully used sortagging as a method of protein-specific fluorescent labeling by conjugated glycine-containing biotin, enhanced GFP, and Alexa Fluor probes to the transmembrane protein osteoclast differentiation factor in HEK293T cells, without inducing any toxic phenotype to the cell culture [38].

The protein ligation reaction catalyzed by *S. aureus* SrtA is limited to the N-terminal to C-terminal protein joining involving two defined substrates. By comparison, a variety of pilus-specific sortase enzymes identified to date offer the unique advantage as a bioconjugation tool via the formation of an isopeptide bond that is mechanically stable and less susceptible to proteolytic cleavage

[39,40]. Recently, McConnell and colleagues generated a recombinant *C. diphtheriae* sortase enzyme termed '^{Cd}SrtA^{3M}' that is more reactive than ^{Cd}SrtA^{2M} described above. When a substrate protein containing the pilin motif was incubated with GFP harboring a C-terminal LPLTG motif in the presence of ^{Cd}SrtA^{3M}, the mutant enzyme catalyzed the covalent joining of the two recombinant proteins [41]. Furthermore, these authors demonstrated that both sortases ^{Sa}SrtA and ^{Cd}SrtA^{3M} can be used in sequential transpeptidation reactions to modify a protein of interest at distinct sites and with high specificity. Using a small ubiquitin-like modifier (SUMO) engineered to contain an N-terminal pentaglycine peptide and a C-terminal pilin motif, ^{Cd}SrtA^{3M} was first used to catalyze the addition of a fluorescein isothiocyanate (FITC) tag harboring the LPLTG motif to the lysine residue of the pilin motif. ^{Sa}SrtA was then used to conjugate Alexa Fluor 546 harboring the LPATG motif to SUMO via pentaglycine [41]. Because of the high degree of specificity for the ε-amine nucleophile within the pilin motif, protein ligation using pilus-specific sortase enzyme may provide selective labeling [41].

Actinomyces Fimbriae: A Paradigm of Tissue Tropism, Hijacking of Pilus Machinery, and Spatial Positioning of Pili

Heterodimeric Fimbriae of *Actinomyces oris*

Actinomyces are one of the most dominant and earliest colonizing genera of microbes present in the human oral cavity, with *A. oris* (formerly called *Actinomyces naeslundii*) detected in children as young as 1 year old [42,43]. *A. oris* is a major contributor to dental plaque through its ability to coaggregate with other microbial species and thus a key to the genesis of complex biofilms on the surface of teeth and the mucosal epithelia [44–46]. This intrinsic adherence property of *A. oris* is largely attributed to the presence of two distinct fimbrial types: type 1 and type 2 fimbriae. *A. oris* has served as a pioneering model of tissue tropism mediated by Gram-positive pili, as type 1 fimbriae mediate bacterial adherence to the salivary proline-rich proteins normally coating the tooth enamel [47], whereas type 2 fimbriae promote bacterial binding to receptor polysaccharides present on the surface of oral streptococci and various host cells [48–51]. Unlike *C. diphtheriae* and many other Gram-positive bacteria, *A. oris* fimbriae are heterodimeric, containing a tip component and another entity forming the pilus shaft. In the case of type 1 fimbriae, FimP forms the pilus shaft with FimQ located at the tip, and their assembly requires the pilus-specific sortase SrtC1, whose genes are all tightly linked together on the *Actinomyces* genome [52]. The specificity of *Actinomyces* sortases appears to be strict, as the pilus-specific sortase SrtC2 is selectively required for formation of type 2 fimbriae only, which consist of the shaft FimA and tip FimB [53]. Since there are only two components in each fimbria, the last subunit of the shaft pilins should be the pilus base. This has raised an intriguing question of how pilus polymerization in *Actinomyces* or in any other two-component pilus systems, such as *B. cereus* [12] and *Streptococcus suis* [54], is terminated.

Using the type 2 fimbriae of *A. oris* as a prototype, the model of pilus assembly in *Actinomyces* is illustrated in Figure 2 [55]. Similar to what is described above for *C. diphtheriae*, pilin precursors translated in the cytoplasm are transported across the cytoplasmic membrane by the Sec translocon, and post-translocational protein folding is mediated by the disulfide bond machine MdbA, permitting membrane insertion of the pilin precursors [56]. When both tip and shaft pilins are available on the membrane, pilus polymerization is catalyzed by the pilus-specific sortase SrtC2 [57]. Finally, cell wall anchoring of the type 2 fimbriae is mediated by the housekeeping sortase SrtA, a class E sortase [58]. Surprisingly, unlike all known sortases studied to date, *A. oris srtA* is an essential gene. Genetic and biochemical studies to reveal the basis of *srtA* essentiality (Box 2) have serendipitously uncovered the molecular basis of regulated pilus polymerization and spatial positioning of pilus adhesins in *Actinomyces* that could not have been envisioned before (see below).

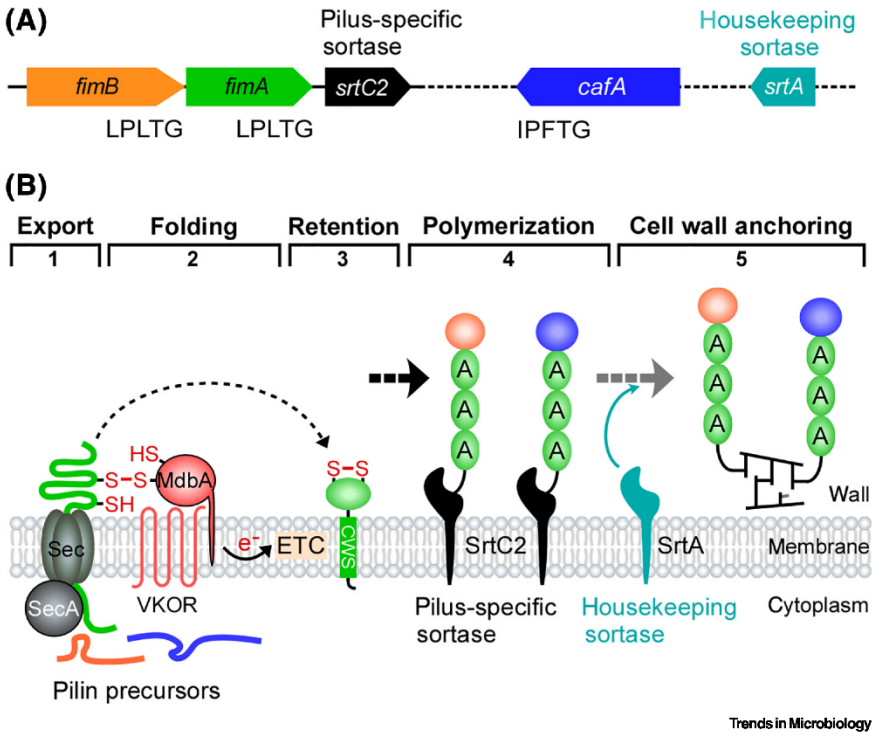

Figure 2. Assembly of the Heterodimeric Type 2 Fimbriae in *Actinomyces oris*. (A) The type 2 fimbriae are encoded by the three-gene operon. Genes encoding the coaggregation factor CafA and the housekeeping sortase SrtA are located elsewhere. (B) Similar to the assembly mechanism of the SpaA pili, assembly of the type 2 fimbriae begins with translocation (Step 1) and post-translocational folding of the shaft pilin FimA and tip pilin FimB mediated by the disulfide bond–forming machine MdbA/VKOR (vitamin K epoxide reductase) coupled to the electron transport chain (ETC) (Step 2). Membrane insertion (Step 3) of these pilins permits pilus polymerization catalyzed by the pilus-specific sortase SrtC2 (Step 4), followed by cell wall anchoring of the pilus polymer catalyzed by the housekeeping sortase SrtA. Reproduced, with permission, from [55].

Coaggregation Factor CafA Illustrates Pilus Hijacking in Gram-Positive Bacteria

The discovery that CafA is the coaggregation factor in *A. oris* has several significant implications, one that provides a paradigm of a surface protein hijacking the pilus assembly machine for pilus display and another a concept of spatial positioning of pilus adhesins for biological functions (see below). As mentioned above, type 2 fimbriae are essential for *A. oris* interactions, or coaggregation, with other oral bacteria, especially oral streptococci, as deletion of *fimA* abrogates coaggregation with *Streptococcus oralis* [57]. Surprisingly, *A. oris* coaggregation could not be blocked by polyclonal antibodies against FimA, nor was the process affected by a deletion

Box 2. Molecular Basis of *srtA* Essentiality in *A. oris*

Unlike all other sortases studied to date, *A. oris srtA* is an essential gene, as deletion of *srtA* has proved to be lethal [58]. A genetic suppressor screen – by Tn*5* transposon mutagenesis – subsequently revealed that *srtA* essentiality is linked to the toxic accrual of a normally cell wall–anchored glycoprotein GspA, an SrtA substrate harboring a cell wall sorting signal with the LAXTG motif. In the absence of *srtA*, glycosylated GspA accumulates in the cytoplasmic membrane, causing lethal 'glyco-stress' accompanied by expansion of the cell envelope and cell growth arrest [58]. An *lcp* mutant devoid of the glycosyltransferase LcpA and unable to glycosylate GspA [73] is one of 13 identified suppressor mutants, so is a *gspA* mutant lacking the cell wall sorting signal that permits membrane insertion prior to cell wall anchoring [58]. This illustrates the power of forward genetic analysis and the continued utility of isolating genetic suppressors in unveiling the intricacies of microbial genetic mechanisms.

