May 2017

Targeting of Flavobacterium Johnsoniae Proteins for Secretion By the Type IX Secretion System

Surashree Sunil Kulkarni
University of Wisconsin-Milwaukee

TARGETING OF *FLAVOBACTERIUM JOHNSONIAE* PROTEINS FOR SECRETION BY THE TYPE IX SECRETION SYSTEM

by

Surashree S. Kulkarni

A Dissertation Submitted in

Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

in Biological Sciences

at

The University of Wisconsin-Milwaukee

May 2017

ABSTRACT

TARGETING OF *FLAVOBACTERIUM JOHNSONIAE* PROTEINS FOR SECRETION BY THE TYPE IX SECRETION SYSTEM

by

Surashree S. Kulkarni

The University of Wisconsin-Milwaukee, 2017
Under the Supervision of Dr. Mark J. McBride

Flavobacterium johnsoniae and many related bacteria secrete proteins across the outer membrane using the type IX secretion system (T9SS). Proteins secreted by T9SSs have amino-terminal signal peptides for export across the cytoplasmic membrane by the Sec system and carboxy-terminal domains (CTDs) targeting them for secretion across the outer membrane by the T9SS. Most but not all T9SS CTDs belong to family TIGR04183 (type A CTDs). This thesis focuses on the functional characterization of diverse CTDs for secretion by the *F. johnsoniae* T9SS. Fusion of the CTDs from *F. johnsoniae* RemA, AmyB, and ChiA to the foreign protein sfGFP that had a signal peptide at the amino terminus (SP-sfGFP) resulted in secretion across the outer membrane. In each case approximately 80 to 100 amino acids from the extreme carboxy-terminus was needed for efficient secretion. Several type A CTDs from distantly related members of the phylum *Bacteroidetes* functioned in *F. johnsoniae*, supporting secretion of sfGFP by the *F. johnsoniae* T9SS. The *F. johnsoniae* adhesin SprB is propelled rapidly along the cell surface resulting in gliding motility. *F. johnsoniae* SprB requires the T9SS for secretion but lacks a type A CTD. It has a conserved C-terminal domain belonging to family TIGR04131, which we refer to as a type B CTD. Type B CTDs are common in the *Bacteroidetes* but little is known regarding their roles

in secretion. The secretion of the foreign protein sfGFP fused to an N-terminal SP and to C-terminal regions of SprB (SP-sfGFP-CTD_{SprB}) was analyzed. CTDs of 218 AAs or longer resulted in secretion whereas a CTD of 149 AAs did not. *sprF*, which lies downstream of *sprB*, is known to be required for SprB secretion. SP-sfGFP-CTD_{SprB} also required SprF for secretion. Efficient secretion only occurred when SP-sfGFP-CTD_{SprB} and SprF were expressed together. Under these conditions CTDs of 218 AAs and 448 AAs resulted in secretion of soluble sfGFP, whereas longer CTDs (663 and 1182 AAs) resulted in attachment of sfGFP to the cell surface. Most *F. johnsoniae* genes encoding proteins with type B CTDs lie immediately upstream of *sprF*-like genes. The CTD from one such protein, Fjoh_3952, facilitated secretion of sfGFP only when it was coexpressed with its cognate SprF-like protein, Fjoh_3951. Secretion did not occur when SP-sfGFP-CTD_{Fjoh_3952} was expressed with SprF, or when SP-sfGFP-CTD_{SprB} was expressed with Fjoh_3951. The results highlight the need for extended regions of type B CTDs for secretion and cell-surface localization, and the requirement for the appropriate SprF-like protein for secretion. Since type B CTD-containing proteins and associated SprF-like proteins are common among members of the phylum *Bacteroidetes* the unique features required for secretion of these proteins may have broad implications.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1

Chapter 2

LIST OF TABLES

ACKNOWLEDGMENTS

First and foremost, I would like to extend my sincere gratitude towards my advisor, Dr. Mark J. McBride, for his mentorship, guidance, and his inputs on experimental designs. I greatly appreciate his patience and humor in times of difficulty, and would like to thank him for making this journey through graduate school a rounded and fulfilling experience. He will always remain an inspiration to me on how to do science, and importantly how to enjoy doing it. I would also like to extend my gratitude towards my committee members Dr. Daad Saffarini, Dr. Charles Wimpee, Dr. Sonia Bardy and Dr. Ching Hong Yang for their time and guidance for the successful completion of my dissertation. Dr. Daad Saffarini's attention to detail and creative ideas on experimental design helped me immensely. I would like to thank Dr. Sonia Bardy for some great and fun experiment ideas, and also for mentoring me throughout my graduate career. Dr. Charles Wimpee or 'Chuck' as fondly call him helped me navigate through some tough times in graduate school and I am grateful to have found a mentor and a friend in UWM. Dr. Yang gave some excellent ideas on 'control' experiments, which I tried to incorporate into my first paper as well as thesis. I would like to thank the National Science Foundation (NSF), and the Graduate School at the University of Wisconsin, Milwaukee in acknowledging my work and providing financial assistance for my research.

My laboratory was like my second home and the members are like my family. I would like to thank the current lab members: Dr. Yongtao Zhu, Joseph Johnston, Rachel Conrad and Dr. Paul Barbier. I would like to extend my sincerest gratitude towards Yongtao for making plasmid pYT179, which I used for designing experiments that laid the foundation of my thesis. He also constructed Δ*sprF* strain and contributed to helping me make figures for my first paper, on which

he is a co-author. I would also like to thank Colton Brendel who was an undergraduate research student as part of UWM's SURF program and a co-author on my first paper as well. We worked together on cloning of two important plasmids pCB3 and pCB4. Special thanks to Joe and Rachel for always being there for me as friends, listening to all my problems and giving me great advice. It would be amiss of me not to mention my gratitude towards past lab members Dr. Sampada Kharade, and Dr. Abhishek Shrivastava for all their guidance when I first joined the McBride lab.

I would not have been able to finish my doctoral work without the unwavering support, sacrifices and love of my parents Dr. Sunil Kulkarni and Mrs. Supriya Kulkarni. Thank you mom and dad for understanding my passion for science and providing me with all the support, which made it possible for me to reach this destination. I would also like to thank my grandparents, my brother, and sister in law for being role models for me and inspiring me every step of the way. I would like to thank my husband, Unmesh, and his family. I thank him for the times I needed a friend the most, listening to my passionate rants on project success and failures with interest and support. Lastly, I want to thank my friends in Milwaukee, who made this foreign country a home, for loving me for who I am, and being there for me through all the ups and downs.

Chapter 1: Introduction

Flavobacterium johnsoniae a Gram-negative rod shaped bacterium that belongs to the phylum *Bacteroidetes*, is commonly found in soil and freshwater environments (1, 2). *F. johnsoniae* digests macromolecules such as complex polysaccharides and proteins (3), a trait that it shares with many other members of the phylum *Bacteroidetes*. *F. johnsoniae* is a non-pathogenic bacterium unlike some of its relatives, including the fish pathogens *Flavobacterium columnare* and *Flavobacterium psychrophilum*, and the human oral pathogen *Porphyromonas gingivalis* (4-6). *F. johnsoniae* is an excellent model organism to study processes that are shared between many members of the phylum *Bacteroidetes* because of the ease of performing genetic manipulations including gene deletions, transposon mutagenesis, and complementation experiments (7-9).

Members of the phylum *Bacteroidetes* share many unique features that distinguish them from other bacteria. These include unusual transcription and translation signals (10-13) , the ability to transport polysaccharides and oligosaccharides across the outer membrane and digest these internally (14, 15), the production of numerous periplasmic and cell-surface glycoproteins (16), the ability to crawl (glide) rapidly over surfaces using a motility machinery that is restricted to the phylum *Bacteroidetes* (17), and a unique protein secretion system, the type IX secretion system (T9SS) (4, 18). My research has focused on the last two of the features mentioned above, gliding motility and the T9SS.

Gliding motility describes the ability of some bacteria to crawl over surfaces such as agar or glass without the aid of flagella or pili (17). Proton motive force (PMF) appears to power *Flavobacterium* gliding motility since uncouplers that dissipate PMF reversibly block gliding (19-

21). On agar, *F. johnsoniae* forms spreading colonies and individual cells can also attach to and move on glass or Teflon at speeds of 2 to 5 μm/sec (22). *Myxococcus xanthus* and *Mycoplasma mobile* are also extensively studied for their gliding motilities. However, there are no similar motility genes between these three gliding bacteria (23, 24). *M. xanthus* exhibits 'social gliding' (also referred to as twitching motility), which relies on ATP-powered type IV pilus extension and retraction. It also displays 'adventurous gliding' that relies on cytoplasmic motors that use PMF as energy source to drive motility (23). *M. mobile*, in contrast, appears to move like a centipede with the aid of 'legs' that protrude from the outside of the cells. The 'legs' bind to surfaces and cells move due to attachment and release of the legs from the substratum. The *M. mobile* gliding motor is not known but it is powered by ATP hydrolysis, unlike *F. johnsoniae* gliding motility (24).

Using genetic and genomic approaches, some of the moving components of the *F. johnsoniae* gliding machinery were identified. Among these is a major cell-surface adhesin, SprB, which is a massive protein approximately 660 kDa in molecular mass. On electron microscopic analysis SprB was observed as thin filaments projecting from the outer membrane of wild-type *F. johnsoniae* cells and was absent in an *sprB* mutant suggesting that the filaments are composed of SprB (21). Immunodetection using anti-sera against SprB revealed uneven distribution of the protein along the cell surface (Fig. 1). *sprB* mutant cells form round non-spreading colonies on agar surfaces because of defects in gliding motility (Fig. 2) (25). Studies with polystyrene spheres coated with anti-SprB antibodies indicated that SprB is rapidly propelled along the cell surface (25). Immunofluorescence using anti-SprB antibodies confirmed that SprB moves along the entire length of the cell and that it appears to follow a helical track (Fig. 3) (26). There are other motility adhesins, many of which share some similarity with SprB. One of these, RemA, is propelled along

the cell surface similar to SprB, and has a SUEL-like lectin domain. This domain interacts with polysaccharides and contributes to cell-aggregation and group motility (27).

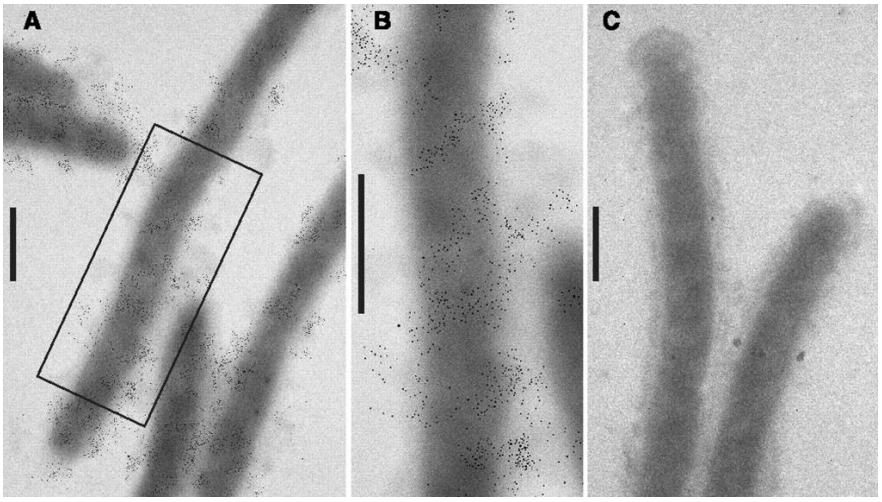

Fig. 1. Localization of SprB by immunoelectron microscopy. Bars = 0.5 μm. (A) Cells of wild-type *F. johnsoniae* FJ1. (B) Higher magnification of a wild-type cell in panel A. (C) Cells of *sprB* mutant FJ156. (25)

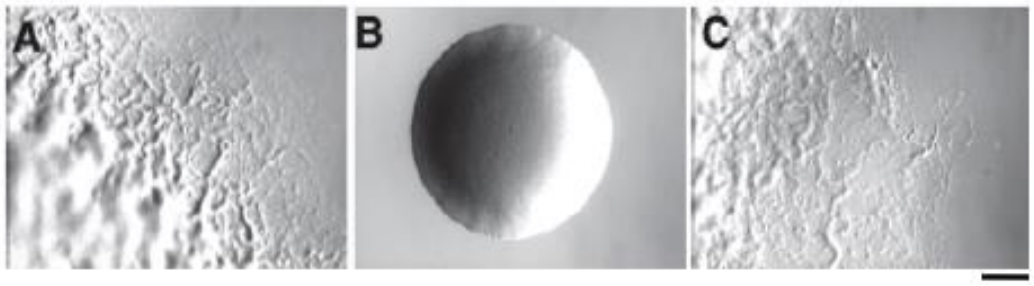

Fig. 2. Photomicrographs of *F. johnsoniae* colonies. (A) wild-type *F. johnsoniae* FJ1 (B) *sprB* mutant FJ156 (C) FJ156 complemented with pSN60 which carries *sprB*. Bar in panel C = 0.5 mm and applies to all panels. (25)

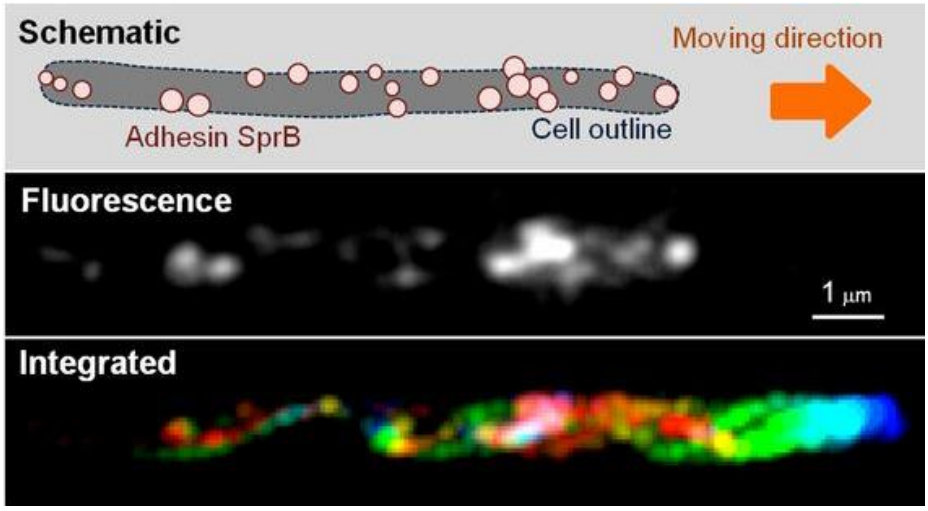

Fig. 3. Helical loop-like motion of SprB observed by immunofluorescence microscopy. The fluorescent signals in a gliding cell were recorded at 0.1-s intervals for 2 s, colored from red (time 0) to blue (2 s), and integrated into a single image (Bottom). (26)