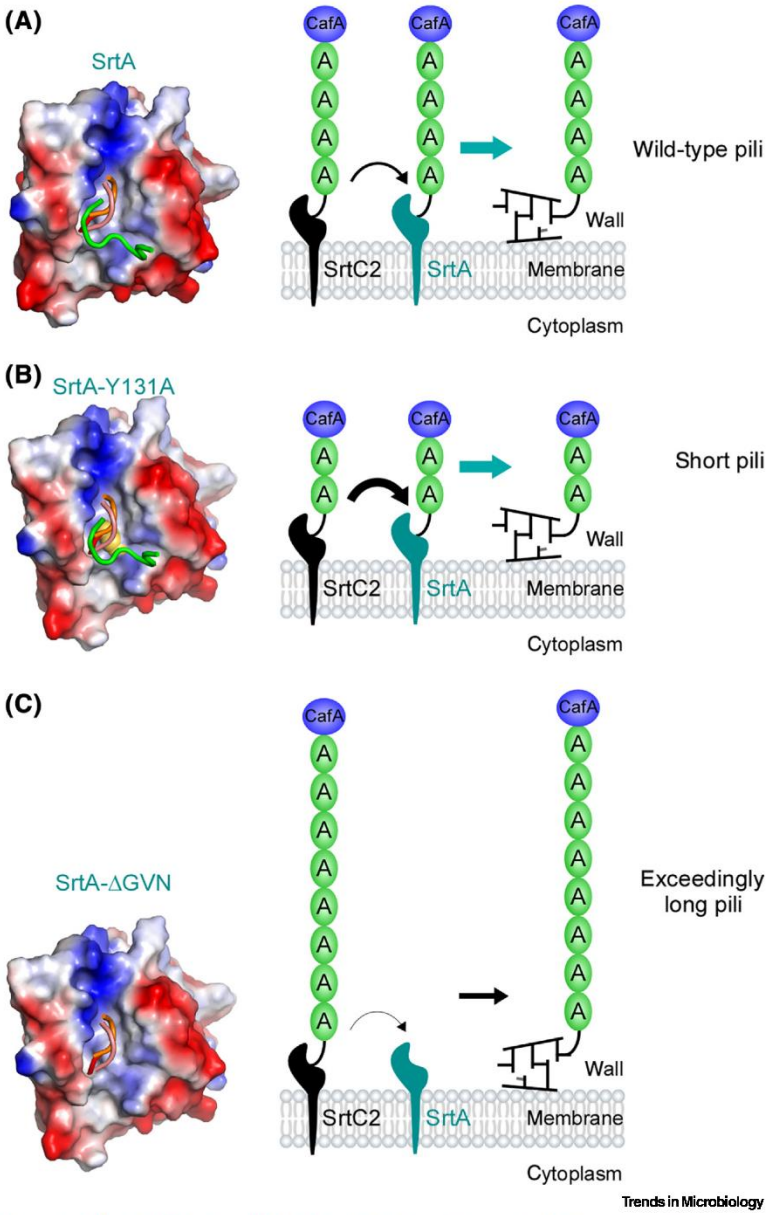

Figure 3. A Model of Pilus Length Modulated by Sortase Enzymes in *Actinomyces oris*. X-ray crystallization revealed the structural features of the housekeeping sortase SrtA: the tyrosine residue Y131, and the tripeptide loop GVN. (A) In the wild-type MG1, balanced activities of the pilus-specific sortase SrtC2 and the housekeeping sortase SrtA produce type 2 fimbriae with typical length. (B) Alanine substitution of Y131 enhances SrtA sortase activity for the LPLTG motif of FimA, interfering with pilus polymerization, resulting in premature cell wall anchoring of short pili. (C) Mutation or deletion of the GVN loop reduces SrtA preference for the LPLTG motif of FimA, leading to continuous polymerization by SrtC2 and generating exceedingly long fimbriae that can be anchored to the cell wall by this pilus-specific sortase enzyme. Reproduced, with permission, from [60].

of *fimB*, the gene that encodes the type 2 fimbrial tip pilin FimB [59]. Thus, a hunt was on for discovering the responsible adhesive principle that defied molecular genetic identification strategies used so far. One potential scenario was that the FimA shaft contains some other protein to mediate coaggregation. This prompted a systematic elimination of each individual LPXTG-containing surface protein encoded in the *Actinomyces* genome. Indeed, among the 14-candidate surface protein–encoding genes successfully deleted, one displayed a clear-cut coaggregation defect on its own [59]. The implicated gene product, thus named CafA, was subsequently proved to be the long-sought-after coaggregation factor by biochemical experiments: Antibodies against CafA captured type 2 fimbriae and blocked bacterial coaggregation. Electron microscopic analyses revealed that CafA localizes at the pilus tip, forming a distinct pilus structure with shaft pilin FimA. Intriguingly, the CWSS sequence of CafA is strikingly similar to that of FimB, leading to the hypothesis that some Gram-positive surface proteins may hijack a pilus assembly machine via molecular mimicry to be displayed at the pilus tip [59]. As significant as this may be for advances in oral bacterial biology and therapeutic intervention, the broader implication of whether the general mechanism of pilus hijacking is more widespread remains to be investigated.

Spatial Positioning of Pilus Adhesins

Over a decade ago, it was speculated that Gram-positive adhesins appended at the pilus tip mediate the initial bacterial encounter with host cells due to the extended nature of pili [3]. Because pilus lengths vary greatly within individual pilus types and among various types of pili, it is important to know whether pilus-mediated adhesion processes depend on pilus length and whether and how pilus length is controlled in Gram-positive bacteria. A breakthrough in this problem came from the observation that *A. oris* mutants lacking the housekeeping sortase *srtA* produced exceedingly long pili, as might be expected, but surprisingly, they failed to adhere to oral streptococci [58]. This was puzzling, since the coaggregation factor CafA was still abundantly detected at the tips of these long pili [60]. Through a series of probing experiments, Chang and colleagues demonstrated that as the pilus length was shortened by inducing expression of *srtA*, coaggregation could be restored, supporting the notion that the enzymatic activity of the housekeeping sortase SrtA is a key determinant of pilus length modulation. This new insight leads to the important question of whether SrtA activity is subject to regulation functionally or genetically. X-ray crystallization revealed that SrtA harbors two structural elements: a conserved tyrosine residue Y131 and a GVN tripeptide loop that may be of regulatory significance. Indeed, alanine substitution of Y131 residue resulted in the production of shorter pili and defective coaggregation by *A. oris*, whereas mutations of the GVN loop led to assembly of extremely long pili and no coaggregation by the mutant bacteria [60], the phenotype similar to *srtA* depletion [58].

These results led to a mechanistic model that Y131 mutations alter the preference of the class E sortase SrtA, which normally recognizes the LAXTG motif, toward the LPXTG motif present in the FimA pilin subunits. As a result, pilus polymerization is terminated by SrtA-catalyzed cell wall anchoring of the FimA polymer, leading to the display of short pili on the cell surface. Conversely, in the case of the GVN motif, its mutations diminished SrtA's preference for the LAXTG motif, making SrtA's capacity limited for cell wall anchoring. As a consequence, pilus polymerization continues unperturbed, leading to extremely long pili (Figure 3). Consistent with this model, the deletion of *gspA*, which codes for one of the most abundant SrtA substrates with the LAXTG motif, resulted in normal assembly of FimA pili in the GVN mutation background and enabled positive coaggregation by the mutant strain. Together, these structural genetic findings provide compelling grounds to posit that the housekeeping sortase functions as a molecular ruler for pilus polymerization and, as such, a positive effector of bacterial coaggregation and virulence.

Concluding Remarks

Collective efforts during the last decade dissected the molecular assembly mechanisms of Gram-positive pili and probed their roles in bacterial pathogenesis and their use in the development of vaccines. While pilus vaccines have yet to emerge in the clinical arena, we now have made great strides in the basic biology and have a clearer view of pilus biogenesis in Gram-positive bacteria. A common feature in these monoderms is the biphasic mode of pilus assembly by distinct steps of enzymatic catalysis involving two sortases whereby pilus polymerization catalyzed by pilus-specific sortase is followed by cell wall anchoring of pili promoted by the housekeeping sortase. Regardless of the sortase enzymes involved, the basic principle of these transpeptidation reactions in the polymerization phase is the same: the enzymatic cleavage of a substrate and covalent linkage of the cleaved substrate to a nucleophilic acceptor. This transpeptidation reaction generates an isopeptide bond that is mechanically strong and can resist a potential unfolding force up to 690 pN [39]. This unique property of isopeptide bonding, via lysine and threonine residues, is protease resistant and offers a versatile tool in protein engineering and bioconjugation [41].

Where do we go from here? In spite of differences with their Gram-negative counterparts in the manner of assembly, the heteromeric pili of Gram-positive bacteria play significant roles in bacterial physiology and virulence as those of Gram-negative bacteria. Today, however, many fundamental questions regarding this are waiting to be addressed (see Outstanding Questions). Given the importance of these questions and the genetic and biochemical versatility of *C. diphtheriae* and *A. oris* as model organisms and their importance in significant human conditions, we believe these two systems will continue as fertile and attractive experimental models of pilus biogenesis in Gram-positive bacteria for some time to come.

Outstanding Questions

In the actinobacteria *C. diphtheriae* and *A. oris*, the membrane-bound disulfide bond–forming machine MdbA promotes post-translocational folding of pilins. Given that no major protein-folding machines are linked to pilus assembly in Firmicutes, how do these organisms solve the protein-folding problem in pilus assembly?

In a heterodimeric pilus system such as *A. oris*, the last shaft pilin acts as a pilus base and stop signal, and the housekeeping sortase appears to control pilus polymerization and hence pilus length. How does the housekeeping sortase indiscriminately recognize this base pilin from the rest? This raises an intriguing possibility that pilus polymerization and termination may depend on the stoichiometric availability of pilin substrates and sortase enzymes at the pilusosome. If so, does this require additional factors?

How are the tip pilins FimB and CafA in *A. oris* involved in pilus assembly? What mechanisms govern how a tip pilin is spatiotemporally incorporated only at the tips of pili?

How do surface proteins with the LPXTG motif such as CafA in *A. oris* hijack the sortase machine to be incorporated into the pilus tip?

Pili are found in the culture medium, especially in late log phase and stationary phase. What are the roles of secreted pili, or are they products of cell wall turnover?

Acknowledgements

We would like to dedicate this review to our late former mentor and colleague Dr Olaf Schneewind, who discovered the cell wall anchoring pathway of Gram-positive bacterial surface proteins. His amazing insights and warm friendship will be sorely missed. We thank our laboratory members for critical review of the manuscript and for discussion. Work in the Ton-That laboratory related to Gram-positive pili has been supported by the National Institute of Dental and Craniofacial Research (NIDCR)/National Institutes of Health (NIH) under award numbers DE025015 and DE017382 (H. T-T.).

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Chapter 4: A Conserved Signal-Peptidase Antagonist Modulates Membrane Homeostasis of Actinobacterial Sortase Critical for Surface Morphogenesis

4.1 Introduction

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* 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 glycol-stress (19). LepB2 is one of two signal peptidases in *A. oris* (21). Critically, a non-polar, 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 **s**ortase-**a**ssociated **f**actor), encoded by a gene immediately downstream of the housekeeping sortase *srtA* in *A. oris* (19). This transmembrane protein is highly conserved in Actinobacteria (see Appendix Figure A-1) – 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 Figure 4.1) 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 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 new paradigm for future investigations in other bacteria of this phylum, many of which are human commensals and pathogens

4.2 Materials and Methods

4.2.1 Bacterial strains, plasmids, and media

Bacterial strains and plasmids used in this study are listed in Appendix Table A-2. *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 37ºC 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.

4.2.2 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 Appendix A (16, 23).

4.2.3 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 37ºC to mid-log 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).

4.2.4 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 mid-log phase at 37ºC 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 10mM 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 minutes at 37ºC. 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).

4.2.5 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 (20mM Tris-HCl pH: 7.4, 150mM NaCl, 1mM $CaCl_2$) in a 1:1 ratio, and agitated by gentle rotational shaking. Coaggregation was recorded by a FluorChem Q (Protein Simple).