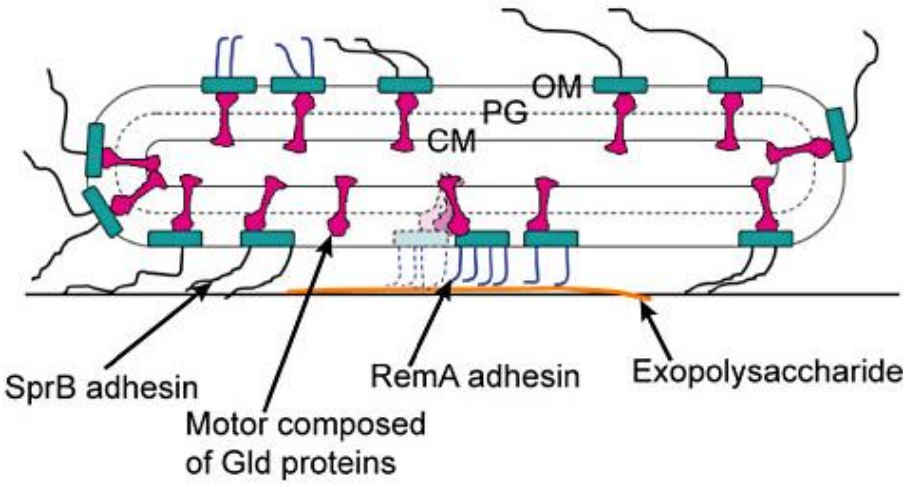

Fig. 4. Model of *F. johnsoniae* gliding motility. Gld proteins anchored in the cell envelope presumably form the motors that propel SprB and RemA along the cell surface. Exopolysaccharides secreted by the cells provide a substratum and interaction points for the adhesin RemA. CM- cytoplasmic membrane; OM- outer membrane; PG- peptidoglycan. (27)

Analysis of mutants completely or partially defective in gliding led to the identification of nineteen motility genes that are divided into two categories: *gld* and *spr* (7, 9, 28-33). Mutations in *gld* genes result in complete loss of motility while *spr* gene mutations result in less severe motility defects. Gld and Spr proteins are thought to be components of the motility machinery that propels SprB and RemA along the cell-surface (Fig. 4). Some of these proteins are also part of the newly discovered protein secretion apparatus, the T9SS.

Gram-negative bacteria employ protein secretion systems to deliver proteins across the outer membrane. Some secreted proteins are released in soluble form, whereas others become attached to the cell surface. Secreted proteins are involved in various bacterial processes such as motility, adhesion, evasion of host immune responses, and digestion of macromolecules such as polysaccharides and proteins. Protein secretion systems enable the bacteria to survive in various ecological niches and provide obvious competitive advantages. There are six well-known bacterial secretion systems, classified from Type I to Type VI (Fig. 5). The type-VII secretion system, also known as the chaperone-usher pathway (34, 35), and the type-VIII secretion system, the extracellular nucleation-precipitation pathway involved in secretion and assembly of curli amyloid fibers (36) are also well known, but are not usually referred to using the 'type' designations. Another secretion system, the ESX protein export system, was discovered in members of the Gram-positive genus *Mycobacterium* (37). Secretion systems related to the ESX system have also been suggested to occur in other groups of bacteria (38). Type I, III, IV and VI secretion systems transport proteins directly from the cytoplasm to the outside. Type II, V, VII, VIII and IX secretion systems rely on the Sec or Tat systems to export protein across the cytoplasmic membrane and only carry out the final secretion across the outer membrane. The secreted proteins are anchored to the outer membrane or released in the surrounding medium (34).

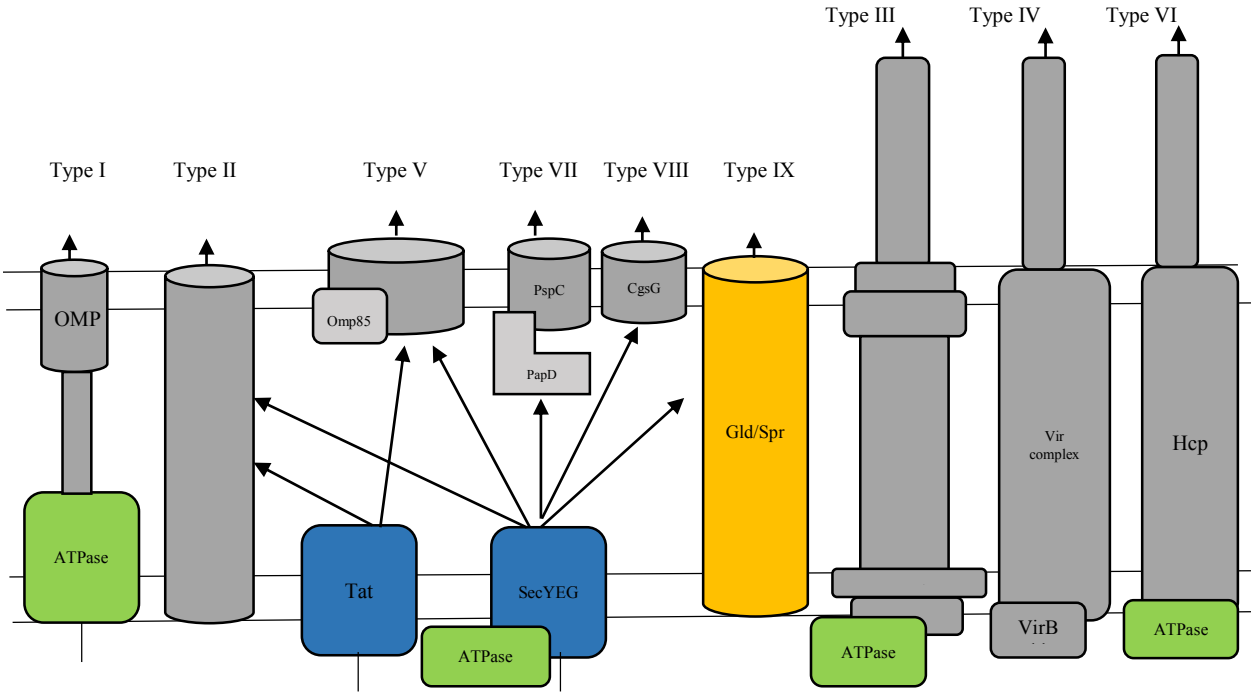

Fig. 5. Diagram depicting Type I to Type IX secretion systems. Protein export pathway from cytoplasm to periplasm is carried out by the Sec and Tat protein export machineries (blue) in type II, V, VII, VIII and IX secretion systems. The type I, III, IV, and VI secretion systems transport proteins directly from the cytoplasm to the outside. (Modified from (38)).

The type IX secretion system or T9SS was recently discovered in both motile and non-motile members of the phylum *Bacteroidetes*. It has been extensively studied in *F. johnsoniae* and in the non-motile oral pathogen *P. gingivalis* (4, 18). The *F. johnsoniae* T9SS is essential for secretion of cell surface motility adhesins SprB and RemA and also for secretion of proteins not associated with motility, such as the extracellular chitinase ChiA the amylase AmyB, and numerous proteases (39, 40). The *P. gingivalis* T9SS is involved in secretion of virulence factors such as the gingipain proteases (4). The components of the T9SS are unique and are not found outside of this phylum (Fig 6). The *F. johnsoniae* Gld and Spr proteins GldK, GldL, GldM, GldN, SprA, SprE and SprT are components of the T9SS (41) and have counterparts in the *P. gingivalis* T9SS (PorK, PorL, PorM, PorN, *sov*, PorW, and PorT respectively) (4).

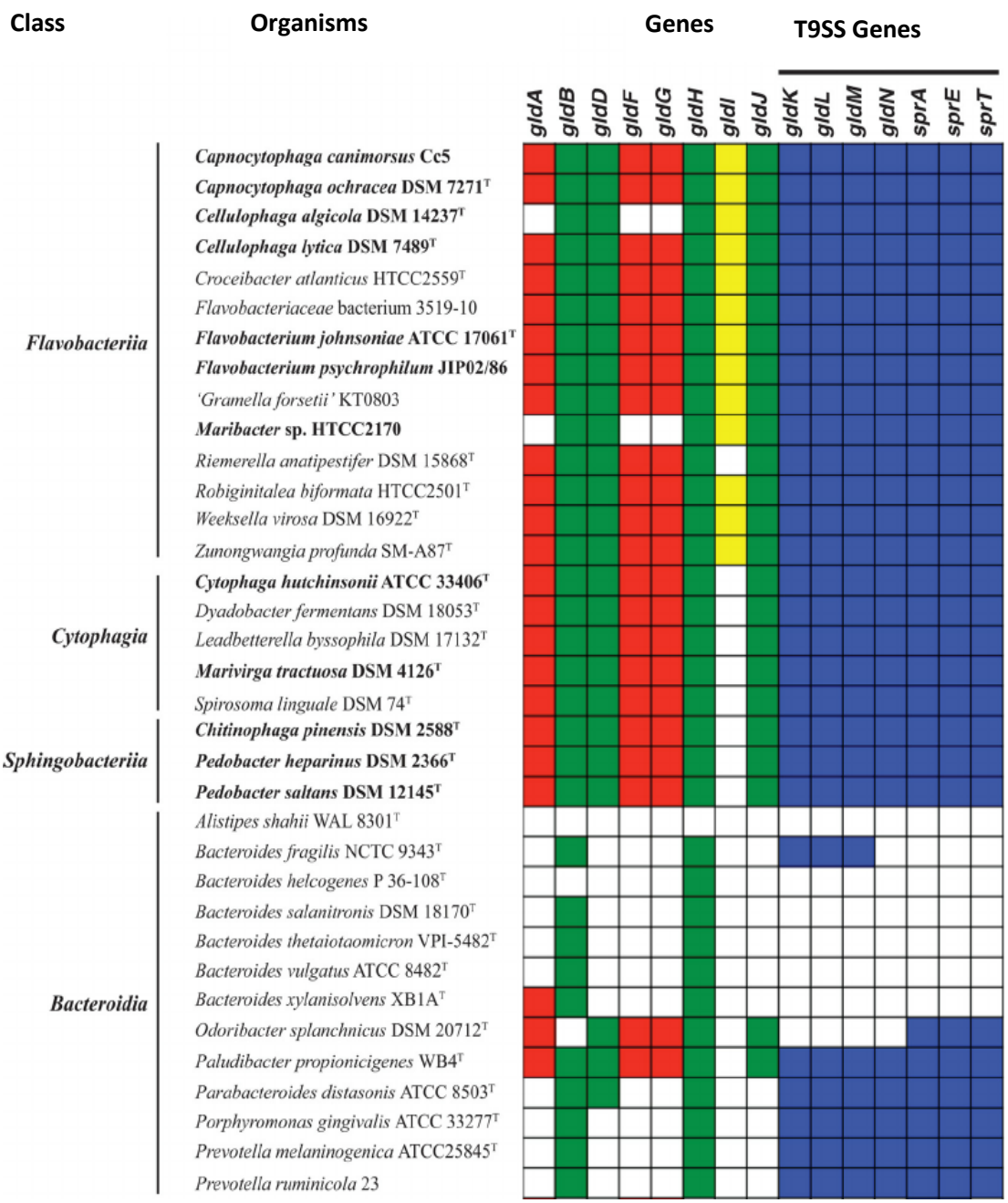

Fig. 6. Distribution of T9SS and gliding motility genes among members of the phylum *Bacteroidetes*. A colored square indicates the presence of an ortholog and a white square indicates the absence of an ortholog. Colors of squares correspond to genes encoding: red, ABC transporter components; yellow, peptidylprolyl isomerases; blue, T9SS components; green, other proteins required for gliding. Blue and green colored squares are core gliding motility genes. Adapted from (17). *Rhodothermus marinus* and *Salinibacter ruber* were removed from the figure because they have now been assigned to a new phylum, *Rhodothermeota* (42, 43).

Recent studies demonstrated that *P. gingivalis porP*, *porK porL*, *porM* and *porN* are co-transcribed (44). Biochemical analyses revealed that the proteins encoded by these five genes are distributed in the cell envelope with PorL and PorM localized to the inner membrane, PorK and PorN associated with the inner face of the outer membrane, and the β-barrel protein PorP spanning the outer membrane. Negative stain electron microscopic analyses revealed the structural details suggesting that PorK and PorN form a large ring on the periplasmic side of the outer membrane (45). With the aid of bacterial two hybrid and co-immune precipitation assays, it was found that PorL and PorM interact with each other likely through their transmembrane helices. PorM also has a periplasmic domain that interacts with PorK, PorN and PorP (Fig. 7) (44).

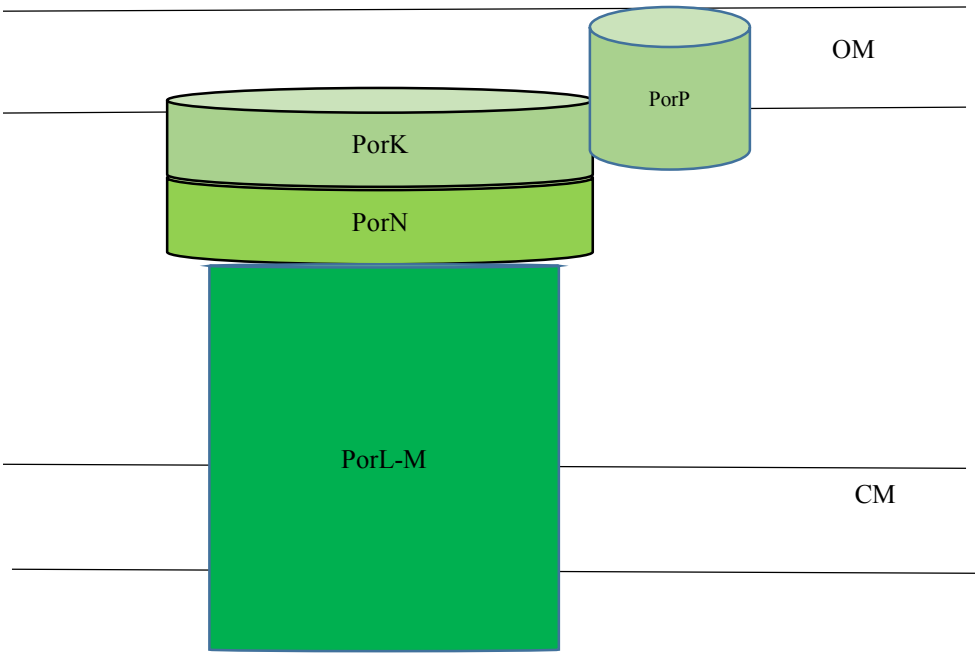

Fig. 7. Schematic representation of the PorKLMNP T9SS core complex. The model highlights the localizations of the T9SS proteins in the cell envelope. (Modified from (44)).