4.2.6 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 hours 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 minutes, washed 3 to 5 times with water, de-stained, and dissolved in 30% acetic acid for 5 minutes, and quantified by measuring absorbance at 580 nm.

4.2.7 Bacterial two-hybrid

Cells of the *E. coli* adenylate cyclase deficient strain BTH101 were grown at 30°C to mid-log 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 uL of electrocompetent cells. Transformations were conducted via electroporation in pre-chilled 1-mm gap cuvettes under the following conditions: 2.5 kV, 25 μF capacitance, 100 Ω resistance. Cells were allowed to recover for 2 hours 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. 4-uL aliquots of each cell suspension was spotted onto MacConkey agar plates supplemented with 1% w/v maltose, 50μg/mL kanamycin, and 100μg/mL ampicillin and incubated at 30°C for up to 72 hours prior to imaging.

To quantify BACTH interaction, a ß-galactosidase assay was followed as previously described (25, 26). BTH101 cells grown overnight in LB supplemented with 0.5mM 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, 1mM $MgSO_4$, 0.05M β-mercaptoethanol, pH 7.0) and lysed by the addition of chloroform and SDS. Ortho-Nitrophenyl-ß-galactoside (ONPG) was added to cell lysate and incubated 35 minutes at 30ºC before quenching by the

addition of Na_2CO_3. OD_{420nm} was recorded and Miller units calculated using the equation, Miller units = 1000 * [(OD_{420nm} / (OD_{600nm} of culture * volume of culture in mL * reaction time in mins)]. Experiments were performed in triplicate and statistical analysis was determined by *t*-test using GraphPad Prism.

4.2.8 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.

4.3 Results

4.3.1 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 (Figure 4.1A). 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* (Figure 4.1A and Appendix Figure A-1). Interestingly, in *Bifidobacterium dentium*, a SafA-like domain is fused to the C-terminus of the housekeeping

sortase (Figure 4.1A and Appendix Figure A-1), further supporting the idea of co-evolution and functional relationship between SrtA and SafA.

To elucidate the function of SafA in *A. oris*, we first generated a non-polar, 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 polymerase chain reaction (qRT-PCR). As shown in Figure 4.1B, 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) (Figure 4.1C; 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 ($SrtA_m$) (Figure 4.1C; 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 (Figure 4.1B), 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 (Figure 4.1D; 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 (Figure 4.1D; 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 (Figure 4.1D; last 4 lanes). The results suggest that SafA might block proteolytic cleavage and secretion 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 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 (Figure 4.1E). 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 that are anchored to the cell wall by pilus-specific sortase SrtC2 (20) – both phenotypes similar

to that of *srtA* disruption (Figure 4.1F) 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 (Appendix Figure A-2). Importantly, ectopic expression of *safA* from *A. oris*, *C. diphtheriae* and *C. matruchotii* successfully rescued the defects of the *safA* mutant (Figure 4.1F and Appendix Figure A-3).

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 wildtype *A. oris* and *Streptococcus oralis* interact and form visible clumps of bacteria. As shown in Figure 4.1G, 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 (Figure 4.1G and Appendix Figure A-3). 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 wildtype, we sought to determine if loss of *safA* alters the ability of *A. oris* to form mono-species biofilms *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* (Figure 4.1H-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.

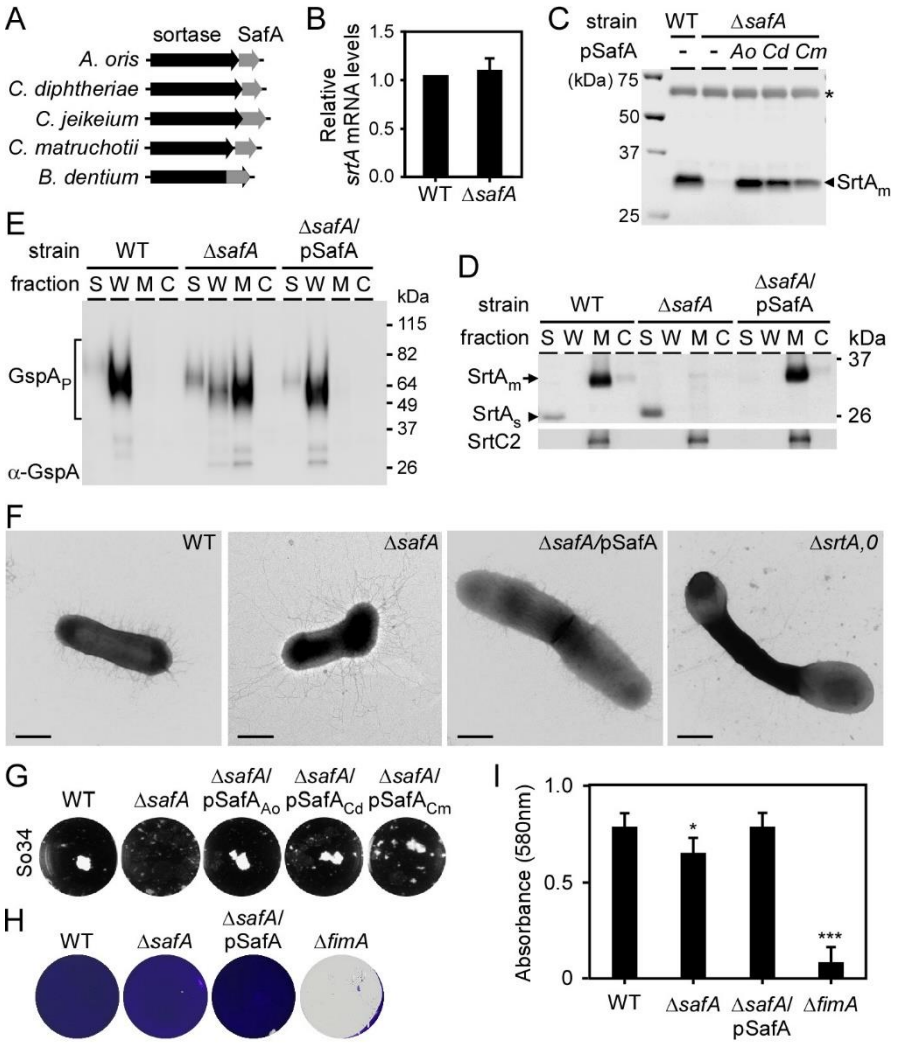

Figure 4.1 A conserved membrane protein, SafA, is required for membrane localization of the housekeeping sortase SrtA, cell morphology, surface assembly, and biofilm formation. (**A**) Presented are genetic loci coding for the housekeeping sortase (black) and a conserved membrane protein, SafA (grey), 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 standard deviation. 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 mid-log 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 non-specific band (asterisk) serving as loading control. (**D**-**E**) Equivalent cells of indicated strains grown to mid-log 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**) Mid-log 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 indicates 0.5 μm. (**G**) Equal cell numbers of indicated *A. oris* strains and *S. oralis* So34 were mixed in co-aggregation 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; *, $p < 0.05$; ***, $p < 0.001$.

4.3.2 The transmembrane SrtA protein contains a non-canonical signal peptide whose cleavage is blocked by SafA

The results shown in Figure 4.1D above suggest that SrtA might be subjected 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 (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 (Figure 4.2A). All of these features are typical of a bacterial signal peptide sequence (27). We also found similar domains in the N-terminal sequence of the housekeeping sortase SrtF in *C. diphtheriae*, but not in *S. aureus* SrtA (Figure 4.2A), which reportedly does not harbor a signal peptide (28).

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 (Figure 4.2B; 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 (29, 30), 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 (Figure 4.2B; lanes S57P). Replacement of the potential cleavage site AXA motif with FFF residues also enhanced membrane retention (Figure 4.2B; 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 (Figure 4.2A, highlighted in light blue) completely prevented SrtA cleavage and enhanced membrane localization of SrtA in the absence of SafA (Figure 4.2B; lanes Δ13). A similar phenotype was observed when this region was replaced by 13 amino acids from *S. aureus* (Figure 4.2B; 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 (Figure 4.2B; lanes *Cd*13). This establishes that that 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} (Figure 4.2A; H6), and this construct ($SrtA_{H6}$) was introduced in the same strain Δ(*srtA*/*safA*/*gspA*) (Figure 4.2C). Compared to wildtype SrtA,

$SrtA_{H6}$ was similarly processed (Figure 4.2C). Using this H_6-engineered SrtA, we purified the cleaved SrtA fragment from the culture supernatant by affinity chromatography (Figure 4.2D) and analyzed the cleaved sequence by N-terminal Edman degradation sequencing as previously described (21). The result (Table S1) proved that the cleavage takes place between residues A^{56} and S^{57} as predicted (Figure 4.2A).

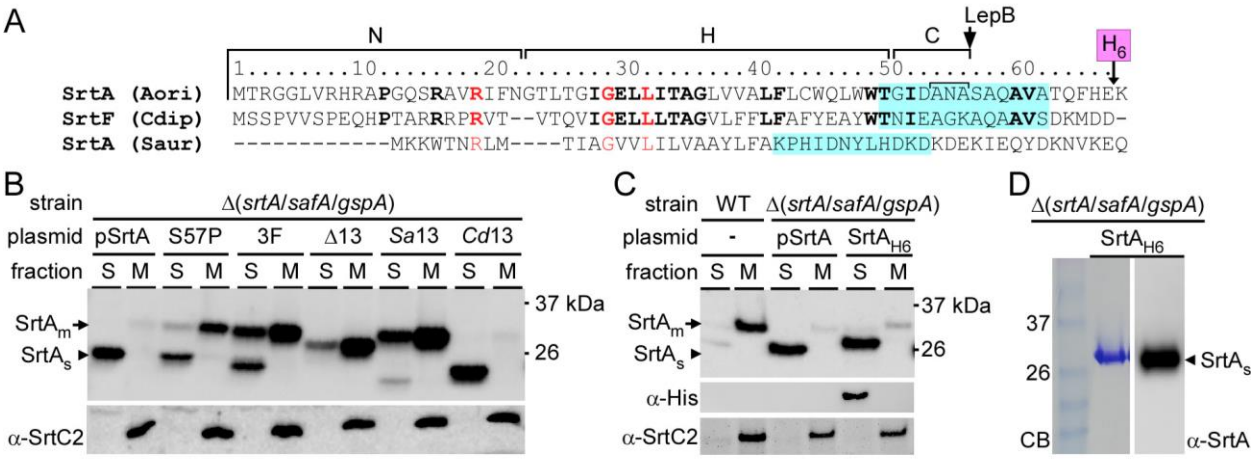

Figure 4.2 The housekeeping sortase SrtA harbors a cleavable signal peptide sequence. (**A**) *A. oris* SrtA appears to contain a signal peptide sequence with a positively charged N-region, hydrophobic (H), and a C-region consisting of a conserved AXA motif (bracket) predicted to be cleaved by the signal 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.

4.3.3 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 Figure 4.3A, 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 secretion into the medium. Further, in contrast to the Δ*safA* mutant, in which SrtA is largely cleaved and secreted into the medium (see Figure 4.1D), the Δ*safA*/Δ*lepB2* double mutant displayed mostly unprocessed SrtA on the membrane (Figure 4.3A; 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 (Figure 4.3A; last six lanes). Clearly, LepB2 is the signal peptidase that processes and secretes 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 wild-type cell morphology, although it produced less pili (Figure 4.3B). This is consistent with our previous report that establishes the role of LepB2 in pilus assembly (21); of note, deletion of *lepB2* 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 (Figure 4.3B). Furthermore, expression of the catalytically inactive LepB2 mutants, S101A or K169A, in Δ*safA*/Δ*lepB2*

phenocopied this double mutant (Figure 4.3B). 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.

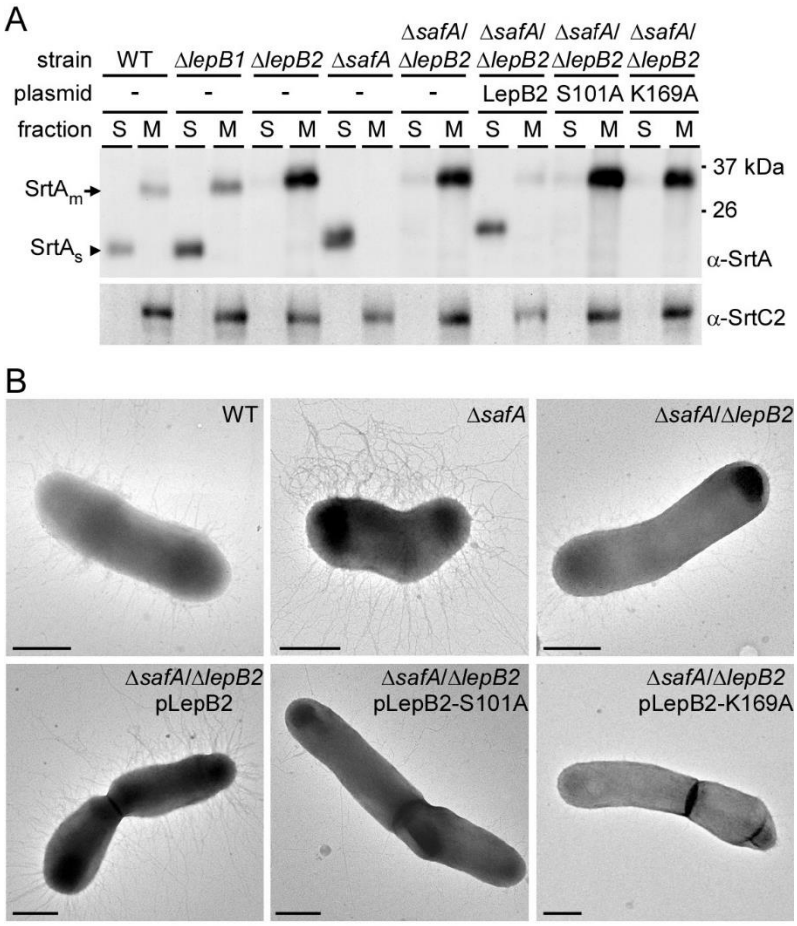

Figure 4.3 SafA prevents SrtA from cleavage by the signal peptidase LepB2. (**A**) Indicated strains, including strains expressing wild type LepB2 or its catalytically inactive mutants (S101A and K169A), were analyzed by immunoblotting with α-SrtA and α-SrtC2. (**B**) Cells of indicated strains were analyzed by electron microscopy as described in Figure 4.1F; scale bars of 0.5 μm.

4.3.4 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 towards the cytoplasm and the C-terminus toward the exoplasm (see TMHMM 2.0 Server, http://www.cbs.dtu.dk/services/TMHMM/) (Figure 4.4A). 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 (Figure 4.4B). Analysis of these fusion constructs demonstrated that they functionally complemented the Δ*safA* mutant and were able to restore membrane localization of SrtA (Figure 4.4C). 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 (Appendix Figure A-4). 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 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 Appendix Figure A-3 and Figure 4.4C, 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 (Figure 4.4D).

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 (26, 31). 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 ß-galactosidase activity. As shown in Figure 4.4F, 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 (Figure 4.4E-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 (Appendix Figure A-1 and Figure 4.4E). 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 (Figure 4.1), the recombinant wild type His-tagged SafA was membrane embedded and enabled membrane localization of SrtA (Figure 4.4G, 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 (Figure 4.4G; lanes $SafA_1$ and $SafA_2$, respectively). Immunoblotting for the His-tag revealed 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 (Figure 4.4G; 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 (Figure 4.4F). We infer that in *A. oris*, the intra-membranous SrtA and SafA interact with each other and that the exoplasmic mini-motif 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.

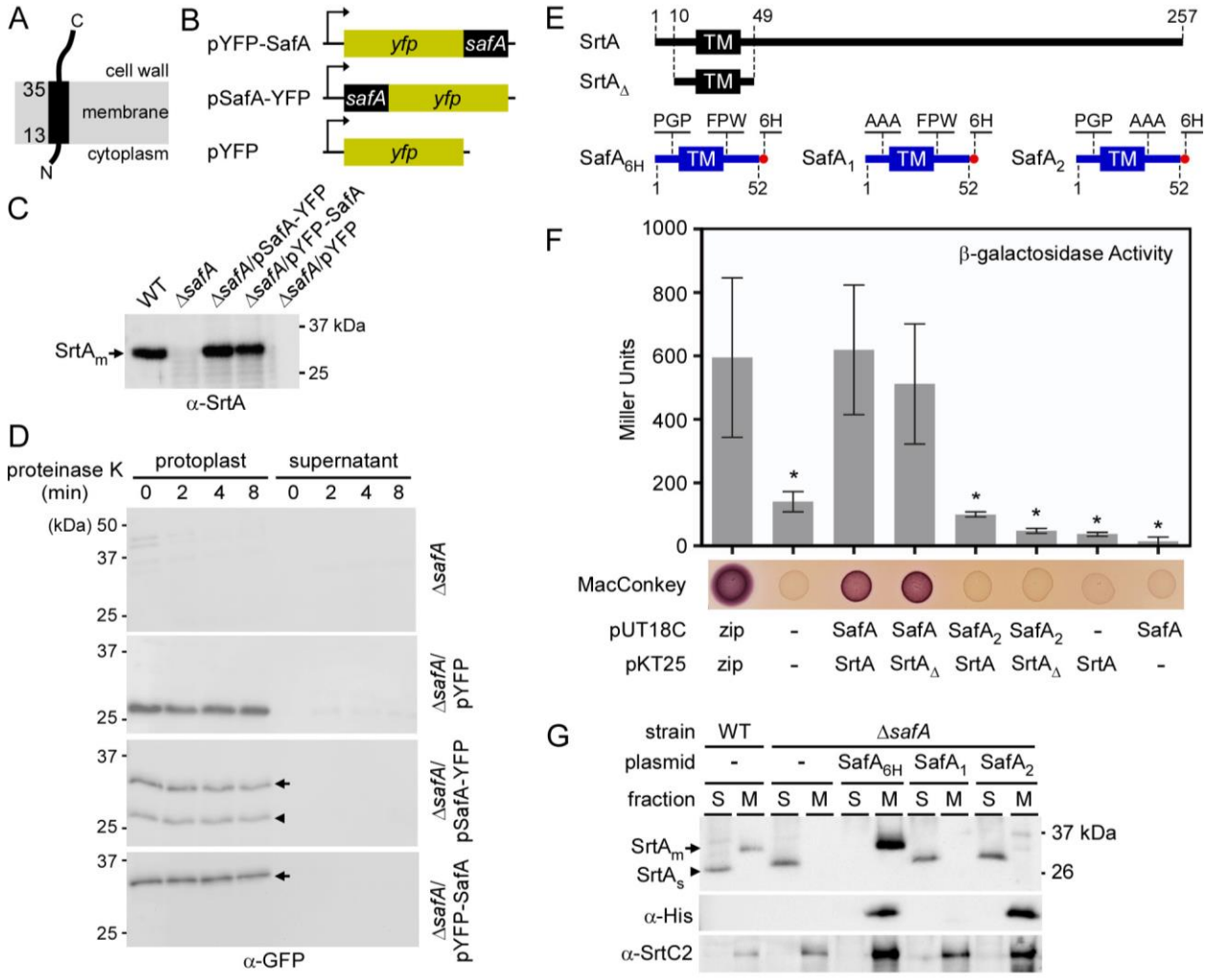

Figure 4.4 Conserved residues within SafA are essential for interaction with SrtA. (**A**) Membrane topology of SafA is predicted by TMHMM (32), with the N-terminus facing the cytoplasm and the C-terminus towards the cell wall. (**B**) Shown are recombinant plasmids expressing yellow fluorescent proteins (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**) Mid-log 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 co-expressed in *E. coli* BTH101 cells. SrtA-SafA protein interaction was determined by MacConkey agar plating or quantified by ß-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 Figure 4.3A.

4.4 Discussion

Short open reading frames (ORFs) coding for small proteins in bacteria have been overlooked in traditional systematic genome annotations and comparative genomics (33). 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 (34). 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 (Figure 4.1A). We readily unveiled a functional connection between the two proteins – SrtA and SafA. While *safA* deletion did not affect *srtA* expression (Figure 4.1B), this mutation induced processing of membrane-bound SrtA, resulting in secretion of a fraction of the processed sortase (Figure 4.1C-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 secretion 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* (Figure 4.1). 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 (Figure 4.1D). 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 (Figure 4.1). 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 (Figure 4.1G). Because biofilm formation requires the fimbrial shaft FimA (16), it is expected that that *safA* mutant should form mono-species biofilms and indeed this was the case (Figure 4.1H-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, 35). Our compelling evidence that SrtA is cleaved in the *safA* mutant (Figure 4.1), combined with the fact that the signal peptidase LepB2 is somehow linked to the lethality of *srtA* deletion (19), led us to re-analyze the sequence of the first 65 amino acids of SrtA manually, hence unveiling a typical tripartite domain of a signal peptide in this sequence (Figure 4.2A). A combination of mutational, biochemical and genetic analyses subsequently established that SrtA harbors a bona fide signal sequence and revealed the actual cleavage site (Figure 4.2 and Appendix Table A-1), which is processed by LepB2, one of two signal peptidases that are encoded by the organism (Figure 4.3).

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 mini-motif of SafA in the exoplasmic face of the membrane (Figure 4.4). 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 (Figure 4.4).