The proteins secreted by T9SSs have N-terminal signal peptides that allow export across the cytoplasmic membrane via the Sec machinery and rely on the components of the T9SS for secretion across the outer membrane (39, 46, 47). They also have conserved C-terminal domains (CTDs), which act as targeting signals for secretion across the outer membrane through the T9SS (17, 39, 46-48). The CTDs are cleaved during secretion and the mature proteins either attach to the cell surface (4, 41, 46, 49) or are released in soluble form (39, 40). In *P. gingivalis* CTD cleavage is thought to be carried out by PorU, which has been described as a peptidase (50). The CTDs of T9SS-secreted proteins differ in sequence and belong to at least two different TIGRFAM families, TIGR04183 and TIGR04131 (39, 47). The conserved CTDs allow in-silico predictions of proteins secreted by the T9SSs of the members of the phylum *Bacteroidetes*. Secretion of many of these proteins has been verified experimentally in *P. gingivalis*, *Cytophaga hutchinsonii* and *F. johnsoniae* (39, 46).

The first evidence of CTD targeting of proteins in *F. johnsoniae* was observed with the ChiA CTD. Fusing 105 amino acids of $ChiA_{CTD}$ to mCherry resulted in secretion of mCherry into the culture medium (40). *F. johnsoniae* T9SS is predicted to secrete 53 CTD- containing proteins including SprB and RemA. RemA belongs to TIGR04183 (type A CTD) and SprB belongs to TIGR04131 (type B CTD) (39). However, the exact roles of the CTDs in targeting SprB and RemA were unknown. *P. gingivalis* RgpB and HBP35 type A CTDs have been analyzed in detail. Deletion of the CTD of RgpB results in accumulation of the protein in the periplasm (51, 52). Fusing HBP35 CTD to GFP resulted in its cell surface localization and glycosylation (53). The CTDs are thought to be cleaved by PorU. Mutation of *porU* resulted in accumulation of RgpB at the cell surface with an intact CTD (50-52). Analyses of truncated versions of RgpB indicated

that the C-terminal 72 amino acids are necessary for secretion and outer membrane attachment (51). The crystal structure of the RgpB CTD was recently resolved and was found to have an Ig-like fold with seven antiparallel β- strands organized in two β- sheets packed against each other in a β- sandwich (54).

The SprB CTD is different in sequence, belonging to protein domain family TIGR04131 (type B CTDs). Proteins with type B CTDs are common in members of the *Bacteroidetes*. A transposon insertion in *sprB* resulted in the production of truncated SprB protein lacking the C-terminal 34 amino acids. This protein failed to be secreted to the cell surface (25). This indicated a possible role of the type B CTD in aiding secretion of SprB through the T9SS. However, there has not been a detailed study on the involvement of type B CTDs in secretion in any bacterium. This thesis is focused on understanding the mechanism of targeting of proteins for secretion by the *F. johnsoniae* T9SS (Fig. 8).

Specifically, this thesis describes the functional characterization of two type A CTDs (RemA, AmyB), two type B CTDs (SprB and Fjoh_3952) and ChiA CTD, which differs in sequence from type A and type B CTDs. Chapter 2 deals primarily with the type A CTDs, including those of RemA, AmyB. ChiA CTD is also examined in chapter 2. A slightly modified form of chapter 2 was published in the Journal of Bacteriology. Chapter 3 provides the first detailed study of the role of type B CTDs (SprB and Fjoh_3952) in protein secretion. It also examines the role of SprF as an adapter or chaperone involved in this process. A manuscript describing these data is currently in preparation. Three appendices follow the main text. Appendix 1 describes the use of the *F. johnsoniae* $ChiA_{CTD}$ to target a foreign protein for secretion by the T9SS of the fish pathogen *F. columnare*. This is part of a large collaborative study to describe the role of the T9SS

in *F. columnare* virulence and a manuscript describing this work is being prepared for publication. Appendix 2 describes the phenotypes associated with *F. johnsoniae* cells carrying mutations in the *F. johnsoniae* genes related to *E. coli* chemotaxis genes *cheB* and *cheR*. Appendix 3 describes the use of a transposon mutagenesis screen to identify novel genes linked to gliding motility.

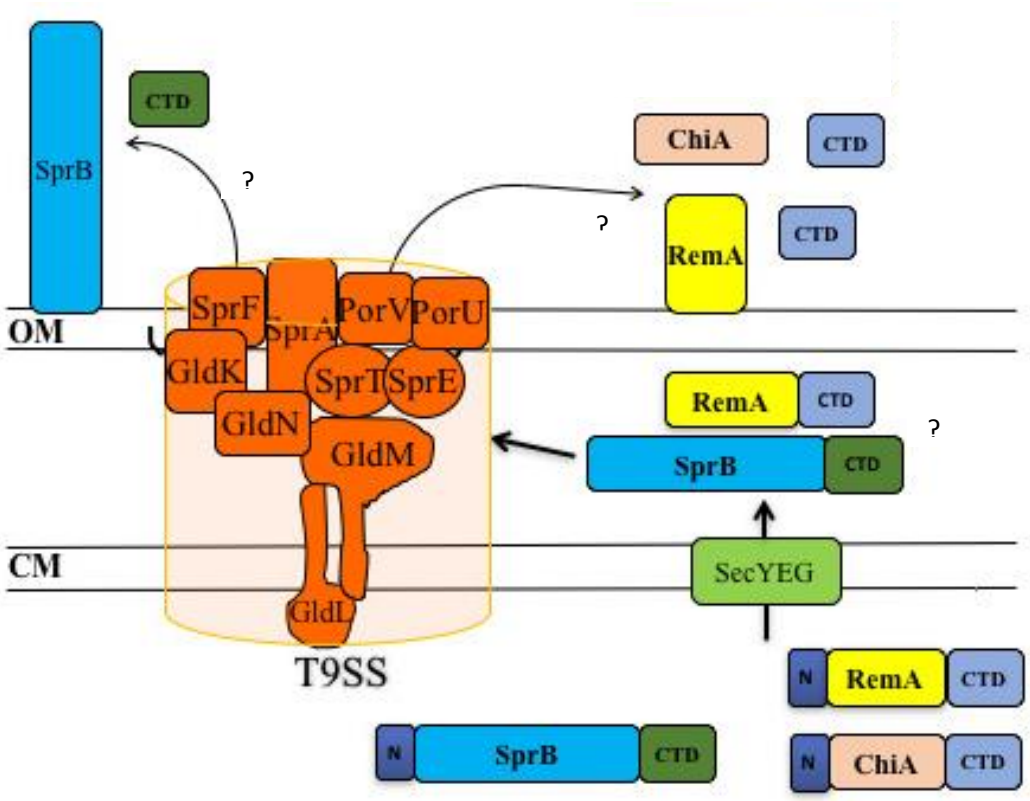

Fig. 8. Members of the genus *Flavobacterium*, and many related bacteria, secrete proteins across the outer membrane using the type IX secretion system (T9SS core proteins in orange). Proteins secreted by T9SSs have amino-terminal signal peptides (N) for export across the cytoplasmic membrane by the Sec system, and carboxy-terminal domains (CTDs) targeting them for secretion across the outer membrane by the T9SS. Most T9SS CTDs belong to either family TIGR04183 (type A CTDs; blue) or TIGR04131 (type B CTDs; dark green). The CTDs are cleaved off during or after secretion of the effector proteins.

References:

1. Larsbrink J, Zhu Y, Kharade SS, Kwiatkowski KJ, Eijsink VG, Koropatkin NM, McBride MJ, Pope PB. 2016. A polysaccharide utilization locus from *Flavobacterium johnsoniae* enables conversion of recalcitrant chitin. Biotechnol Biofuels 9:260.
2. Sack EL, van der Wielen PW, van der Kooij D. 2011. *Flavobacterium johnsoniae* as a model organism for characterizing biopolymer utilization in oligotrophic freshwater environments. Appl Environ Microbiol 77:6931-8.
3. McBride MJ, Xie G, Martens EC, Lapidus A, Henrissat B, Rhodes RG, Goltsman E, Wang W, Xu J, Hunnicutt DW, Staroscik AM, Hoover TR, Cheng YQ, Stein JL. 2009. Novel features of the polysaccharide-digesting gliding bacterium *Flavobacterium johnsoniae* as revealed by genome sequence analysis. Appl Environ Microbiol 75:6864-75.
4. Sato K, Naito M, Yukitake H, Hirakawa H, Shoji M, McBride MJ, Rhodes RG, Nakayama K. 2010. A protein secretion system linked to bacteroidete gliding motility and pathogenesis. Proc Natl Acad Sci U S A 107:276-81.
5. Declercq AM HF, Van den Broeck W, Bossier P, Decostere A. 2013. Columnaris disease in fish: a review with emphasis on bacterium-host interactions. Vet Res 44.
6. Starliper CE. 2011. Bacterial coldwater disease of fishes caused by *Flavobacterium psychrophilum*. Journal of Advanced Research 2:97-108.
7. Braun TF, Khubbar MK, Saffarini DA, McBride MJ. 2005. *Flavobacterium johnsoniae* gliding motility genes identified by mariner mutagenesis. J Bacteriol 187:6943-52.
8. Rhodes RG, Pucker HG, McBride MJ. 2011. Development and use of a gene deletion strategy for *Flavobacterium johnsoniae* to identify the redundant gliding motility genes *remF*, *remG*, *remH*, and *remI*. J Bacteriol 193:2418-28.
9. Agarwal SD HD, McBride MJ. 1997. Cloning and characterization of the *Flavobacterium johnsoniae* (*Cytophaga johnsonae*) gliding motility gene, *gldA*. Proc Natl Acad Sci U S A 94:12139-12144.
10. Vingadassalom D, Kolb A, Mayer C, Rybkine T, Collatz E, Podglajen I. 2005. An unusual primary sigma factor in the *Bacteroidetes* phylum. Mol Microbiol 56:888-902.
11. Wegmann U HN, Carding SR. 2013. Defining the bacteroides ribosomal binding site. Appl Environ Microbiol 79:1980-9.
12. Chen S, Kaufman MG, Bagdasarian M, Bates AK, Walker ED. 2010. Development of an efficient expression system for *Flavobacterium* strains. Gene 458:1-10.
13. Chen S BM, Kaufman M, Walker E. 2007. Characterization of strong promoters from an environmental *Flavobacterium hibernum* strain by using a green fluorescent protein-based reporter system. . Appl Environ Microbiol 73:1089–1100.
14. Anderson KL. SA. 1989. Genetic evidence that outer membrane binding of starch is required for starch utilization by *Bacteroides thetaiotaomicron*. J Bacteriol 171:3199-204.
15. Martens EC, Koropatkin NM, Smith TJ, Gordon JI. 2009. Complex glycan catabolism by the human gut microbiota: the *Bacteroidetes* Sus-like paradigm. J Biol Chem 284:24673-7.
16. Coyne MJ, Fletcher CM, Chatzidaki-Livanis M, Posch G, Schaffer C, Comstock LE. 2013. Phylum-wide general protein O-glycosylation system of the *Bacteroidetes*. Mol Microbiol 88:772-83.

17. McBride M, and Zhu Y. 2013. Gliding motility and Por secretion system genes are widespread among members of the phylum *Bacteroidetes*. J Bacteriol 195:170-8.
18. Sato K, Sakai E, Veith PD, Shoji M, Kikuchi Y, Yukitake H, Ohara N, Naito M, Okamoto K, Reynolds EC, Nakayama K. 2005. Identification of a new membrane-associated protein that influences transport/maturation of gingipains and adhesins of *Porphyromonas gingivalis*. J Biol Chem 280:8668-77.
19. Duxbury T HB, Marshall KC. 1980. Acetate acts as a protonphore and differentially affects bead movement and cell migration of the glinding bacterium *Cytophaga johnsonae* (*Flavobacterium johnsoniae*). Microbiology 143:3693-3701.
20. Wolkin RH PJ. 1986. Phage adsorption and cell adherence are motility-dependent characteristics of the gliding bacterium *Cytophaga johnsonae*. J Gen Microbiol 132:355-367.
21. Nakane D. SK, Wada H., McBride M. J., Nakayama K. . 2013. Helical flow of surface protein required for bacterial gliding motility. . Proc Natl Acad Sci USA 110:11145–11150.
22. Liu J, McBride MJ, Subramaniam S. 2007. Cell surface filaments of the gliding bacterium *Flavobacterium johnsoniae* revealed by cryo-electron tomography. J Bacteriol 189:7503-6.
23. Velicer GJ, Vos M. 2009. Sociobiology of the myxobacteria. Annu Rev Microbiol 63:599-623.
24. Miyata M. 2010. Unique centipede mechanism of Mycoplasma gliding. Annu Rev Microbiol 64:519-37.
25. Nelson SS, Bollampalli S, McBride MJ. 2008. SprB is a cell surface component of the *Flavobacterium johnsoniae* gliding motility machinery. J Bacteriol 190:2851-7.
26. McBride MJ, Nakane D. 2015. *Flavobacterium* gliding motility and the type IX secretion system. Curr Opin Microbiol 28:72-7.
27. Shrivastava A, Rhodes RG, Pochiraju S, Nakane D, McBride MJ. 2012. *Flavobacterium johnsoniae* RemA is a mobile cell surface lectin involved in gliding. J Bacteriol 194:3678-88.
28. Braun TF, McBride MJ. 2005. *Flavobacterium johnsoniae GldJ* is a lipoprotein that is required for gliding motility. J Bacteriol 187:2628-37.
29. Hunnicutt DW, McBride MJ. 2001. Cloning and characterization of the *Flavobacterium johnsoniae* gliding motility genes *gldD* and *gldE*. J Bacteriol 183:4167-75.
30. Hunnicutt. DW KM, McBride MJ. 2002. Mutations in *Flavobacterium johnsoniae gldF* and *gldG* disrupt gliding motility and interfere with membrane localization of GldA. J Bacteriol 184.
31. Hunnicutt. DW MM. 2000. Cloning and characterization of the *Flavobacterium johnsoniae* gliding motility genes, *gldB* and *gldC*. J Bacteriol 182.
32. Nelson SS, Glocka PP, Agarwal S, Grimm DP, McBride MJ. 2007. *Flavobacterium johnsoniae* SprA is a cell surface protein involved in gliding motility. J Bacteriol 189:7145-50.
33. McBride MJ BT. 2004. GldI is a lipoportein that is required for *Flavobacterium johnsoniae* gliding motility and chitin utilization. J Bacteriol 186.
34. Chagnot C, Zorgani MA, Astruc T, Desvaux M. 2013. Proteinaceous determinants of surface colonization in bacteria: bacterial adhesion and biofilm formation from a protein secretion perspective. Front Microbiol 4:303.

35. Busch A, Waksman G. 2012. Chaperone-usher pathways: diversity and pilus assembly mechanism. Philos Trans R Soc Lond B Biol Sci 367:1112-22.
36. Barnhart MM, Chapman MR. 2006. Curli biogenesis and function. Annu Rev Microbiol 60:131-47.
37. Ligon LS, Hayden JD, Braunstein M. 2012. The ins and outs of *Mycobacterium tuberculosis* protein export. Tuberculosis (Edinb) 92:121-32.
38. Green ER, Mecsas J. 2016. Bacterial Secretion Systems: An Overview. Microbiol Spectr 4.
39. Kharade SS, McBride MJ. 2015. *Flavobacterium johnsoniae* PorV is required for secretion of a subset of proteins targeted to the type IX secretion system. J Bacteriol 197:147-58.
40. Kharade SS MM. 2014. The *Flavobacterium johnsoniae* chitinae ChiA is required for chitin utilization and is secreted by the type IX secretion system. J Bacteriol 196:961-970.
41. Shrivastava A JJ, Barren JM, McBride MJ. 2012. *Flavobacterium johnsoniae* GldK, GldL, GldM, and SprA are required for secretion of the cell suraface gliding motility adhesins SprB and RemA. J Bacteriol 195:3201-12.
42. Munoz R, Rossello-Mora R, Amann R. 2016. Revised phylogeny of *Bacteroidetes* and proposal of sixteen new taxa and two new combinations including Rhodothermaeota phyl. nov. Syst Appl Microbiol 39:281-96.
43. Hahnke RL, Meier-Kolthoff JP, Garcia-Lopez M, Mukherjee S, Huntemann M, Ivanova NN, Woyke T, Kyrpides NC, Klenk HP, Goker M. 2016. Genome-Based Taxonomic Classification of *Bacteroidetes*. Front Microbiol 7:2003.
44. Vincent MS, Canestrari MJ, Leone P, Stathopulos J, Ize B, Zoued A, Cambillau C, Kellenberger C, Roussel A, Cascales E. 2017. Characterization of the *Porphyromonas gingivalis* Type IX Secretion Trans-envelope PorKLMNP Core Complex. J Biol Chem 292:3252-3261.
45. Gorasia DG, Veith PD, Hanssen EG, Glew MD, Sato K, Yukitake H, Nakayama K, Reynolds EC. 2016. Structural Insights into the PorK and PorN Components of the *Porphyromonas gingivalis* Type IX Secretion System. PLoS Pathog 12:e1005820.
46. Veith PD, Nor Muhammad NA, Dashper SG, Likic VA, Gorasia DG, Chen D, Byrne SJ, Catmull DV, Reynolds EC. 2013. Protein substrates of a novel secretion system are numerous in the *Bacteroidetes* phylum and have in common a cleavable C-terminal secretion signal, extensive post-translational modification, and cell-surface attachment. J Proteome Res 12:4449-61.
47. Sato K, Yukitake H, Narita Y, Shoji M, Naito M, Nakayama K. 2013. Identification of *Porphyromonas gingivalis* proteins secreted by the Por secretion system. FEMS Microbiol Lett 338:68-76.
48. Nguyen KA, Travis J, Potempa J. 2007. Does the importance of the C-terminal residues in the maturation of RgpB from *Porphyromonas gingivalis* reveal a novel mechanism for protein export in a subgroup of Gram-Negative bacteria? J Bacteriol 189:833-43.
49. Gorasia DG, Veith PD, Chen D, Seers CA, Mitchell HA, Chen YY, Glew MD, Dashper SG, Reynolds EC. 2015. *Porphyromonas gingivalis* Type IX Secretion Substrates Are Cleaved and Modified by a Sortase-Like Mechanism. PLoS Pathog 11:e1005152.
50. Glew MD, Veith PD, Peng B, Chen YY, Gorasia DG, Yang Q, Slakeski N, Chen D, Moore C, Crawford S, Reynolds EC. 2012. PG0026 is the C-terminal signal peptidase of a novel secretion system of *Porphyromonas gingivalis*. J Biol Chem 287:24605-17.

51. Seers CA, Slakeski N, Veith PD, Nikolof T, Chen YY, Dashper SG, Reynolds EC. 2006. The RgpB C-terminal domain has a role in attachment of RgpB to the outer membrane and belongs to a novel C-terminal-domain family found in *Porphyromonas gingivalis*. J Bacteriol 188:6376-86.
52. Slakeski N, Seers CA, Ng K, Moore C, Cleal SM, Veith PD, Lo AW, Reynolds EC. 2011. C-terminal domain residues important for secretion and attachment of RgpB in *Porphyromonas gingivalis*. J Bacteriol 193:132-42.
53. Shoji M, Sato K, Yukitake H, Kondo Y, Narita Y, Kadowaki T, Naito M, Nakayama K. 2011. Por secretion system-dependent secretion and glycosylation of *Porphyromonas gingivalis* hemin-binding protein 35. PLoS One 6:e21372.
54. de Diego I, Ksiazek M, Mizgalska D, Koneru L, Golik P, Szmigielski B, Nowak M, Nowakowska Z, Potempa B, Houston JA, Enghild JJ, Thogersen IB, Gao J, Kwan AH, Trewhella J, Dubin G, Gomis-Ruth FX, Nguyen KA, Potempa J. 2016. The outer-membrane export signal of *Porphyromonas gingivalis* type IX secretion system (T9SS) is a conserved C-terminal beta-sandwich domain. Sci Rep 6:23123.

Chapter 2. Diverse C-terminal sequences involved in *Flavobacterium johnsoniae* protein secretion.

This chapter is a modified version of a paper in Journal of Bacteriology (in press; https://doi.org/10.1128/JB.00884-16). This chapter includes some of the online supplemental materials of the paper integrated into it.

Abstract

Flavobacterium johnsoniae and many related bacteria secrete proteins across the outer membrane using the type IX secretion system (T9SS). Proteins secreted by T9SSs have amino-terminal signal peptides for export across the cytoplasmic membrane by the Sec system and carboxy-terminal domains (CTDs) targeting them for secretion across the outer membrane by the T9SS. Most but not all T9SS CTDs belong to family TIGR04183 (type A CTDs). We functionally characterized diverse CTDs for secretion by the *F. johnsoniae* T9SS. Attachment of the CTDs from *F. johnsoniae* RemA, AmyB, and ChiA to the foreign protein sfGFP that had a signal peptide at the amino terminus resulted in secretion across the outer membrane. In each case approximately 80 to 100 amino acids from the extreme carboxy-termini was needed for efficient secretion. Several type A CTDs from distantly related members of the phylum *Bacteroidetes* functioned in *F. johnsoniae*, supporting secretion of sfGFP by the *F. johnsoniae* T9SS. *F. johnsoniae* SprB requires the T9SS for secretion but lacks a type A CTD. It has a conserved C-terminal domain belonging to family TIGR04131, which we refer to as a type B CTD. The CTD of SprB was required for its secretion, but attachment of C-terminal regions of SprB of up to 1182 amino acids to sfGFP failed to result in secretion. Additional features outside of the C-terminal region of SprB may be required for its secretion.

Introduction

Many members of the phylum *Bacteroidetes* secrete proteins across the outer membrane using the type IX secretion system (T9SS) (1, 2). The components of the T9SS were first identified and characterized in *Porphyromonas gingivalis* and *Flavobacterium johnsoniae* (3, 4) and have more recently been studied in other members of the phylum *Bacteroidetes* (5-7). Components of the secretion system include GldK, GldL, GldM, GldN, SprA, SprE, SprT, PorU, and PorV (referred to as PorK, PorL, PorM, PorN, sov, PorW, PorT, PorU, and PorV respectively in *P. gingivalis*). *F. johnsoniae* also has a paralog of GldN called GldO, and deletion of the genes encoding both proteins is required for complete loss of secretion (8). An additional component, PorZ, was also recently described in *P. gingivalis* (9). Most of the proteins listed above are unique to members of the phylum *Bacteroidetes*. Some of the components of the T9SS were first identified in *F. johnsoniae* as proteins required for gliding motility (10, 11) and were later realized to function in protein secretion in both *F. johnsoniae* and in *P. gingivalis* (3, 12, 13). The *F. johnsoniae* cell-surface motility adhesins SprB and RemA require the T9SS for their delivery across the outer membrane (3, 13, 14). Rapid movement of SprB and RemA along the cell surface is responsible for cell movement (15, 16). Mutations in the T9SS genes prevent proper assembly of the motility apparatus. Some components of the T9SS may also have more direct functions in motility (13, 17). Most but not all bacteria that have T9SSs exhibit gliding motility. *P. gingivalis* is one of the exceptions; it has a T9SS but is nonmotile.

Proteins secreted by T9SSs have N-terminal signal peptides allowing export across the cytoplasmic membrane by the Sec system (2, 18, 19). They also typically have conserved C-terminal domains (CTDs) that are thought to target them to the T9SS for secretion across the outer

membrane (2, 18-20). Once delivered across the outer membrane some proteins are released in soluble form (18, 21) whereas others are modified and attached to the cell surface (2, 3, 13, 22). The CTDs are removed during or after secretion. In *P. gingivalis* this is thought to involve PorU which has been described as a C-terminal signal peptidase (23). The secretion process and attachment of secreted proteins to the cell surface is somewhat reminiscent of Gram positive bacterial sortase-mediated secretion and cell-surface localization, although the proteins involved are not related in sequence (22). T9SS CTDs typically fall into one of two protein domain families corresponding to TIGR04183 (here referred to as type A CTDs) and TIGR04131 (here referred to as type B CTDs) (18, 19). The conserved CTDs allow predictions to be made regarding the number of proteins secreted by the T9SSs of individual bacterial species. For example, *F. johnsoniae* and *Cytophaga hutchinsonii* are predicted to secrete 53 and 147 proteins respectively using their T9SSs (7, 13). Secretion of many of these proteins has been verified experimentally (2, 18). These predictions may underestimate the actual number of secreted proteins since some proteins secreted by T9SSs, such as *F. johnsoniae* ChiA, have CTDs that are required for secretion but that exhibit no obvious sequence similarity to either type A or type B CTDs (21).

T9SS CTDs have been functionally characterized for two *P. gingivalis* proteins, the gingipain protease RgpB, and the hemin-binding protein HBP35 (20, 24-26). RgpB and HBP35 both have type A CTDs. HBP35 required the C-terminal 22 AAs for secretion and cell surface attachment (25). Deletion of this region or modification of a conserved lysine within this region resulted in decreased secretion. Attachment of C-terminal regions to GFP allowed secretion of the foreign protein GFP by *P. gingivalis*. Analysis of CTDs from *F. johnsoniae* has been limited to a single protein, the soluble chitinase ChiA (21). As indicated above, ChiA requires the T9SS for secretion but it lacks a recognizable conserved CTD. Nevertheless, attachment of the C-terminal

105 AAs of ChiA to the foreign protein mCherry resulted in secretion of mCherry from the cell (21). Here we functionally examine the C-terminal regions of five diverse *F. johnsoniae* proteins that require the T9SS for secretion. These included two type A CTDs, two type B CTDs, and the ChiA CTD. We also demonstrate that some CTDs from distantly related members of the phylum *Bacteroidetes* can function with the *F. johnsoniae* T9SS.

Materials and Methods

Bacterial strains, plasmids and growth conditions. *F. johnsoniae* ATCC 17061^T strain UW101 was the wild-type strain used in this study (27-29). *F. johnsoniae* strains were grown in Casitone-yeast extract (CYE) medium at 30°C, as previously described (30). *Escherichia coli* strains were grown in Luria-Bertani medium (LB) at 37°C (31). Strains and plasmids used in this study are listed in Table 1, and primers are listed in Table 2. Antibiotics were used at the following concentrations when needed: ampicillin, 100 μg/ml; kanamycin, 30 μg/ml; erythromycin, 100 μg/ml; streptomycin, 100 μg/ml; and tetracycline, 20 μg/ml.

Generation of plasmids that express sfGFP with signal peptides at the N-terminus and with regions of *F. johnsoniae* T9SS CTDs at the C-terminus. A plasmid expressing the N-terminal signal peptide of RemA fused to sfGFP, which was in turn fused to the C-terminal 97 amino acids of RemA (SP_{RemA}-sfGFP-CTD_{RemA}) was constructed as follows. A 511-bp fragment spanning the *remA* promoter, start codon and N-terminal signal peptide-encoding region was amplified using Phusion DNA polymerase (New England Biolabs, Ipswich, MA) and primers 1269 (engineered KpnI site) and 1270 (engineered BamHI site). This fragment was inserted into the KpnI and BamHI sites of pCP23 to generate pYT40. A 711-bp region of sfGFP without stop codon was amplified from pTB263 using primers 1389 (engineered BamHI site) and 1427 (engineered

GGSGGGSG linker and XbaI site). This fragment was inserted into the BamHI and XbaI sites of pYT40, generating pYT179. To introduce the 97-amino acid-CTD-encoding region of *remA*, 339-bp was amplified using primer 1488 (engineered XbaI site) and 1489 (engineered SphI site). The product was cloned into pYT179, to generate pSK30. A similar construct in a vector with a different antibiotic resistance marker (*ermF*) was obtained by amplifying the fragment encoding SP_{RemA}-sfGFP-CTD_{RemA} from pSK30 using primers 1269 and 1489, and inserting this into the SalI site of pMM105.A that had been made blunt using end-conversion mix from the perfectly-blunt cloning kit (Novagen, Madison, WI), to generate pSK97. Orientation of the insert was confirmed by restriction enzyme digestion and by DNA sequencing.