Together, these results lead us to propose a model for how SafA modulates SrtA function in envelope morphogenesis (Figure 4.5). According to this model, SafA and SrtA are normally co-localized and embedded within the membrane via their respective trans-membrane domains. This co-localization 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 (Figure 4.5A). In the absence of SafA, or when the FPW motif is mutated, the signal peptide of SrtA is unmasked, enabling LepB2 to process SrtA (Figure 4.5B). As 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 (Figure 4.5B). 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 co-immunoprecipitation 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 (Figure 4.1 and Figure 4.3), whereas the majority of SrtA is cleaved in the *safA* mutant (Figure 4.1). 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 co-localization on the membrane, that together dictate SrtA's membrane abundance, cleavage and secretion. 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 trans-membrane 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 (Figure 4.1 and Figure 4.3) 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 (Appendix Figure A-1), which further supports the idea of co-evolutionary 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* (36), 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. 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.

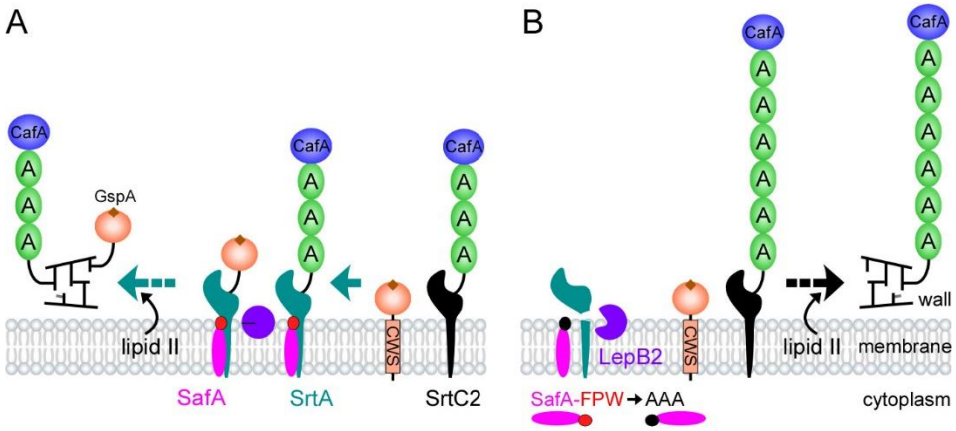

Figure 4.5 A working model of SafA-mediated antagonism of signal peptidase. (**A**-**B**) See text for details.

4.5 References

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Chapter 5 Conclusions and Impact

5.1 Summary of Research Findings

The membrane associated sortase transpeptidase enzymes were first discovered in *Staphylococcus aureus* with the archetype being SrtA (1, 2). Since their initial discovery, a multitude of sortase enzymes have since been discovered and characterized in a number of Gram-positive bacterial species including *Corynebacterium diphtheriae, Actinomyces oris, Streptococcus gordonii, Streptococcus pyogenes, Bacillus cereus* and *Enterococcus faecalis* (3). To date, sortases have been separated into six major classes based upon function, sequence homology, and substrate preference (4). Although sortases have been identified in various other Gram-positive bacterial species, *Corynebacterium diphtheriae* and *Actinomyces oris* have served as particularly fruitful models of sortase mediated surface morphogenesis and pilus assembly. The mechanism in which sortases catalyze reactions is well established, as is their roles in pilus assembly, protein anchoring, and virulence (5-7). One aspect of this mechanism which remains elusive is the regulation of sortase activity. In an attempt at elucidating possible regulation mechanisms, previous studies have demonstrated that the housekeeping sortase SrtA in *A. oris* displays an unprecedented role in regulating pilus length at anchoring, as mutations within sortase can result in either premature or delayed pilus anchoring which results in pili that are shorter or longer than wild-type respectively (8). These observations led to the hypothesis that additional factors may serve to modulate sortase anchoring activity, and thus we turned to the bacterial genome in an attempt at identifying these factors.

Traditionally, small proteins within the bacterial genome have remained overlooked and understudied (9). With the study described here, we successfully characterize a novel small transmembrane protein which modulates sortase activity through a novel paradigm of signal

peptidase-mediated cleavage of the housekeeping sortase. Through this work we also highlight the importance of these small often overlooked peptides within the bacterial genome. Here we utilize the model system of *Actinomyces oris* to identify and elucidate the function of the conserved transmembrane peptide SafA. We demonstrate that *safA* is encoded immediately downstream of the housekeeping sortase *srtA*. Interestingly, we found that deletion of *safA* results in phenotypes consistent with deletion of *srtA*, however *srtA* expression remains un-altered. We do however demonstrate that SafA functions to modulate SrtA membrane localization, and thus regulate SrtA function by interacting with SrtA to protect it from cleavage by the signal peptidase LepB2. Additionally, we utilized Edman degradation amino acid sequencing to identify the precise site in which SrtA is cleaved by LepB2. Within this study we establish the membrane topology of SafA and using mutational analysis and the Bacterial Adenylate Cyclase Two-Hybrid system identified a conserved domain of SafA which is demonstrated to be essential for its interaction with SrtA. Further experiments in which we aimed to utilize co-immunoprecipitation of SrtA and SafA failed in the presence of various crosslinkers. Based upon our findings, we hypothesize that this observed interaction between SrtA and SafA is transient in nature, and that SafA primarily serves to block LepB2 from accessing the identified SrtA cleavage site. Given that with study we establish a novel paradigm of sortase membrane localization modulation, further investigation into this mechanism is needed to establish the precise regulation and function of these processes.

The study described herein, revealing a sortase-associated factor, SafA, provides the first mechanism of the modulation of surface assembly via membrane homeostasis of the housekeeping sortase SrtA. As we have demonstrated both the evolutionary and functional conservation of SafA in Actinobacteria, this establishes a novel and relevant paradigm in bacterial pathogenesis and provides a new class of promising targets for inhibiting Actinobacteria virulence. In addition to

establishing a paradigm of cell surface morphogenesis in Gram-positive cell surface morphogenesis, these studies also demonstrate a novel paradigm in which small proteins are demonstrated to function as signal peptidase agonists by blocking signal peptide processing.

5.2 Future Studies

5.2.1 Exploring the evolutionary and functional conservation of SafA in *Bifidobacterium* species

With the discoveries presented herein, an additional question as to the purpose and evolutionary advantage of signal peptidase-mediated cleavage of the housekeeping sortase arises. As we have previously stated, the Actinobacteria species *Bifidobacterium dentium* does not harbor a separate SafA homolog, but rather a C-terminal domain of the housekeeping sortase which appears homologous to SafA. Additionally, one may attempt to elucidate if fusing SafA to the C-terminus of SrtA in *A. oris* may provide full protection of the SrtA signal peptide sequence and thus fully inhibit cleavage by LepB2. Based upon evolutionary lineages, it appears that the orders of Actinomycetales and Bifidobacteriales diverged from one another yet remain closely related. We posit that housekeeping sortases initially harbored the SafA-like C-terminal domain to protect the signal peptide sequence of the sortase from processing by signal peptidases, yet Actinobacteria evolved this domain to consist of two separate reading frames to more precisely modulate the anchoring of proteins to the cell wall. In support of this hypothesis, we demonstrate that deletion of SafA does not alter the expression of SrtA (Figure 4.1B), however this does lead to the question of the mechanism of the precise regulation of when SafA is protecting SrtA, and when SrtA is to be cleaved and released from the cell membrane. We favor the hypothesis that under conditions in which it is advantageous to avoid protein anchoring, such as when pili are being polymerized, SrtA

is released from the bacterial membrane due to LepB2-mediated cleavage. Once the pilus has reached an optimal length, SafA protects SrtA from cleavage, thus allowing SrtA to be maintained in the membrane compartment long enough to catalyze the covalent anchoring of the pilus to the cell wall.

5.2.2 Elucidating the precise mechanism of regulation of SafA

Given our hypothesis that Actinobacteria evolved to include SafA as its own reading frame, it begs the question as to the precise dynamics of SrtA cleavage. We hypothesize that the modulation of SrtA membrane localization is regulated by stoichiometric ratios of the three proteins of interest SrtA, SafA, and LepB2. To further explore this hypothesis future studies may utilize an *in vitro* lipid micelle system in which the precise ratios of each component can be regulated to recapitulate both the cleavage and SafA-mediated protection of SrtA. If successful, these studies could reveal the precise relative amount of LepB2 which is necessary to displace SafA from its binding partner SrtA, and thus provide more insight into this novel paradigm.

5.2.3 Determining the conservation of SafA-mediated modulation of surface morphogenesis in Actinobacteria using *Corynebacterium diphtheriae* as an experimental model

Corynebacterium diphtheriae has served as a fruitful model for elucidating the role of sortases in both pilus assembly and pilus anchoring. *C. diphtheriae* was the organism in which pilus-specific sortases were identified which led to the first model of sortase mediated pilus assembly in which the pilus specific sortase, SrtA, catalyzes polymerization of the SpaABC pili, which are then ultimately anchored by way of the housekeeping sortase SrtF (10, 11). Similar to as we have demonstrated in *A. oris*, the gene locus of the housekeeping sortase *srtF* in *C.*

diphtheriae does contain a SafA homolog immediately downstream of the sortase gene (Figure 4.1A). Here, we demonstrate that SafA appears to be evolutionarily conserved across Actinobacteria (Appendix Figure A-1). In support of this hypothesis, we demonstrate that SafA homologs from *Corynebacterium diphtheriae* and *Corynebacterium matruchotii* can rescue defects associated with *safA* deletion in *A. oris* (Figure 4.1G). Furthermore, inclusion of the putative C-region of the SrtF signal peptide sequence into SrtA from *A. oris* does result in cleavage of this peptide when ectopically expressed in *A. oris* (Figure 4.2A-B). Based on these data, the conservation of the signal peptide-mediated cleavage of housekeeping sortases in *C. diphtheriae* warrants further study. Thus, this dissertation should serve as a template for future studies to determine if SrtF is indeed cleaved by the native signal peptidase in *C. diphtheriae*. To follow the outline set forth herein, SrtF and SafA of *C. diphtheriae* can be cloned into the bacterial adenylate cyclase two-hybrid system to determine if they too interact in a similar manner as SrtA and SafA in *A. oris*.

5.2.4 Determining role of SafA in mediating bacterial virulence

A final area of study for future work with SafA may be to establish its role in mediating bacterial virulence. Sortases have served as an attractive target for inhibiting bacterial virulence and also may serve as viable vaccine components (2, 12, 13). With these presented findings in which SafA modulates sortase localization and therefore its activity, SafA may serve as an essential virulence factor for Actinobacteria infection, and thus inhibitors of SafA may prove to be an attractive avenue for disrupting Actinobacteria pathogenesis. Currently there are no suitable virulence models for *A. oris* infection, however our laboratory has established multiple animal-based virulence models to study *C. diphtheriae* virulence. Firstly, we have established two *Caenorhabditis elegans* models of infection in which worms infected with *C. diphtheriae*

demonstrate decreased survival and the formation of a deformed anal region when compared to those infected by *E. coli* (14). Additionally, we have established a rodent infection model in Guinea pigs in which previous studies conducted by our laboratory have demonstrated that mutants of *C. diphtheriae* which produce no pili are avirulent as compared to those infected by wild-type strains (15). Therefore, to elucidate if SrtF or SafA appear to have a direct role in mediating bacterial virulence, these model systems can be utilized in future studies. If successful these studies may provide evidence of SafA serving as a virulence factor and provide a new class of peptides to be targeted to limit bacterial virulence.

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Appendix A: Supporting Information for a Conserved Signal-Peptidase Antagonist Modulates Membrane Homeostasis of Actinobacterial Sortase Critical for Surface Morphogenesis

A-1: Materials and Methods

A-1.1: pSafA$_{Ao}$, pSafA$_{Cd}$, and pSafA$_{Cm}$.

The primer pair rpsJ-F/R and specific primer pairs safA-F/R (Table S3), were used to PCR-amplify the *A. oris rpsJ* promoter and the *safA* open reading frame (ORF) from *A. oris*, *C. diphtheriae*, or *C. matruchotii*, respectively, while appending appropriate restriction sites to each DNA fragment. Amplified fragments were digested with corresponding restriction enzymes (KpnI and NdeI for the *rpsJ* promoter, NdeI and EcoRI for *A. oris* and *C. diphtheriae safA*, or KpnI and HpaI for the *rpsJ* promoter, HpaI and EcoRI for *C. matruchotii safA*), and the digested promoter and *safA* fragments were ligated into the *E. coli/Actinomyces* shuttle vector pJRD215 precut with KpnI and EcoRI. The cloned sequences were confirmed by DNA sequencing.

A-1.2: pSafA$_1$ and pSafA$_2$.

The primer pairs, rpsJ-F/R and safA-F/R (Table S3), were used to PCR-amplify the *A. oris rpsJ* promoter and the *safA* ORF from *A. oris*, respectively, while appending appropriate restriction sites for cloning into pHTT117 (Table S2) at KpnI and EcoRI sites. The resulting plasmid was used as template for site-directed mutagenesis according to a published protocol (1), using primer pairs, SafA(PGP/AAA)-F/R and SafA(FPW/AAA)-F/R, to generate PGP or FPW to AAA mutations, respectively. The resulting mutations were verified by DNA sequencing.

DNA fragments encompassing the *A. oris rpsJ* promoter and *safA* with mutations were subcloned into pJRD215.

A-1.3: pYFP, pSafA-YFP and pYFP-SafA.

The promoter region of *fimQ* was generated by PCR with the primer pair Pcom-fimQ-F/Pcom-fimP-R (2) and digested with KpnI and NdeI. The digested *fimQ* promoter was ligated into pJRD215 precut with KpnI and NdeI, resulting in pJRD-PfimQ. The primer pair rpsJ-F/R and safA-F/R (Table S3), was used to PCR-amplify the *A. oris rpsJ* promoter, which was digested with KpnI and NdeI, prior to ligation into pJRD215 precut with the same enzymes, resulting in pJRD-PrpsJ. The *yfp* gene was amplificated with primers yfp-F/R from pK-PIM-YFP as template (3). The *yfp* amplicon was digested with NdeI and XbaI and ligated into pJRD215-PfimQ precut with the same enzymes to generate pYFP. For constructing pSafA-YFP, the primer pairs C-safA-F/R and C-YFP-F/R were used to PCR-amplify the *safA* and *yfp* genes, respectively. The *safA* and *yfp* fragments were digested with NdeI/KpnI and KpnI/XbaI, respectively, prior to ligating into the NdeI and XbaI sites of pJRD-PrpsJ to generate pSafA-YFP. To construct pYFP-SafA, the *yfp* and *safA* fragments were-PCR amplified with primer pairs N-YFP-F/R and N-safA-F/R, and then digested with NdeI/XbaI and XbaI/EcoRI, respectively. The digested *yfp* and *safA* fragments were ligated into pJRD-PrpsJ pre-treated with same enzymes. All cloned sequences were verified by DNA sequencing.

A-1.4: pSrtA-S57P, pSrtA-3F, pSrtA-Δ13, pSrtA-Sa13 and pSrtA-Cd13.

The *srtA* coding sequence and *rpsJ* promoter region were amplified from pSrtA (4) with primers prpsJ-F and C-SrtA-R. The PCR product was digested with EcoRI and HindIII and ligated

into pHTT177 precut with the same enzymes, resulting in pHTT-SrtA. To generate SrtA mutants, pHTT-SrtA was used as template for site-directed mutagenesis with mutations incorporated into the 5' end of the synthesized primers (Table S3) according to a published procedure (1). The PCR products were purified by gel extraction and phosphorylated to facilitate re-ligation of the amplicon into circular plasmids, which were then transformed into *E. coli* DH5α. Mutant *srtA* fragments were verified by DNA sequencing, prior to subcloning into pJRD215 at BamHI and EcoRI sites.

A-1.5: pSrtA$_{H6}$.

pHTT-SrtA generated above was used as a template for inverse PCR amplification using primers Re-srtA-(his6)-3F and Re-srtA-(his6)-3R, while appending a 6xHis-Tag to the C-terminus of SrtA. The obtained PCR product was circulated to generate pHTT-SrtA$_{H6}$, which was further verified by DNA sequencing. A region encompassing the rpsJ promoter and this *srtA* with H_6 insertion was subcloned into pJRD215 at BamHI and EcoRI sites.

A-1.6: SrtA and SafA constructs for BACTH.

According to a previously established method (5, 6), srtA and safA fragments were cloned in-frame with adenylate cyclase subunits present on BACTH vectors by PCR amplification in which the 5' primer contained BamHI site and the 3' primer contains KpnI restriction sites. The resulting PCR products and empty vectors (pUT18C or pKT25) were digested by BamHI and KpnI prior to ligation. Resulting ligation reactions were transformed into DH5α *E. coli*, and colonies containing pUT18C or pKT25 were selected for by plating on LB supplemented with 100μg/mL ampicillin or 50μg/mL kanamycin, respectively. Plasmids were isolated and constructs were

confirmed by DNA sequencing. pUT18C and pKT25 constructs were co-transformed into adenylate cyclase deficient *E. coli* BTH101 and selected for by plating on Macconkey agar supplemented with 1% sucrose, 100 µg/mL ampicillin and 50µg/mL kanamycin.

A-1.7: Generation of deletion mutants in *A. oris*.

A. oris deletion mutants were generated according to a previously established protocol (4, 7, 8). Briefly, 1-kb flanking regions upstream and downstream of a gene of interest were PCR-amplified with appropriate primers (Table S3) and cloned in the deletion vector pCWU2 (4). The generated plasmid was electroporated into *A. oris* CW1 cells, and cells with the plasmid integrated into the bacterial chromosome, integrants, were selected by HIA plates supplemented with 50 µg/mL kanamycin. An integrant was used to inoculate a culture without antibiotics to facilitate a second recombination event leading to wild type or mutant alleles, which were selected on HIA plates containing 2-deoxy-D-galactose (2-DG). Deletion mutants were verified by PCR and immunoblotting. Single deletion mutants were then used to make double and triple mutants.

A-2: Tables and Figures

Appendix Table A-1: Edman degradation sequencing data

Cycle	Amino acid (pmol)																		
	Asp (D)	Asn (N)	Ser (S)	Gln (Q)	Thr (T)	Gly (G)	Glu (E)	His (H)	Ala (A)	Arg (R)	Tyr (Y)	Pro (P)	Met (M)	Val (V)	Trp (W)	Phe (F)	Ile (I)	Lys (K)	Leu (L)
1	5.667	0.996	48.53	11.92	4.94	14.83	4.563	1.687	12.26	5.767	3.315	2.117	0.865	7.04	0.244	1.198	2.361	1.964	2.578
2	4.503	1.518	9.75	4.312	4.028	16.82	3.901	1.334	75.05	4.875	2.767	2.592	0.605	4.869	0.26	2.133	3.378	2.258	5.62
3	5.041	1.669	5.572	42.27	4.41	18.34	9.276	1.909	29.26	5.77	3.006	4.708	0.513	11.08	0.176	2.281	3.224	2.878	4.643
4	5.413	2.06	4.917	21.73	5.657	20.72	7.034	2.181	57.95	6.257	3.339	4.933	0.635	7.472	0.38	2.656	3.486	3.566	4.337
5	6.633	2.082	5.203	11.65	7.699	23.79	6.453	2.904	42.58	7.767	3.963	5.142	1.027	32.14	0.488	4.052	3.747	4.066	4.925
6	7.611	2.854	5.462	10.17	8.509	25.88	6.938	3.915	51.8	7.681	4.304	4.99	1.198	24.77	0.585	4.62	3.966	4.21	5.669
7	8.742	3.117	5.83	9.322	9.856	27.78	8.151	4.545	45.1	8.528	4.731	5.322	1.345	15.54	0.627	6.697	4.098	4.432	6.305
8	9.574	3.373	6.288	18.42	10.73	30.4	10.23	6.204	36.46	7.213	5.155	5.852	1.573	12.03	0.65	7.362	4.215	4.613	6.943
9	10.58	2.82	6.871	22.02	11.46	33.98	12.43	7.797	33.34	9.832	5.714	6.689	1.82	11.17	0.59	15.07	4.417	5.057	7.485
10	11.67	4.293	7.668	20.41	12.43	37.96	13.95	13.39	33.61	8.084	6.141	7.765	2.038	12.21	0.669	19.71	4.833	5.729	8.49

Appendix Table A-2: Bacterial strains and plasmids used

Strain & Plasmid	Description	Reference
Strain		
A. oris MG1	Wild type *A. oris*	(9)
A. oris CW1	Δ*galk*; an isogenic derivative of MG1	(8)
A. oris WU36	A conditional *srtA* deletion mutant	(4)
A. oris AR4	Δ*fimA*; an isogenic derivative of CW1	(4)
A. oris WU49	Δ*gspA*-Δ*srtA;* a double mutant lacking *gspA* and *srtA*	(4)
A. oris WU12	Δ*safA*; an isogenic derivative of CW1	This Study
A. oris WU49b	Δ*gspA* Δ*srtA ΔsafA;* isogenic derivative of MG1	This Study
A. oris WU42	Δ*lepB2*; lacking *lepB2*	(10)
A. oris WU50	Δ*lepB1*; lacking *lepB1*	(10)
A. oris WU47	Δ*lepB2*-Δ*safA*; lacking *lepB2* and *safA*	This Study
E. coli BTH101	An adenylate cyclase deficient strain used for a bacterial two hybrid assay	(6)
S. oralis So34	Cell surface receptor RPS positive	(11)
Plasmid		
pHTT177	A derivative of pUC19; Kan^R	(12)
pCWU2	A derivative of pHTT177 for generating deletion mutants	(8)
pCWU2-Δ*safA*	A derivative of pCWU2 for deletion of *safA*	This Study
pJRD215	*E. coli*/*Actinomyces* shuttle vector; Kan^R and Sm^R	(13)
pCWU10	A derivative of pJRD215; Kan^R	(10)
pUT18C	A vector containing the T18 fragment of adenylate cyclase; Amp^R	(6)
pKT25	A vector containing the T25 fragment of adenylate cyclase; Kan^R	(6)
pUT18C-Zip	A derivative of pUT18C expressing a leucine zipper protein fused in frame to the T18 fragment; Amp^R	(6)
pKT25-Zip	A derivative of pKT25 expressing a leucine zipper protein fused in frame to the T25 fragment; Kan^R	(6)
pUT18C-SafA	A derivative of pUT18C expressing SafA fused in frame to the T18 fragment	This Study
pUT18C-$SafA_1$	A derivative of pUT18C-SafA with PGP to AAA mutation	This Study
pUT18C-$SafA_2$	A derivative of pUT18C-SafA with FPW to AAA mutation	This Study
pKT25-SrtA	A derivative of pKT25 expressing SrtA fused in frame with the T25 fragment	
pKT25-$SrtA_\Delta$	A derivative of pKT25 expressing the N-terminal SrtA (residues 10-49) fused in frame with the T25 fragment	This Study