A plasmid expressing full length RemA with sfGFP inserted immediately after the N-terminal signal peptide was also constructed. For this purpose, the 4383-bp region spanning all of *remA* including the stop codon, but lacking the region encoding the N-terminal signal peptide was amplified using primers 1271 (engineered XbaI site) and 1272 (engineered XbaI site). This fragment was inserted into XbaI site of pYT179 and the orientation of insertion was confirmed by sequencing, generating pYT180. pSK37, expressing the RemA N-terminal signal peptide fused to sfGFP without CTD_{RemA}, was constructed as a control. To construct this plasmid, the gene encoding sfGFP was amplified using primers 1389 (engineered BamHI site) and 1390 (engineered XbaI site and introduced stop codon) and introduced into BamHI- and XbaI-digested pYT40. A similar construct in a vector with a different antibiotic resistance marker (*ermF*) was obtained by amplifying the fragment encoding SP_{RemA}-sfGFP from pSK37 using primers 1269 and 1390, and inserting this into the SalI site of pCP11 that had been made blunt using end-conversion mix from the perfectly-blunt cloning kit to generate pSK96. Orientation of the insert was confirmed by restrictrion enzyme digestion and by DNA sequencing. Fragments encoding CTD_{RemA} regions of

87 amino acids and 62 amino acids were cloned in pYT179, generating plasmids pSK71 and pSK81 respectively. A 258-bp region encoding 85 amino acids near the C-terminus of RemA but lacking the C-terminal 12 amino acids was also cloned into pYT179, generating plasmid pSK79. Similarly, a plasmid producing SP_{RemA}-sfGFP fused to the C-terminal 97 amino acids of RemA in which a conserved lysine of RemA was replaced with an alanine (K1432A) was constructed. For this purpose, a 300-bp fragment was amplified using primers 1488 (engineered XbaI site) and 1962 (engineered SphI site and engineered alanine codon). This fragment was inserted into pYT179, generating pSK91.

The RemA signal peptide was replaced by the cytochrome-C (Fjoh_1634) signal peptide to determine if the signal peptide from a normally periplasmic protein would be sufficient to allow CTD-mediated secretion by the T9SS. A 396-bp region spanning the promoter and N-terminal signal sequence of Fjoh_1634 was amplified using primer 1946 (engineered KpnI site) and 1947 (engineered BamHI site). This fragment was inserted into the KpnI and BamHI sites of pSK30, generating plasmid pSK84.

Constructs expressing SP_{RemA}-sfGFP fused to CTD-containing regions of *F. johnsoniae* ChiA, AmyB (Fjoh_1208), SprB, and the SprB-like protein Fjoh_3952 were also generated as follows. A 735-bp region of sfGFP was amplified from pTB263 using primer 1389 (engineered BamHI site) and 1427 (engineered XbaI site). This fragment was cloned into the BamHI and XbaI sites of pSSK52 (21), generating pCB3, which encodes sfGFP fused to the 105 amino acid C-terminal region of ChiA. Similarly, fragments encoding CTD_{ChiA} regions of 79 amino acids and 62 amino acids were cloned in pCB3, generating plasmids pSK89 and pCB4 respectively. For *F. johnsoniae* AmyB, a 390-bp region encoding the C-terminal 99 amino acids was inserted into the

XbaI and SphI sites of pYT179, generating plasmid pSK82. Similarly, regions encoding the C-terminal 73 amino acids and 59 amino acids were also inserted into the XbaI and SphI sites of pYT179, generating plasmids pSK85 and pSK86 respectively. For *F. johnsoniae* SprB, 300-bp, 657-bp, and 3,549-bp regions encoding the C-terminal 99, 218, and 1182 amino acids were amplified and inserted into the XbaI and SphI sites of pYT179, generating plasmids pSK93, pSK56, and pSK62 respectively. For the *F. johnsoniae* SprB-like protein Fjoh_3952, a 687-bp region encoding the C-terminal 228 amino acids was inserted into the XbaI and SphI sites of pYT179, generating pSK58.

Generation of plasmids expressing SP_{RemA}-sfGFP fused to regions of *Cellulophaga algicola*, *C. hutchinsonii*, and *P. gingivalis* T9SS CTDs. A 417-bp region encoding the C-terminal 108 amino acids of Celal_2532 (AmyA) was amplified from the *C. algicola* DSM 14237 genome using primer 1885 (engineered XbaI site) and 1886 (engineered SphI site). This fragment was inserted into pYT179, generating pSK65. A 294-bp region encoding the C-terminal 97 amino acids of CHU_1335 (Cel9B) was amplified from the *C. hutchinsonii* ATCC 33406 genome using primer 1925 (engineered XbaI site) and 1926 (engineered SphI site). This fragment was inserted into pYT179, generating pSK76. A 339-bp region encoding the C-terminal 103 amino acids of PGN_1466 (RgpB) was amplified from the *P. gingivalis* ATCC 33277 genome using primer 1923 (engineered XbaI site) and 1924 (engineered SphI site). This fragment was inserted into pYT179, generating pSK75.

Microscopic observation of binding of cells to protein G-coated polystyrene spheres to detect surface-localized SprB or sfGFP. Cells were grown in MM (32) at 25˚C without shaking. 1 µl of purified anti-SprB (16) or anti-GFP (0.5 mg per ml; GeneScript), 0.5-µm-diameter

protein G-coated polystyrene spheres (1 µl of a 0.1% stock preparation; Spherotech Inc., Livertyville, IL), and bovine serum albumin (BSA) (1 µl of 1% solution) were added to 7 µl cells. The mixture was introduced into a tunnel slide prepared as described previously (14) using Nichiban NW-5 double sided tape (Nichiban Co, Tokyo, Japan) to hold a glass coverslip over a glass slide. Samples were incubated for 3 min and observed using an Olympus BH-2 phase-contrast microscope. Images were recorded using a Photometrics Cool-$SNAP_{cf}^2$ camera and analyzed using Metamorph software. Images were recorded for 30s and 100 randomly selected cells were examined for the presence of spheres that remained attached to the cells during this time.

Analysis of secretion of SP_{RemA}-sfGFP-CTD_{RemA} during different stages of growth. 200 µl *F. johnsoniae* cells (OD_{600}~1) carrying pSK30 expressing SP_{RemA}-sfGFP-CTD_{RemA} were inoculated in 50 ml CYE containing tetracycline (10 µg/ml) in side arm flasks and incubated at 25°C with shaking. In exponential phase (approximately 10 h) and stationary phase (approximately 22 h) 1 ml cultures were taken from the flasks and centrifuged at 22,000 x g for 15 min. In each case the cells (pellet) and the spent medium (supernatant) were flash frozen in dry ice ethanol baths and stored at -80°C until needed. The pellet and spent media samples were thawed, cells were suspended in the original culture volume of phosphate-buffered saline consisting of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, and 2 mM KH_2PO_4 (pH 7.4), and both samples were subjected to SDS-PAGE and Western blot analysis to detect sfGFP as described below. Cells prepared in the same way were also examined for levels of the T9SS proteins GldK, GldL, GldM, GldN, SprA, and SprE using antisera specific for each.

Western blot analyses. *F. johnsoniae* cells were grown to early stationary phase in CYE at 25°C with shaking. Cells were pelleted by centrifugation at 22,000 x g for 15 min, and the culture supernatant (spent medium) and cell pellet were separated. For whole-cell samples, the cells were suspended in the original culture volume of phosphate-buffered saline. Equal amounts of spent medium and whole cells were boiled in SDS-PAGE loading buffer for 10 min. Proteins were separated by SDS-PAGE, and Western blot analyses were performed as previously described (8). Equal amounts of each sample based on the starting material were loaded in each lane. For cell extracts this corresponded to 10 μg protein, whereas for spent medium this corresponded to the equivalent volume of spent medium that contained 10 μg cell protein before the cells were removed. For detection of sfGFP by Western blotting, anti-GFP antibodies (0.5 mg per ml) were used at a dilution of 1:3,000.

For detection of full length SprB, cells were mixed with SDS-PAGE loading buffer and incubated at 98˚C for 10 min. Proteins (25 μg protein was loaded per lane) were separated on 3 to 8% Criterion XT Tris-acetate acrylamide gels (Bio-rad, Hercules, CA) and detected by Western blotting using antibodies against SprB at a dilution of 1:2500.

GldK, GldL, GldM, GldN, SprA, and SprE were detected by Western blot as previously described (8, 11, 13, 33). SprF was detected similarly, using polyclonal antibodies against SprF peptides that were produced by Biomatik Corporation (Cambridge, Ontario, Canada) at a dilution of 1:4,000. In each case, 10 μg of proteins were loaded per lane.

Proteinase K treatment of cells to determine the localization of sfGFP-SprB fusion protein. Cells of *F. johnsoniae* were grown in CYE at 25°C with shaking. Cells were collected, washed and suspended in 20 mM sodium phosphate-10 mM $MgCl_2$ (pH 7.5) and diluted to an

OD_{600} reading of 1.5. To examine sfGFP-CTD_{SprB}, proteinase K was added to the intact cells to a final concentration of 1 mg/ml and incubated at 25°C with gentle inverting. At various times, 150 µl of cells were sampled, 10 mM phenylmethylsulfonyl fluoride was added and the samples were boiled for 1 min to stop digestion. SDS-PAGE loading buffer was added and the samples were boiled for another 7 min. Equal volumes were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes, and proteins were detected with anti-serum against GFP. As a positive control for digestion of sfGFP by proteinase K an identical cell sample was lysed using a French pressure cell, unbroken cells and debris were removed by centrifugation, and proteinase K was added as above. Control samples that were not exposed to proteinase K were also included.

Genome analyses. Genome sequences were analyzed for T9SS genes that encode proteins that belong to appropriate TIGRFAM multiple sequence alignment families (34). This was accomplished using the Integrated Microbial Genomes (IMG version 4.0.1, https://img.jgi.doe.gov/) Function Profile Tool to examine the genomes for sequences predicted to encode orthologs of GldK (TIGR03525), GldL (TIGR03513), GldM (TIGR03517), GldN (TIGR03523), and SprA (TIGR04189). The genomes were also examined for genes encoding proteins with type A CTDs (TIGR04183) and type B CTDs (TIGR04131) in the same way. In each case the trusted cutoffs assigned by The J. Craig Venter Institute (JCVI) were used to identify family members. These cutoffs allow identification of the vast majority of family members with vanishingly few false positives (34).

Table 1. Strains and plasmids used in this study.

Strain	Description[a]	Source or reference
***E. coli* strains**		
DH5αmcr	Strain used for general cloning	Life Technologies (Grand Island, NY, USA)
HB101	Strain used with pRK2013 for triparental conjugation	(35, 36)
***F. johnsoniae* strains**		
FJ1	wild type *F. johnsoniae* ATCC 17061^T	(10)
CJ1827	*rpsL2*; Sm^r 'wild-type' *F. johnsoniae* strain used in construction of deletion mutants	(37)
CJ2122	Δ*gldK*	(13)
CJ2157	Δ*gldL*	(13)
CJ2262	Δ*gldM*	(13)
CJ1631A	Δ*gldN-gldO*	(8)
CJ2302	Δ*sprA*	(13)
FJ149	*sprE*	(33)
CJ2518	Δ*sprF*	(38)
KDF002	*sprT*	(3)
CJ2116	Δ*porU*	(18)
CJ2130	Δ*porV*	(18)

CJ1922	Δ*sprB*	(37)
CJ1984	Δ*remA*	(14)
FJ117	*sprB HimarEm2* mutant	(16)
FJ156	*sprB HimarEm2* mutant	(16)

Plasmid	**Description**	**Source or reference**
pCB3	735-bp sfGFP without stop codon amplified and cloned into pSSK52. Encodes SP_{ChiA}-sfGFP-$CTD_{ChiA(105AA)}$; Ap^r (Tc^r)	This study
pCB4	440-bp region encoding 62 amino acids of CTD_{ChiA} inserted into pCB3. Encodes SP_{ChiA}-sfGFP-$CTD_{ChiA(62AA)}$; Ap^r (Tc^r)	This study
pCP11	*E. coli-F. johnsoniae* shuttle plasmid; Ap^r (Em^r)	(30)
pCP23	*E. coli-F. johnsoniae* shuttle plasmid; Ap^r (Tc^r)	(39)
pMM105.A	*E. coli-Capnocytophaga canimorsus* shuttle plasmid; Ap^r (Em^r)	(40)
pRK2013	Helper plasmid for triparental conjugation; IncP Tra^+ Km^r	(36)
pRR48	1294-bp fragment spanning *sprF* inserted into pCP23; Ap^r (Tc^r)	(41)
pSK30	339-bp region encoding 97 amino acids of CTD_{RemA} inserted into pYT179. Encodes SP_{RemA}-sfGFP-$CTD_{RemA(97AA)}$; Ap^r (Tc^r)	This study
pSK37	SP_{RemA}-sfGFP with stop codon cloned into pYT40. Encodes SP-sfGFP; Ap^r (Tc^r)	This study

pSK56	657-bp region encoding 218 amino acids of CTD_{SprB} inserted into pYT179. Encodes SP-sfGFP- $CTD_{SprB(218AA)}$; Ap^r (Tc^r)	This study
pSK58	687-bp region encoding 228 amino acids of CTD_{Fjoh_3952} inserted into pYT179. Encodes SP-sfGFP- $CTD_{Fjoh\_3952(228AA)}$; Ap^r (Tc^r)	This study
pSK62	3549-bp region encoding 1182 amino acids of CTD_{SprB} inserted into pYT179. Encodes SP-sfGFP- $CTD_{SprB(1182AA)}$; Ap^r (Tc^r)	This study
pSK65	417-bp region encoding 108 amino acids of CTD_{Celal_2532} inserted into pYT179. Encodes SP-sfGFP- CTD_{Celal_2532}; Ap^r (Tc^r)	This study
pSK71	312-bp region encoding 87 amino acids of CTD_{RemA} inserted into pYT179. Encodes SP-sfGFP- $CTD_{RemA(87AA)}$; Ap^r (Tc^r)	This study
pSK75	339-bp region encoding 103 amino acids of CTD_{PGN_1466} inserted into pYT179. Encodes SP-sfGFP- CTD_{PGN_1466}; Ap^r (Tc^r)	This study
pSK76	294-bp region encoding 97 amino acids of CTD_{CHU_1335} inserted into pYT179 Encodes SP-sfGFP- CTD_{CHU_1335}; Ap^r (Tc^r)	This study
pSK79	258-bp region encoding 85 amino acids near the C-terminus of RemA but lacking the C-terminal 12 amino acids inserted into pYT179. Encodes SP-sfGFP- $CTD_{RemA(lacking\ final\ 12\ AA)}$; Ap^r (Tc^r)	This study
pSK81	234-bp region encoding 62 amino acids of CTD_{RemA} inserted into pYT179. Encodes SP-sfGFP- $CTD_{RemA(62AA)}$; Ap^r (Tc^r)	This study
pSK82	390-bp region encoding 99 amino acids of CTD_{AmyB} inserted into pYT179. Encodes SP-sfGFP- $CTD_{AmyB(99AA)}$; Ap^r (Tc^r)	This study
pSK84	396-bp fragment spanning the Fjoh_1634 promoter, start codon, and the N-terminal signal peptide-encoding region	This study