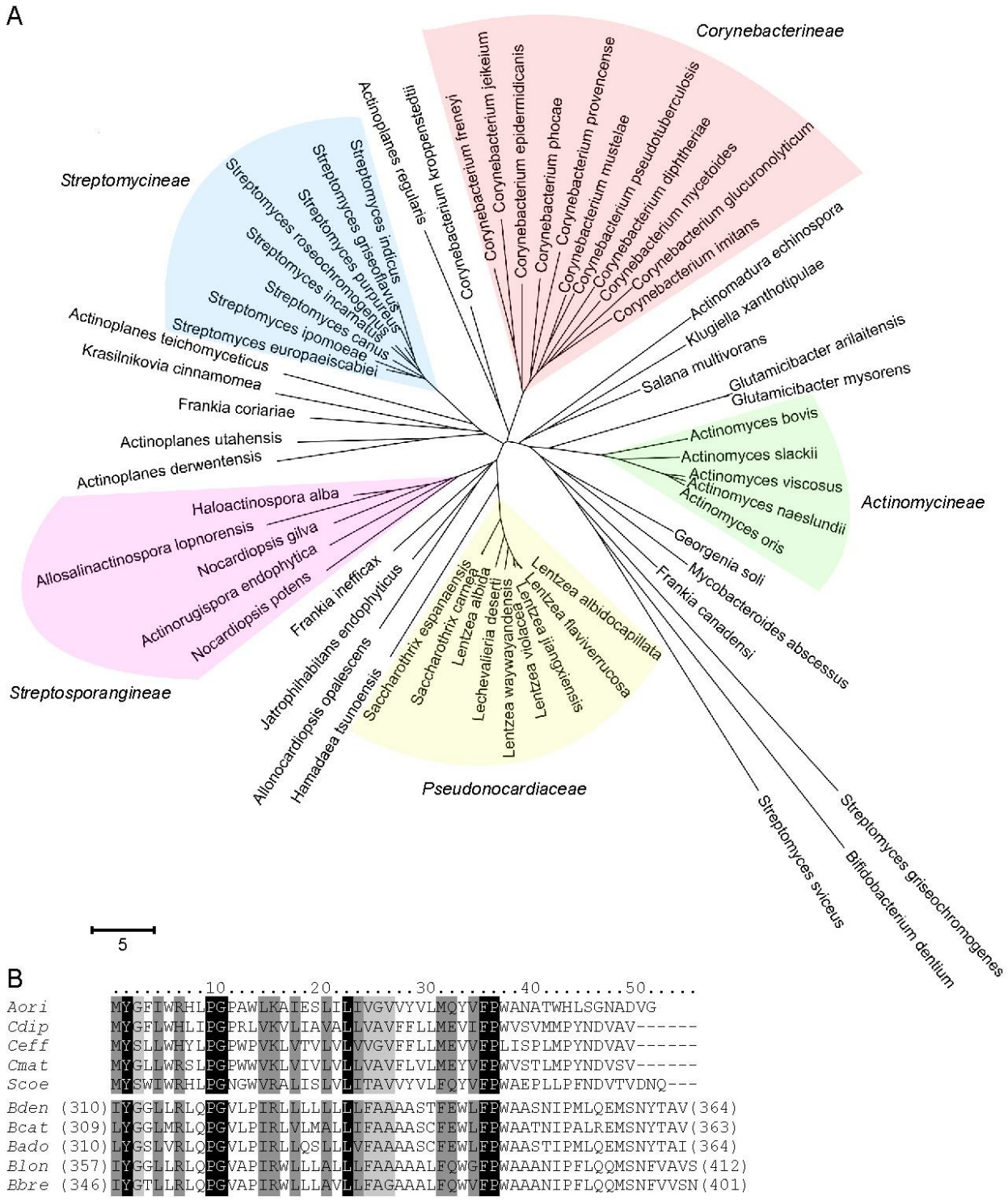
pSrtA	A derivative of pJRD215 constitutively expressing SrtA	(4)
$pSafA_{H6}$	A derivative of pJRD215 constitutively expressing *A. oris* SafA with a 6xHistidine tag at the C-terminus	This Study
$pSafA_{Ao}$	pJRD215 constitutively expressing *A. oris* SafA	This Study
$pSafA_{Cd}$	pJRD215 constitutively expressing *C. diphtheriae* SafA	This Study
$pSafA_{Cm}$	pCWU10 constitutively expressing *C. matruchotii* SafA	This Study
$pSafA_1$	A derivative of $pSafA_{H6}$ expressing *A. oris* $SafA_{H6}$ with PGP residues mutated to AAA	This Study
$pSafA_2$	A derivative of $pSafA_{H6}$ expressing *A. oris* $SafA_{H6}$ with FPW residues mutated to AAA	This Study
pSafA-YFP	pJRD215 constitutively expressing SafA fused at its C-terminus with YFP	This Study
pYFP-SafA	pJRD215 constitutively expressing SafA fused at its N-terminus with YFP	This Study
pYFP	pJRD215 constitutively expressing cytoplasmic YFP	This Study
pSrtA-S57P	A derivative of pSrtA with S57P mutation	This Study
pSrtA-3F	A derivative of pSrtA with the AXA motif replaced by FFF	This Study
pSrtA-Δ13	A derivative of pSrtA with deletion of 13 residues encompassing the cleavage site	This Study
pSrtA-*Sa*13	A derivative of pSrtA with the *A. oris* 13 residues replaced with an analogous sequence from *S. aureus* SrtA	This Study
pSrtA-*Cd*13	pJRD215 expressing SrtA with the *A. oris* 13 residues replaced with the *C. diphtheriae* homologous sequence	This Study
pLepB2	pJRD215 constitutively expressing LepB2	(10)
pLepB2-S101A	A derivative of pLepB2 expressing LepB2 with S101A mutation	(10)
pLepB2-K169A	A derivative of pLepB2 expressing LepB2 with K169A mutation	(10)

Appendix Table A-3: Primers used in this study

Primer	Sequence	Used For
safA-up-F	GGCGGAATTCACCAGCGCGGTGAGGCGGTGTCCT	pCWU2-Δ*safA*
safA-up-R	GGCGGGTACCAGGACCCGGCAGGTGCCGCCAGATG	pCWU2-Δ*safA*
safA-down-F	GGCGGGTACCGTCGGCTGACCGGCGGCCCGTCAG	pCWU2-Δ*safA*

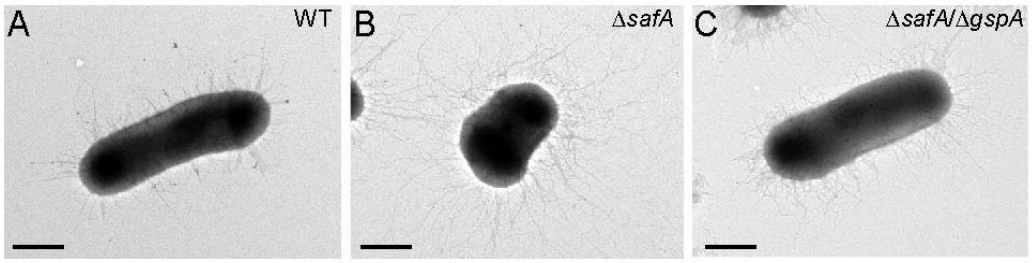
safA-down-R	GGCGTCTAGACGGCCGACCCGCGCCTGGTCAACG	pCWU2-Δ*safA*
rpsJ-F	GGCGGGATCCCGCCCGAGCGCGGGGACCAGT	*rpsJ*-promoter
rpsJ-R	GGCGCATATGGGCGCCTAACCTCTCTTGTACTTG	*rpsJ*-promoter
safA-R_{H6}	GGCGGAATTCTCAGTGGTGGTGGTGGTGGTGGCCG ACGTCGGCGTTGCCG GAC	$pSafA_{H6}$
SafA-F	GGCGCATATGATGTACGGCTTCATCTGGCGGCAC	$pSafA_{Ao}$
SafA-R	GGCGGAATTCAGGCCTGACGGGCCGCCGGTCAG	$pSafA_{Ao}$
$SafA_{C.dip}$-F	GGCGCATATGATGTATGGATTTTTGTGGCATCTC	$pSafA_{Cd}$
$SafA_{C.dip}$-R	GGCGGAATTCTCCAAGCAGCAGAGGCGGTAGGC	$pSafA_{Cd}$
PrpsJ-KpnI-5	AAAAAGGTACCCGCCCGAGCGCGGGG	$pSafA_{Cm}$
PrpsJ-$safA_{c.mat}$-3	GGGAACGCCATAATAATCCATACATCTTGTTGCCT CCTTAGCAGGGTGC	$pSafA_{Cm}$
$safA_{c.mat}$-5	GCAACAAGATGTATGGATTATTATGGCGTTCCCTG CCTGGG	$pSafA_{Cmt}$
HpaI-$safA_{c.mat}$-3	AAAAAAGTTAACGGCCCAATGCCTACACGGACAC G	$pSafA_{Cm}$
$SafA_1$-5	GCGGCTGCTGCGTGGCTCAAGGCCATTGAGTCG	$pSafA_1$
$SafA_1$-3	CAGGTGCCGCCAGATGAAGCCGT	$pSafA_1$
$SafA_2$-5	GCCGCCGCG GCCAACGCCACCTGGC ACCTGTCCG	$pSafA_2$
$SafA_2$-3	GACGTACTGCATGAGC ACGTAGAC	$pSafA_2$
C-safA-F(NdeI)	GGCGCATATG ATGTACGGCTTCATCTGGCGGC	pSafA-YFP
C-safA-R(KpnI)	GGCGGGTACCGCCGACGTCG GCGTTGCCGGAC	pSafA-YFP
C-YFP-F(KpnI)	GGCGGGTACCGTGAGCAAGG GCGAGGAGCTGTTC	pYFP-SafA
C-YFP-R(XbaI)	GGCGTCTAGATCACTTGTACAGCTCGTCCATG	pYFP-SafA
YFP-F	GGCGCATATGGTGAGCAAGG GCGAGGAGCTGT	pYFP
YFP-R	GGCGATCGATTCACTTGTAC AGCTCGTCCATG	pYFP
SrtA-S57P-5	CCTGCGCAGGCGGTGGCCACGCAG	pSrtA-S57P
SrtA-S57P-3	GGCGTTGGCGTCAATGCCGGTCCA	pSrtA-S57P
SrtA-3F-5	TTCTTCTTCAGCGCGCAGGCGGTGGCCACGCAG	pSrtA-3F
SrtA(3F)-3	GTCAATGCCGGTCCACCACAGCTGCCAG	pSrtA-3F
SrtA-Δ13-5	ACGCAGTTCCACGAGAAGCAGGTCCAG	pSrtA-Δ13
SrtA-Δ13-3	GGTCCACCACAGCTGCCAGCACAGGAAG	pSrtA-Δ13
SrtA(c-sau)-F	TACCTGCACGACAAGGACACGCAGTTCCACGAGA AGCAGGTCCAG	pSrtA-*Sa*13
SrtA(c-sau)-R	GTTGTCGATGTGCGGCTTGGTCCACCACAGCTGCC AGCACAG	pSrtA-*Sa*13
SrtA-c-cdip-F	GCACAGGCAGCCGTCTCCACGCAGTTCCACGAGA AGCAGGTCCAG	pSrtA-*Cd*13
SrtA-c-cdip-R	CTTGCCTGCCTCGATGTTGGTCCACCACAGCTGCC AGCACAG	pSrtA-*Cd*13