	inserted into pSK30. Encodes SP_{Fjoh_1634}-sfGFP-CTD_{RemA}; Ap^r (Tc^r)	
pSK85	312-bp region encoding 73 amino acids of CTD_{AmyB} inserted into pYT179. Encodes SP-sfGFP- $CTD_{AmyB(73AA)}$; Ap^r (Tc^r)	This study
pSK86	270-bp region encoding 59 amino acids of CTD_{AmyB} inserted into pYT179. Encodes SP-sfGFP- $CTD_{AmyB(59AA)}$; Ap^r (Tc^r)	This study
pSK89	491-bp region encoding 79 amino acids of CTD_{ChiA} inserted into pCB3. Encodes SP_{ChiA}-sfGFP- $CTD_{ChiA(79AA)}$; Ap^r (Tc^r)	This study
pSK91	300-bp region encoding CTD_{RemA} with K1432A mutation inserted into pYT179. Encodes SP-sfGFP-$CTD_{RemA(K1432A)}$; Ap^r (Tc^r)	This study
pSK93	300-bp region encoding 99 amino acids of CTD_{SprB} inserted into pYT179. Encodes SP-sfGFP- $CTD_{SprB(99AA)}$; Ap^r (Tc^r)	This study
pSK96	SP_{RemA}-sfGFP from pSK37 cloned into pCP11. Encodes SP_{RemA}-sfGFP; Ap^r (Em^r)	This study
pSK97	SP_{RemA}-sfGFP-CTD_{RemA} from pSK30 inserted into pMM105.A. Encodes SP_{RemA}-sfGFP-$CTD_{RemA(97AA)}$; Ap^r (Em^r)	This study
pSN48	pCP23 carrying *sprA*; Ap^r (Tc^r)	(11)
pSSK30	pCP23 carrying mcherry; Ap^r (Tc^r)	(21)
pSSK51	484-bp fragment spanning the *chiA* promoter, start codon, and N-terminal signal peptide-encoding region inserted into pSSK30. Encodes SP_{ChiA}-mCherry; Ap^r (Tc^r)	(21)
pSSK52	566-bp region encoding 105 amino acids of CTD_{ChiA} inserted into pSSK51. Encodes SP_{ChiA}-mCherry-CTD_{ChiA}; Ap^r (Tc^r)	(21)
pTB263	Plasmid expressing fluorescent protein sfGFP; Ap^r	(42)

pYT40	511-bp fragment spanning the *remA* promoter, start codon, and the N-terminal signal peptide-encoding region inserted into pCP23; Ap^r (Tc^r)	This study
pYT179	735-bp sfGFP amplified without stop codon and cloned into pYT40. Encodes SP_{RemA}-sfGFP; Ap^r (Tc^r)	This study
pYT180	4383-bp fragment encoding 1386 amino acids of the C-terminus of RemA inserted in pYT179. Encodes SP_{RemA}-sfGFP-$CTD_{RemA(1386AA)}$; Ap^r (Tc^r)	This study

[a]Antibiotic resistance phenotypes are as follows: ampicillin, Ap^r; erythromycin, Em^r; streptomycin, Sm^r; tetracycline, Tc^r. The antibiotic resistance phenotypes given in parentheses are those expressed in *F. johnsoniae* but not in *E. coli*. The antibiotic resistance phenotypes without parentheses are those expressed in *E. coli* but not in *F. johnsoniae*.

Table 2. Primers used in this study.

1269	5' GCTAGGGTACCACGTTCCTGATAGGCACAAAAATGC 3'; forward primer used in construction of pYT40; KpnI site underlined
1270	5' GCTAGGGATCCGCCATTAGTTGGCATTCCAGGAAAA 3'; reverse primer used in construction of pYT40; BamHI site underlined
1389	5' GCTAGGGATCCTCTAAAGGTGAAGAACTGTTCACCG 3'; forward primer used in construction of pSK37 and pYT179; BamHI site underlined
1390	5' GCTAGGCATGCTTATTTGTAGAGCTCATCCATGCCG 3'; reverse primer used in construction of pSK37; SphI site underlined
1399	5' GCTAGTCTAGAACAGATACGAAAGATTATTACATCGAG 3'; forward primer used in construction of pSK93; XbaI site underlined
1400	5' GCTAGGCATGCTTATCTGTATAAAGTGAAATGTCCAAC 3'; reverse primer used in construction of pSK56; SphI site underlined
1404	5' GCTAGGCATGCTCACCTAATACAATAACTAACCTC 3'; reverse primer used in construction of pSSK52; SphI site underlined
1427	5' GCTAGTCTAGAGCAACGATAGCTTATTTTAAAAACAAT 3'; forward primer used in construction of pSK89; XbaI site is underlined
1488	5' GCTAGTCTAGAGATCGTTTTGCACTTCGTTACACT 3'; forward primer used in construction of pSK30; XbaI site underlined
1489	5' GCTAGGCATGCCTTACTTGGCAAATGGATTTTTTA 3'; reverse primer used in construction of pSK30; SphI site underlined
1599	5' GCTAGTCTAGAGCAACGATAGCTTATTTTAAAAACAAT 3'; forward primer used in construction of pSK89; XbaI site underlined
1600	5' GCTAGTCTAGAGCTTATGCAGCTTATTTCGCATCACAA 3'; forward primer used in construction of pSSK52; XbaI site underlined
1771	5' GCTAGGGATCCCTAACCCGACTATCATAGAACCGAC 3'; forward primer used in construction of pYT314; BamHI site underlined
1772	5' GCTAGGTCGACTGTTGTTACAGCCATGAGTACTAAGG 3'; reverse primer used in construction of pYT314; SalI site underlined
1773	5' GCTAGGTCGACTCGATTAGTAACTGTCCTTGTACGC 3'; forward primer used in construction of pYT316; SalI site underlined

1774	5' GCTAGGCATGCTAAAAGTTCAGTTGGCAGTTCTTCG 3'; reverse primer used in construction of pYT316; SphI site underlined
1880	5’ GCTAGGCATGCTGGCGAGGAATTACCTTCTGGTGA 3'; forward primer used in construction of pSK62; XbaI site underlined
1843	5’ GCTAGTCTAGAGTGGTGATTACAATTGATCCAAGC 3'; forward primer used in construction of pSK56; XbaI site underlined
1868	5’ GCTAGTCTAGAGTCGAAGTGCCATCGATTACAGTA 3'; forward primer used in construction of pSK58; XbaI site underlined
1885	5' GCTAGTCTAGAGCTTTAGAGGCTTTTGAAAATGTG 3'; forward primer used in construction of pSK65; XbaI site underlined
1886	5' GCTAGGCATGCTTGTGGGCGTTTCTGAACTATCTC 3'; reverse primer used in construction of pSK65; SphI site underlined
1870	5' GCTAGGCATGCGCTAAGCCATTTTATTGATTTGGA 3'; reverse primer used in construction of pSK58; SphI site underlined
1899	5' GCTAGTCTAGAACATTAGGAACTGGTGATTTTGAG 3'; forward primer used in construction of pSK71; XbaI site underlined
1923	5' GCTAGTCTAGAGAGAGTATCGCTGATGAAACGAAC 3'; forward primer used in construction of pSK75; XbaI site underlined
1924	5' GCTAG GCATGC GCCCTTATTAGAGAATTGCAGTGT 3'; reverse primer used in construction of pSK75; SphI site underlined
1925	5’ GCTAGTCTAGAGTATCGGTAAGTGTGGGAACTCCT 3'; forward primer used in construction of pSK76; XbaI site underlined
1926	5' GCTAGGCATGCCTGTATAGGCTATTCTTTTATAAGGCG 3'; reverse primer used in construction of pSK76; SphI site underlined
1930	5' GCTAGTCTAGAACTTCTGCAAAAGAAAATATTAAAGAA 3'; forward primer used in construction of pSK81; XbaI site underlined
1932	5' GCTAGGCATGCCTATTCAAGATTAACTTTTACAAGCAGCAC 3'; reverse primer used in construction of pSK79; SphI site underlined
1933	5' GCTAGTCTAGAGAACCAACAACTGTTGGAACAGGA 3'; forward primer used in construction of pSK82; XbaI site underlined
1934	5' GCTAGGCATGCCGAATCGAACAATAGCGAACAAGC 3'; reverse primer used in construction of pSK82; SphI site underlined
1940	5' GCTAGTCTAGAGAAGACATTGCTCAGGTTGATGTA 3'; forward primer used in construction of pCB4; XbaI site underlined

1946	5' GCTAGGGTACCGCTTTGAGCATGAATATTGTATCC 3'; forward primer used in construction of pSK84; KpnI site underlined
1947	5' GCTAGGGATCCATCTTGAGCAAATGAAGTTAGGGA 3'; reverse primer used in construction of pSK84; BamHI site underlined
1948	5' GCTAGTCTAGATATCCAAACCCATCTGTAAACAATGAA 3'; forward primer used in construction of pSK85; XbaI site underlined
1949	5' GCTAGTCTAGACCAGAATTGGAAAGCGGAGAC 3'; forward primer used in construction of pSK86; XbaI site underlined
1962	5' GCTAGGCATGCCTATTTAAAGATCACTGCTCTGGTTATCTG 3'; reverse primer used in construction of pSK91; SphI site underline

Results

Prevalence of T9SSs and T9SS-associated CTDs. Genome analyses indicate that T9SSs are found in most but not all members of the phylum *Bacteroidetes* (Table 3). Individual strains of 104 different species with complete genome sequences were analyzed. This included members of 65 genera. Members of 60 genera, and 90 of the 104 species had each of the five core T9SS genes (*gldK*, *gldL*, *gldM*, *gldN*, *sprA*) that have been assigned to TIGRFAM families. Although most members of the *Bacteroidetes* have T9SSs a few do not. For example, most members of the well-studied genus *Bacteroides* lack T9SS genes (Table 3) and use other mechanisms to secrete proteins (1, 43, 44).

The 104 genomes of *Bacteroidetes* species were also analyzed for proteins with predicted T9SS CTDs belonging to TIGR04183 (type A CTDs) and TIGR04131 (type B CTDs). Of the 90 species that had each of the T9SS genes all had proteins with type A CTDs, and all but one (*Arachidicoccus sp.* BS20) also had proteins with type B CTDs. Most of these had many T9SS-CTD-containing proteins. *Fluviicola taffensis* had the highest number of each with 180 proteins with type A CTDs and 50 proteins with type B CTDs. The 14 *Bacteroidetes* species that lacked components of T9SSs were also examined, and none had predicted proteins with type A or type B CTDs except for *Odoribacter splanchnicus*, which indicates presence of 2 type A and 1 type B CTD proteins. The results demonstrate a strong correlation between both types of CTDs and the T9SS.

Members of other phyla of bacteria were also analyzed, including 3777 complete genome sequences (Table 4). None had orthologs of *gldK*, *gldL*, *gldM*, or *gldN*, but seven species had orthologs of *sprA*. These were members of the phyla *Rhodothermaeota*, *Chlorobi*, *Fibrobacteres*,

Ignavabacteriae, and *Gemmatimonadetes*. None of the 3777 non-*Bacteroidetes* bacterial genomes were predicted to encode proteins with type B CTDs but eleven species had predicted proteins with type A CTDs. Interestingly, six of the seven species mentioned above that had SprA each had many proteins with type A CTDs (ranging from 18 to 147), suggesting a link between type A CTDs and the large outer membrane protein SprA. As indicated above there were only five other bacterial species that had any predicted type A CTDs. Each of these had a single example. Three of the apparent CTDs from these five bacteria were not found at the C-terminus, suggesting that they may have been false-positives, or may have functions unrelated to those of the *Bacteroidetes* CTDs. The high prevalence within the phylum *Bacteroidetes* of orthologs of GldK, GldL, GldM, and GldN, and of proteins with type B CTDs, and their complete absence outside of the phylum *Bacteroidetes* is indicative of the extremely low number of false positives obtained using the trusted cutoffs of the TIGRFAM assignments. The near absence of type A CTDs in bacteria lacking SprA also attests to the low level of false positives, and indicates a strong correlation between the presence of the outer membrane protein SprA and proteins with type A CTDs.

Members of the archaea and eukarya were also examined for orthologs of T9SS components. 218 completed archaeal genome sequences and 36 completed eukaryal genomes were analyzed, but none had orthologs of *gldK*, *gldL*, *gldM*, *gldN*, or *sprA*, and none had predicted proteins with type A or type B CTDs. The eukaryotes examined included protists, fungi, plants (*Arabidopsis thaliana*, *Zea mays*) and animals (*Caenorhabditis elegans*, *Danio rerio*, *Homo sapiens*).

Table 3. Prevalence of T9SS genes and CTD-encoding genes in 104 members of the phylum *Bacteroidetes*[a].

	T9SS components					CTDs	
	GldK	GldL	GldM	GldN	SprA	Type A CTD	Type B CTD
Genome	TIGR03525	TIGR03513	TIGR03517	TIGR03523	TIGR04189	TIGR04183	TIGR04131
Class Flavobacteriia							
Aequorivita sublithincola DSM 14238	1	1	1	1	1	112	5
Algibacter sp. HZ22	1	1	1	1	1	55	10
Capnocytophaga canimorsus Cc5	1	1	1	1	1	1	10
Capnocytophaga haemolytica CCUG 32990	1	1	1	1	1	2	6
Capnocytophaga ochracea DSM 7271	1	1	1	1	1	2	8
Capnocytophaga sp. F0383	1	1	1	1	1	2	7
Cellulophaga algicola DSM 14237	1	1	1	1	1	13	16
Cellulophaga lytica DSM 7489	1	1	1	1	1	14	13
Chryseobacterium sp. IHB B 17019	1	1	1	1	1	83	18
Chryseobacterium sp. StRB126	1	1	1	1	1	101	10
Croceibacter atlanticus HTCC2559	1	1	1	1	1	45	8
Dokdonia sp. PRO95	1	1	1	1	1	17	10
Donghaeana dokdonensis DSW-6	1	1	1	1	1	85	17
Elizabethkingia meningoseptica FMS-007	0	0	0	0	0	0	0
Elizabethkingia sp. BM10	0	0	0	0	0	0	0
Flavobacteriaceae bacterium 3519-10	1	1	1	1	1	55	6
Flavobacterium branchiophilum FL-15	1	1	1	1	1	37	10
Flavobacterium columnare ATCC 49512	1	1	1	1	1	35	7
Flavobacterium indicum GPTSA100-9	1	1	1	1	1	43	16
Flavobacterium johnsoniae ATCC 17061	1	1	1	2	1	40	12
Flavobacterium psychrophilum JIP02/86	1	1	1	1	1	38	10
Fluviicola taffensis DSM 16823	1	1	2	2	1	180	50
Gramella forsetii KT0803	1	1	1	1	1	11	7
Krokinobacter diaphorus 4H-3-7-5	1	1	1	1	1	15	10
Lacinutrix sp. 5H-3-7-4	1	1	1	1	1	31	16
Lutibacter profundi LP1	1	1	1	1	1	19	3
Maribacter sp. HTCC2170	1	1	1	1	1	10	13
Muricauda lutaonensis CC-HSB-11	1	1	1	1	1	7	13
Muricauda ruestringensis DSM 13258	1	1	1	1	1	7	13
Myroides profundi D25	1	1	1	1	1	7	12
Myroides sp. A21	1	1	1	1	1	4	7
Ornithobacterium rhinotracheale DSM 15997	1	1	1	1	1	6	2
Owenweeksia hongkongensis DSM 17368	1	1	1	1	1	159	26
Polaribacter sp. MED152	1	1	1	1	1	27	8
Riemerella anatipestifer DSM 15868	1	1	1	1	1	15	1
Robiginitalea biformata HTCC2501	1	1	1	1	1	7	12
Siansivirga zeaxanthinifaciens CC-SAMT-1	1	1	1	1	1	51	10
Weeksella virosa DSM 16922	1	1	1	1	1	36	3
Winogradskyella sp. PG-2	1	1	1	1	1	66	17
Zobellia galactanivorans DsijT	1	1	1	1	1	29	17
Zunongwangia profunda SM-A87	1	1	1	1	1	7	5
Class Cytophagia							
Belliella baltica DSM 15883	1	1	1	1	1	11	4
Cyclobacterium amurskyense KCTC 12363	1	1	1	1	1	20	5
Cyclobacterium marinum DSM 745	1	1	1	1	1	18	7
Cytophaga hutchinsonii ATCC 33406	1	1	2	2	1	118	27
Dyadobacter fermentans DSM 18053	1	1	1	1	1	88	11
Echinicola vietnamensis DSM 17526	1	1	1	1	1	17	9
Emticicia oligotrophica DSM 17448	1	1	1	1	1	31	10
Flexibacter litoralis DSM 6794	1	1	1	3	1	52	11
Hymenobacter sp. APR13	1	1	1	1	1	83	9
Hymenobacter sp. DG25A	1	1	1	1	1	54	6
Hymenobacter sp. PAMC26554	1	1	1	1	1	51	8
Hymenobacter swuensis DY53	1	1	1	1	1	100	8
Leadbetterella byssophila DSM 17132	1	1	1	1	1	17	4
Marivirga tractuosa DSM 4126	1	1	1	1	1	39	11
Persicobacter sp. JZB09	1	1	1	1	1	32	3
Pontibacter akesuensis AKS 1T	1	1	1	1	1	47	11
Pontibacter korlensis X14-1T	1	1	1	1	1	47	11
Rufibacter sp. DG15C	1	1	1	1	1	49	13
Rufibacter tibetensis 1351	1	1	1	1	1	56	11

Runella slithyformis DSM 19594	1	1	1	1	1	33	18
Spirosoma linguale DSM 74	1	1	1	1	1	53	14
Spirosoma radiotolerans DG5A	1	1	1	1	1	50	15
Class Sphingobacteriia							
Algoriphagus sp. M8-2	1	1	1	1	1	16	6
Arachidicoccus sp. BS20	2	1	1	2	1	11	0
Chitinophaga pinensis DSM 2588	1	1	1	1	1	51	36
Haliscomenobacter hydrossis DSM 1100	1	1	1	1	1	144	36
Mucilaginibacter PAMC26640	1	1	1	2	1	7	9
Niabella soli DSM 19437	0	0	0	0	0	0	0
Niastella koreensis DSM 17620	1	1	1	1	1	111	31
Pedobacter cryoconitis PAMC 27485	1	1	1	1	1	3	5
Pedobacter heparinus DSM 2366	1	1	1	1	1	8	13
Pedobacter saltans DSM 12145	1	1	1	1	1	29	10
Pedobacter sp. PACM 27299	1	1	1	1	1	1	9
Saprospira grandis Lewin	1	1	1	3	2	67	16
Solitalea canadensis DSM 3403	1	1	1	1	1	6	18
Sphingobacterium sp. 21	2	1	1	2	1	1	2
Sphingobacterium sp. ML3W	1	1	1	1	0	1	0
Class Bacteroidia							
Alistipes finegoldii DSM 17242	0	0	0	0	0	0	0
Bacteroides cellulosilyticus WH2	1	1	1	1	1	19	1
Bacteroides dorei CL03T12C01	0	0	0	0	0	0	0
Bacteroides fragilis NCTC 9343	0	1	1	0	0	0	0
Bacteroides helcogenes DSM 20613	0	0	0	0	0	0	0
Bacteroides ovatus ATCC 8483	0	0	0	0	0	0	0
Bacteroides thetaiotaomicron VPI-5482	0	0	0	0	0	0	0
Bacteroides vulgatus ATCC 8482	0	0	0	0	0	0	0
Bacteroides xylanisolvens XB1A	0	0	0	0	0	0	0
Barnesiella viscericola DSM 18177	1	1	1	1	1	41	1
Draconibacterium orientale FH5	2	1	1	1	1	23	7
Odoribacter splanchnicus DSM 20712	0	0	0	0	2	2	1
Paludibacter propionicigenes DSM 17365	1	1	1	1	1	10	6
Parabacteroides distasonis ATCC 8503	1	1	1	1	1	7	1
Porphyromonas asaccharolytica DSM 20707	1	1	1	1	1	29	1
Porphyromonas gingivalis ATCC 33277	1	1	1	1	1	17	1
Prevotella dentalis EDSM 3688	1	1	1	1	1	9	1
Prevotella denticola F0289	1	1	1	1	1	8	1
Prevotella enoeca F0113	1	1	1	1	1	4	1
Prevotella fusca W1435	1	1	1	1	1	6	1
Prevotella intermedia 17-2	1	1	1	1	1	19	1
Prevotella melaninogenica ATCC 25845	1	1	1	1	1	14	1
Prevotella ruminicola 23	1	1	1	1	1	1	1
Prevotella sp. F0039	1	1	1	1	1	13	1
Rikenellaceae bacterium M3	0	0	0	0	0	0	0
Tannerella forsythia ATCC 43037	1	1	1	1	1	28	0
	T9SS components					CTDs	
	GldK	GldL	GldM	GldN	SprA	Type A CTD	Type B CTD
	TIGR03525	TIGR03513	TIGR03517	TIGR03523	TIGR04189	TIGR04183	TIGR04131

[a]Only members of the *Bacteroidetes* with completed genome sequences were examined and only one member of each species was used. Occurrence of genes encoding T9SS components or of genes encoding proteins with T9SS-associated CTDs are shown. Red indicates the presence of a gene and the number indicates the number of such genes in the genome. Genes were identified using the Integrated Microbial Genomes (IMG version 4.0.1) Function Profile Tool and using the TIGRFAM terms listed. The trusted cutoffs set by The Institute for Genomic Research were used in each case as indicated in the Methods section of the main text. These may underrepresent the actual number of proteins secreted by T9SSs. For example, more than 30 proteins are thought to be secreted by the *P. gingivalis* T9SS (2), but only 18 were identified above.

Table 4. Prevalence of T9SS genes and CTD-encoding genes in organisms outside of the phylum *Bacteroidetes*[a].

	T9SS components					CTDs	
	GldK	GldL	GldM	GldN	SprA	Type A CTD	Type B CTD
Genome	TIGR03525	TIGR03513	TIGR03517	TIGR03523	TIGR04189	TIGR04183	TIGR04131
Bacteria (non *Bacteroidetes*, 3777 genomes examined)							
Chloroherpeton thallassium ATCC 35110 (Chlorobi)	0	0	0	0	1	30	0
Arthrospira platensis YZ (Cyanobacteria)	0	0	0	0	0	1	0
Leptolyngbya sp. PCC 7376 (Cyanobacteria)	0	0	0	0	0	1	0
Synechococcus sp. JA-2-3B'a(2-13) (Cyanobacteria)	0	0	0	0	0	1	0
Trichodesmium erythraeum IMS101 (Cyanobacteria)	0	0	0	0	0	1	0
Fibrobacter succinogenes S85 (Fibrobacteres)	0	0	0	0	1	33	0
Thermincola potens JR (Firmicutes)	0	0	0	0	0	1	0
Gemmatimonas aurantiaca T-27T (Gemmatimonadetes)	0	0	0	0	1	0	0
Melioribacter roseus P3M (Ignavibacteriae)	0	0	0	0	1	85	0
Ignavibacterium album JCM 16511 (Ignavibacteriae)	0	0	0	0	1	147	0
Rhodothermus marinus DSM 4252 (Rhodothermaeota)	0	0	0	0	2	48	0
Salinibacter ruber DSM 13855 (Rhodothermaeota)	0	0	0	0	1	18	0
Archaea (218 genomes examined)							
No species identified with T9SS genes or CTDs	0	0	0	0	0	0	0
Eukarya (36 genomes examined)							
No species identified with T9SS genes or CTDs	0	0	0	0	0	0	0

[a]3777 completed genomes were examined. Only completed genome sequences were examined and only one member of each species was used. Since the vast majority of species had no genes encoding T9SS proteins or T9SS-associated CTDs, only species with genes encoding T9SS components or genes encoding proteins with T9SS-associated CTDs are shown. Red indicates the presence of a gene and the number indicates the number of such genes in the genome. Genes were identified using the Integrated Microbial Genomes (IMG version 4.0.1) Function Profile Tool and using the TIGRFAM terms listed. The trusted cutoffs set by The Institute for Genomic Research were used in each case as indicated in the Methods section of the main text. The phyla to which the species belong are indicated in parentheses. Note that the CTDs from *A. platensis*, *Leptolyngbya* sp., and *T. erythraeum* were not found at the C-terminus and thus may be false positives.

Characteristics of *F. johnsoniae* type A and type B CTDs. Multiple sequence alignments of *F. johnsoniae* type A and type B CTDs were assembled to identify conserved features (Fig. 1 and Fig. 2). The conserved region of type A and type B CTDs extend approximately 70 to 100 amino acids from the C-termini of the proteins. *F. johnsoniae* type A CTDs have several highly conserved regions, including YPNP at approximately 70 amino acids from the C-terminus, G(I/L/V)Y at approximately 20 amino acids from the C-terminus, and (K/R)XXK followed immediately by the C-terminal residue. These sequences are also conserved in type A CTDs from many other *Bacteroidetes* (data not shown). *F. johnsoniae* type B CTDs are quite different in sequence, with F(T/S)PNGDGXND at approximately 80 amino acids from the C-terminus, IFXR(W/Y)G at approximately 55 amino acids from the C-terminus, and GX(L/F)X(L/I)X(R/K) at the C-terminus.

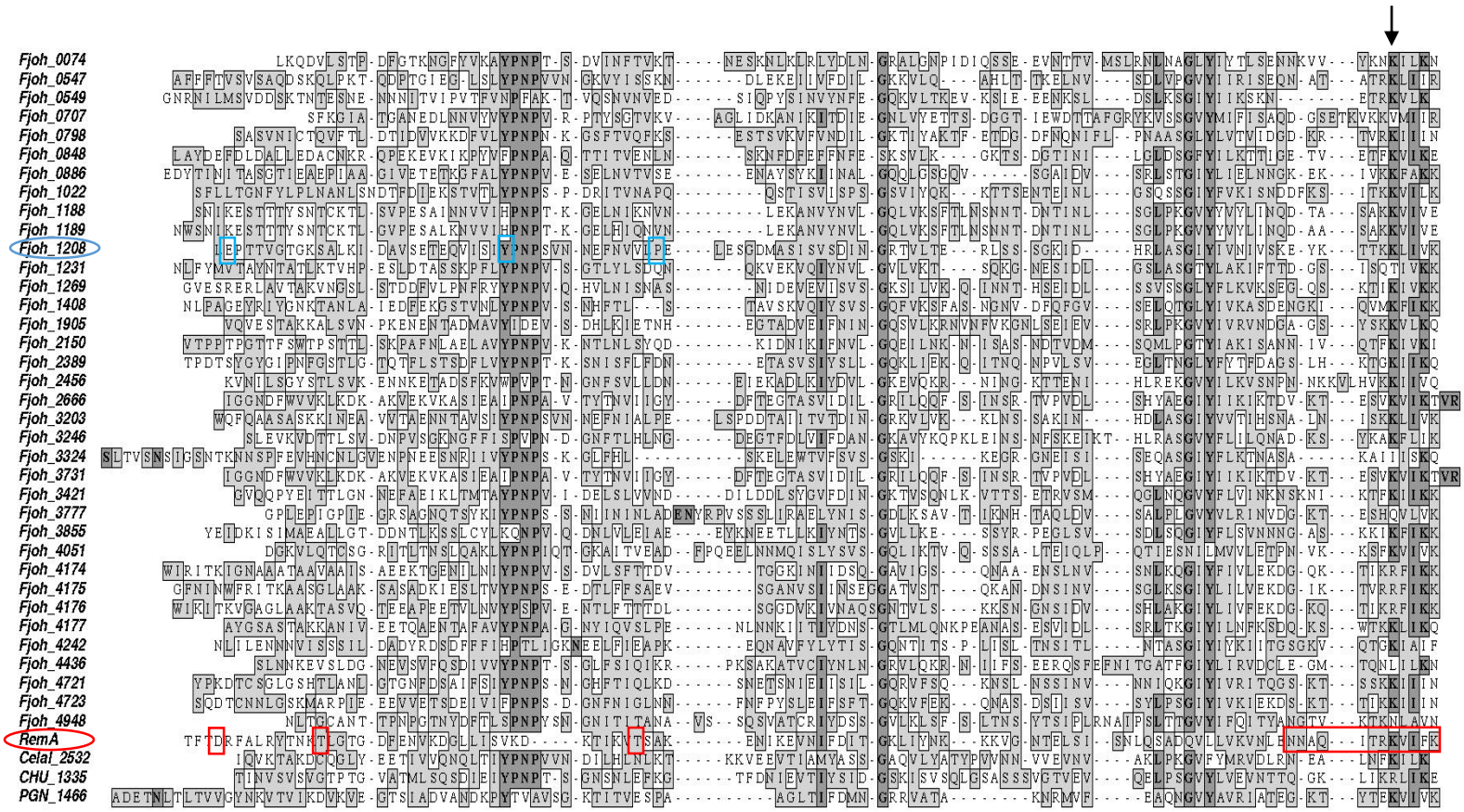

Fig 1. Alignment of the C-terminal 100 amino acids of *F. johnsoniae* proteins that belong to TIGRFAM family TIGR04183 (type A CTD). Protein sequences were aligned using MUSCLE. Dark shading indicates identical amino acids and light shading indicates similar amino acids. The black arrow indicates the conserved lysine that was mutated to alanine in RemA in the experiment shown in Fig. 11B and red rectangular box indicates the last 12 AAs of RemA C terminal region that were deleted in the experiment shown in Fig 11A. Individual red boxed amino acid correspond to those that lie 97, 87 and 62 AAs from the RemA C terminus. Individual blue boxed amino acid correspond to those that lie 99, 73 and 59 AAs from the AmyB C terminus. Proteins from other bacteria that were examined experimentally in this study were Celal_2532 (*C. algicola* AmyA), CHU_1335 (*C. hutchinsonii* Cel9B), and PGN_1466 (*P. gingivalis* RgpB).