Re-srtA-(his6)-3F	TCGCACAAGACCTCCTCTAGTCAT	$pSrtA_{H6}$
Re-srtA-(his6)-3R	CACCACCACCACCACCACCACCGAGCACCCGGCCAGAGTCG	$pSrtA_{H6}$
BamHI-SafA-5	CGCGGATCCAATGTACGGCTTCATCTGGC	pUT18C-SafA
KpnI-SafA-3	CGGGGTACCTCAGCCGACGTCGGCG	pUT18C-SafA
BamH-SrtA-5	CGCGGATCCAATGACTAGAGGAGGTCTTGTG	pKT25-SrtA
KpnI-SrtA-3	CGGGGTACCGTTGACCCCCGGGTCG	pKT25-SrtA
BamHI-$SrtA_{\Delta}$-5	GGGGGATCCACGAGCACCCGGCCAG	pKT25-$SrtA_{\Delta}$
KpnI- $SrtA_{\Delta}$-3	GGGGGTACCTTACCACCACAGCTGCCAGC	pKT25-$SrtA_{\Delta}$
RT-16s-5	GTCGCTAGTAATCGCAGATCAG	RT-PCR
RT-16s-3	GGTGTTGCCGACTTTCATG	RT-PCR
RT-SrtA-5	GTACCTACGGCAACTCCTTC	RT-PCR
RT-SrtA-3	TCACCTTGAACACGTACCAG	RT-PCR

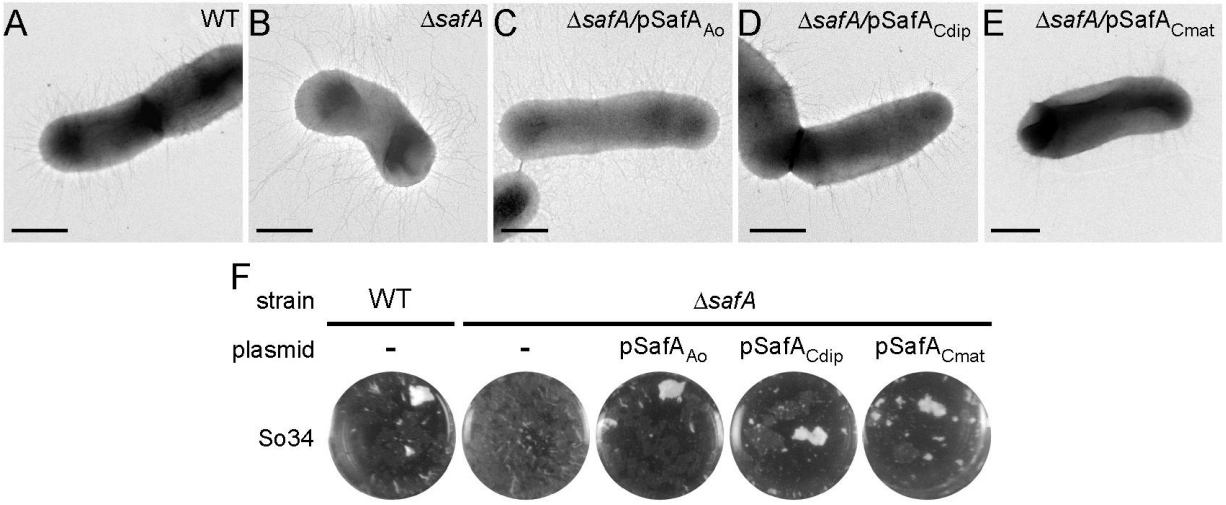


Appendix Figure A-1 Conservation of SafA in Actinobacteria. (**A**) With the *A. oris* SafA amino acid sequence as query, a phylogenetic tree was constructed with the minimum evolutionary algorithm with a bootstrap value of 100 (MEGA X (14)). (**B**) Protein sequence alignment of SafA homologs from *Actinomyces oris* (*Aori*), *Corynebacterium diphtheriae* (*Cdip*), *Corynebacterium efficiens* (*Ceff*), *Corynebacterium matruchotii* (*Cmat*), *Bifidobacterium dentium* (*Bden*), *Bifidobacterium catenulatum* (*Bcat*), *Bifidobacterium adolescentis* (*Bado*), *Bifidobacterium longum*

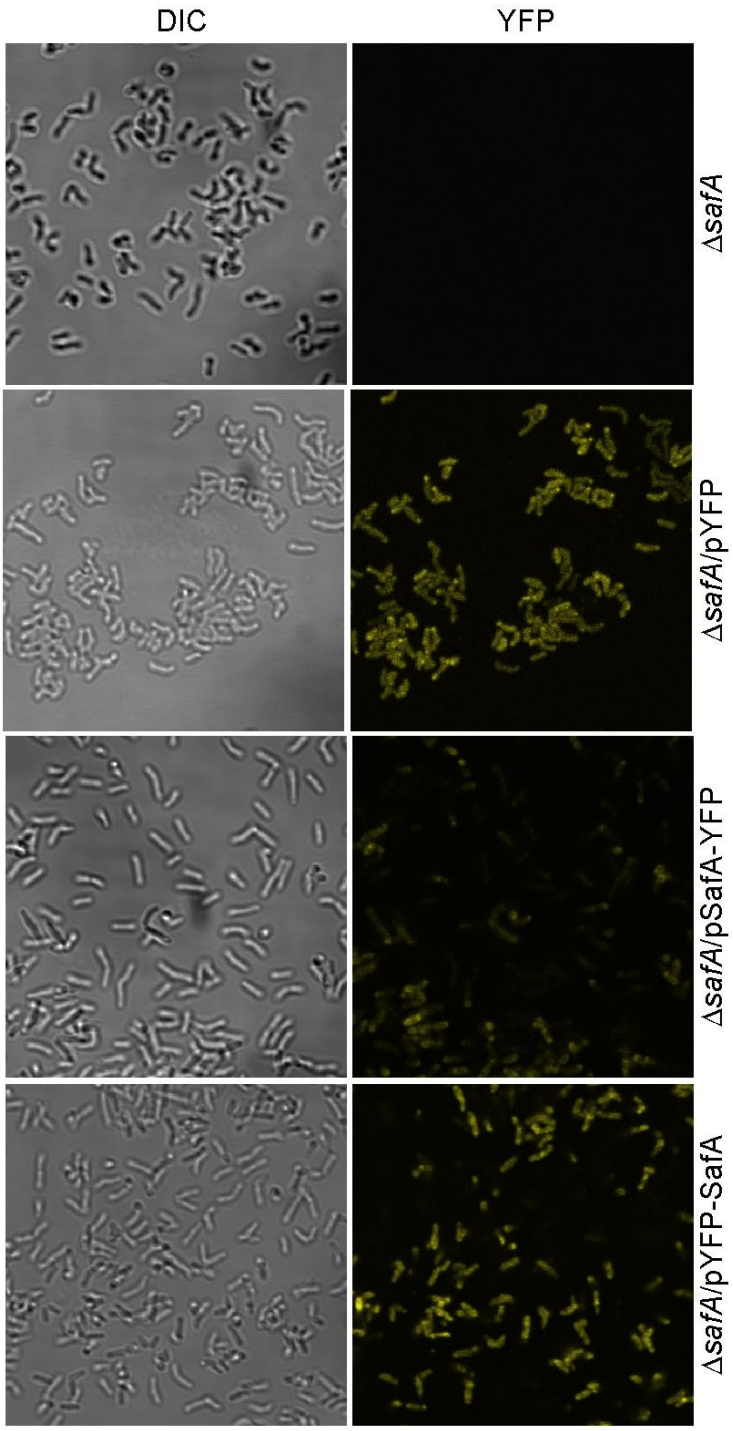
(*Blon*), and *Bifidobacterium breve* (*Bbre*) was performed by Clustal Omega (15). Of note, all *Bifidobacterium* SafA sequences are part of the C-terminus of the housekeeping sortase SrtE proteins, with numbers in parentheses showing the starting and ending positions. Conserved residues are shaded.



Appendix Figure A-2 Electron microscopy of *A. oris* Log-phase cells of indicated strains were analyzed by electron microscopy using negative staining with 1% uranyl acetate. Scale bar indicates 0.5 μm.



Appendix Figure A-3 :**Functionality of Actinobacterial SafA homologs in *A. oris*** (**A**-**E**) Log-phase cells of indicated strains were analyzed by electron microscopy using negative staining with 1% uranyl acetate. Scale bar indicates 0.5 μm. The *A. oris* MG1 (WT), Δ*safA* mutant, and Δ*safA* mutant strains constitutively expressing SafA from *A. oris* (*Ao*), *C. diphtheriae* (*Cd*), or *C. matruchotii* (*Cm*) were analyzed for their ability to aggregate with *S. oralis* So34 in a coaggregation assay as previously reported (16, 17).



Appendix Figure A-4 Determination of SafA membrane topology with fluorescent microscopy. Mid-log phase cells of the Δ*safA* mutant or this strain expressing YFP fused in frame to SafA at the N- (Δ*safA*/pSafA-YFP) or C-terminus (Δ*safA*/pYFP-SafA) were analyzed by differential interference contrast (DIC) and fluorescent microscopy. A cytoplasmic YFP (Δ*safA*/pYFP) was used as control.

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