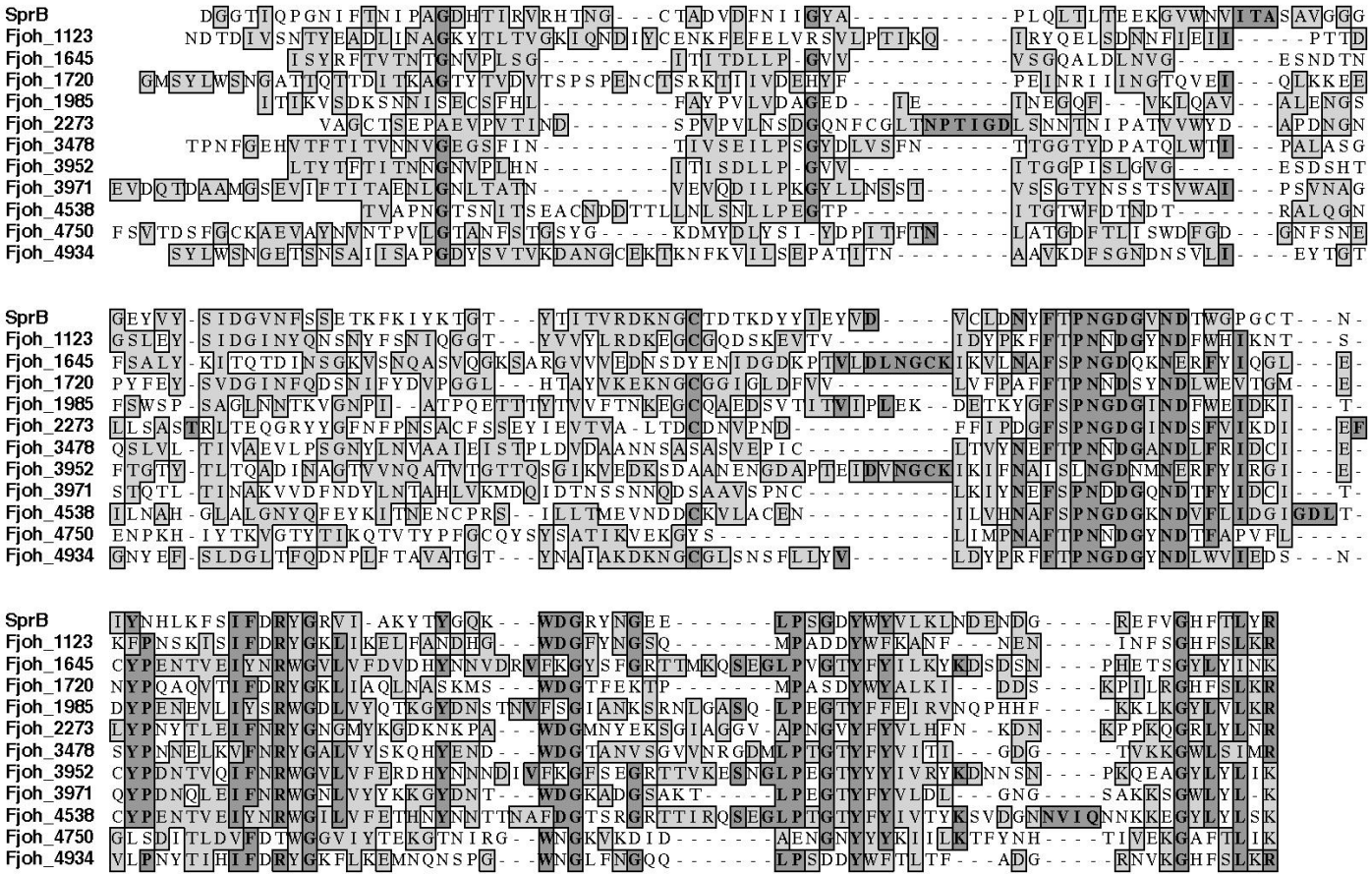

Fig. 2. Alignment of the C-terminal 200 amino acids of *F. johnsoniae* proteins that belong to TIGRFAM family TIGR04131 (type B CTD). Protein sequences were aligned using MUSCLE. Dark shading indicates identical amino acids and light shading indicates similar amino acids. Proteins that were examined experimentally in this study were SprB and Fjoh_3952.

Growth phase of cells affects function of the T9SS. To examine the function of the T9SS we fused the C-terminal 97 amino acids of RemA to the C-terminus of the foreign protein sfGFP (SP_{RemA}-sfGFP-CTD_{RemA}). The recombinant protein also carried the N-terminal signal peptide from RemA to facilitate export across the cytoplasmic membrane by the Sec system. Little sfGFP accumulated in the growth medium of wild type cells growing exponentially in rich media whereas cells from stationary phase (approximately 8 h post exponential phase) exhibited substantial secretion of SP_{RemA}-sfGFP-CTD_{RemA} (Fig. 3). The size of the protein in the culture fluid corresponded to that of sfGFP, suggesting that both the N-terminal signal peptide and the CTD had been removed. The presence of sfGFP in the spent medium was not the result of cell lysis since analysis of total proteins by coomassie staining revealed only trace amounts of protein in the culture fluid (Fig. 4). Further, coexpression of SP-sfGFP and SP-mCherry-CTD_{RemA} allowed secretion of mCherry but not leakage of sfGFP (Fig. 5), indicating that expression of excess CTD-containing protein from plasmid did not cause nonspecific T9SS-mediated leakage of periplasmic proteins. SP_{RemA}-sfGFP-CTD_{RemA} depended on the components of the T9SS for its secretion, since it was not secreted by cells of the Δ*gldNO* T9SS mutant (Fig. 3B).

Mutations in genes encoding other core components of the T9SS (*gldK*, *gldL*, *gldM*, *sprA*, *sprE*, and *sprT*) also resulted in lack of secretion (Fig. 6). The levels of components of the T9SS were examined in an attempt to explain the low level of secretion from exponentially growing cells. Exponentially growing cells had similar levels of GldK, GldL, GldM, GldN, and SprE, as did stationary phase cells, but they had reduced levels of SprA (Fig. 7). SprA is important for secretion, and the low level present in cells growing exponentially in rich media may explain the lack of secretion described above. Although lack of a suitable regulatable promoter prevented overexpression of SprA in exponential cells, increased levels of SprA in stationary phase cells

carrying *sprA* on a plasmid resulted in apparent increased levels of secretion of SP-sfGFP-CTD_{RemA} (Fig 8). Because of the lack of secretion during exponential growth, all subsequent analyses of secretion involved cells grown to approximately 8 h after exponential phase.

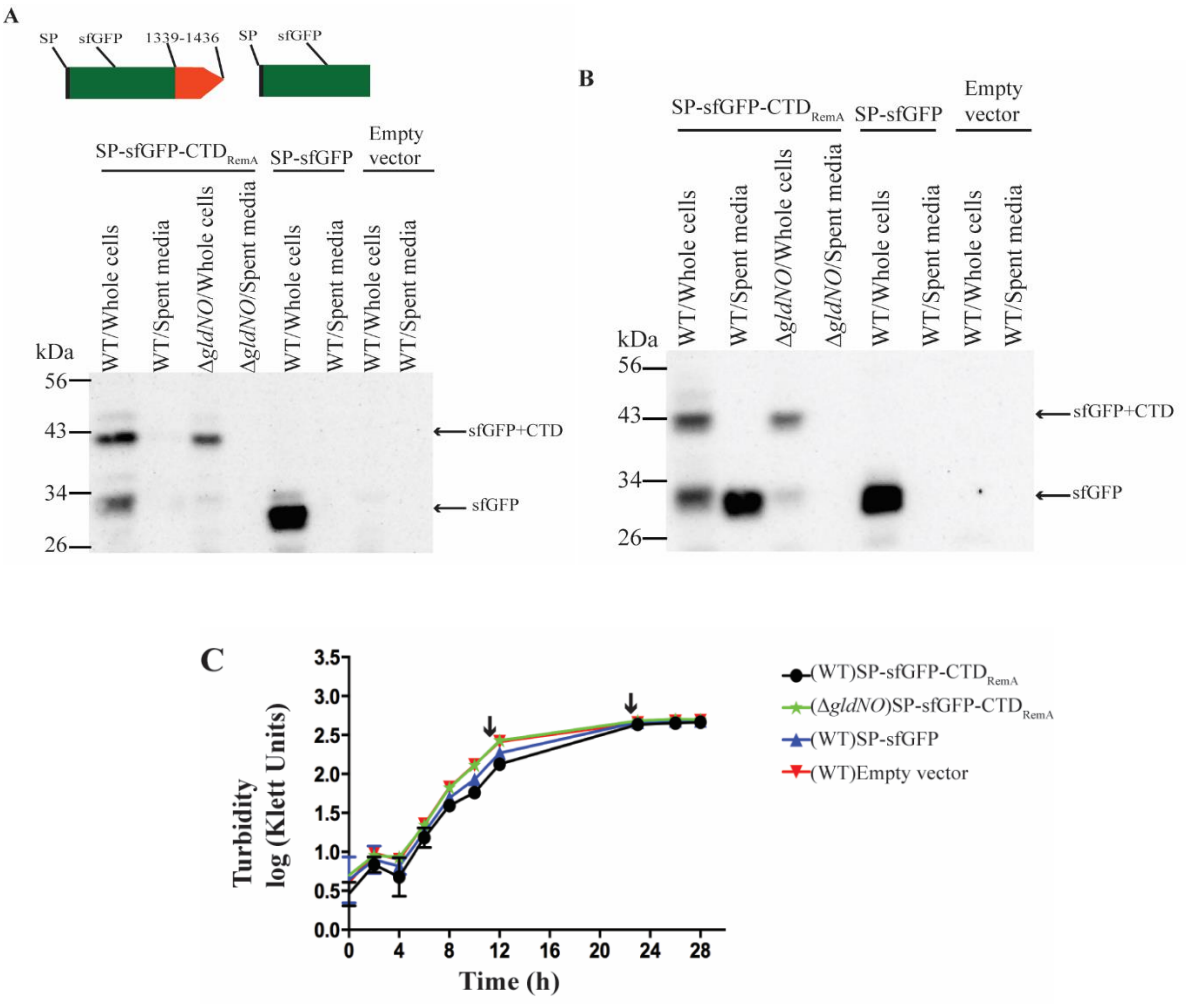

Fig. 3. T9SS-mediated secretion of sfGFP fused to the CTD of RemA**.** Cultures of wild type cells or of the T9SS mutant Δ*gldNO* were incubated in CYE at 25°C with shaking and harvested in exponential phase (10 h) and stationary phase (22 h). Samples were centrifuged and the culture supernatant (spent medium) and intact cells were analyzed for sfGFP by western blot. Cells carried either pCP23 (Empty vector), pSK37 which expresses sfGFP with the N-terminal signal peptide from RemA (SP-sfGFP), or pSK30, which expresses SP-sfGFP fused to the 97-amino acid CTD of RemA (SP-sfGFP-CTD$_{RemA}$). Cartoons at the top indicate the plasmid-encoded proteins and apply to panels A and B, with '1339-1436' indicating the C-terminal 97 amino acids of RemA, and 'SP' indicating signal peptide. Whole cell samples corresponded to 10 µg protein per lane and samples from spent media corresponded to the volume of spent medium that contained 10 µg cell protein before the cells were removed. Samples were separated by SDS-PAGE, and sfGFP was detected using anti-serum against GFP. A) Detection of sfGFP from whole cells and spent media for samples harvested from exponential phase of growth. B) Detection of sfGFP from whole cells and spent media for samples harvested from stationary phase. C) Growth curves, with arrows indicating the points at which cells were harvested. Growth experiments were performed in triplicates and error bars indicate standard deviations.

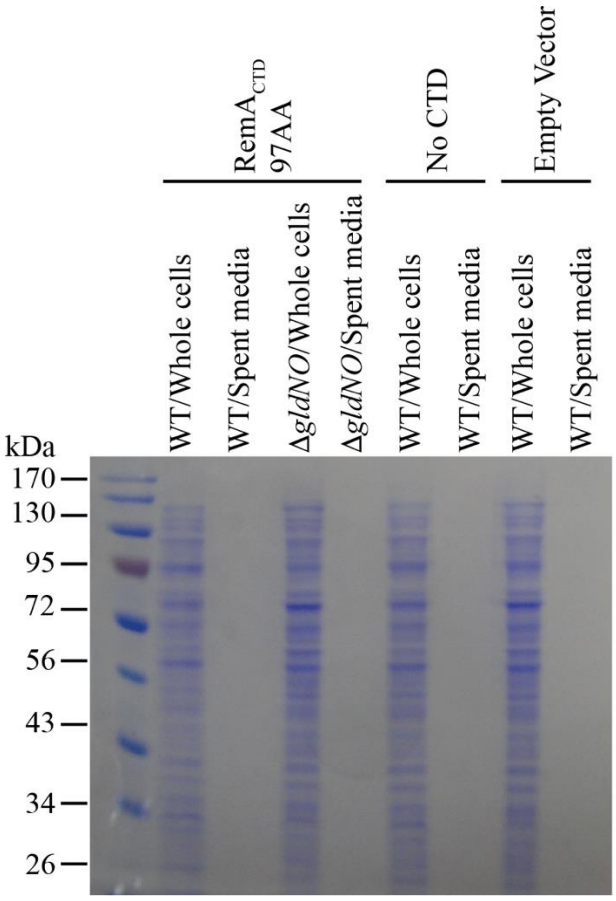

Fig. 4. Analysis of secreted proteins by SDS-PAGE. Cultures of wild type cells or of cells of the T9SS mutant Δ*gldNO* were incubated in CYE at 25°C with shaking and harvested in stationary phase (22 h). 1 ml samples were centrifuged at 22,000 x g for 15 min. The culture supernatant (spent medium) and intact cells were analyzed by SDS-PAGE followed by Coomassie blue staining. Cells carried either pCP23 ('Empty Vector'), pSK37 which expresses sfGFP with the N-terminal signal peptide from RemA (SP-sfGFP; 'No CTD'), or pSK30, which expresses SP-sfGFP fused to the 97-amino acid CTD of RemA (SP-sfGFP-CTD$_{RemA}$; 'RemA$_{CTD}$ 97 AA'). Cell samples corresponded to 10 μg protein per lane and samples from spent media corresponded to the volume of spent medium that contained 10 μg cell protein before the cells were removed.

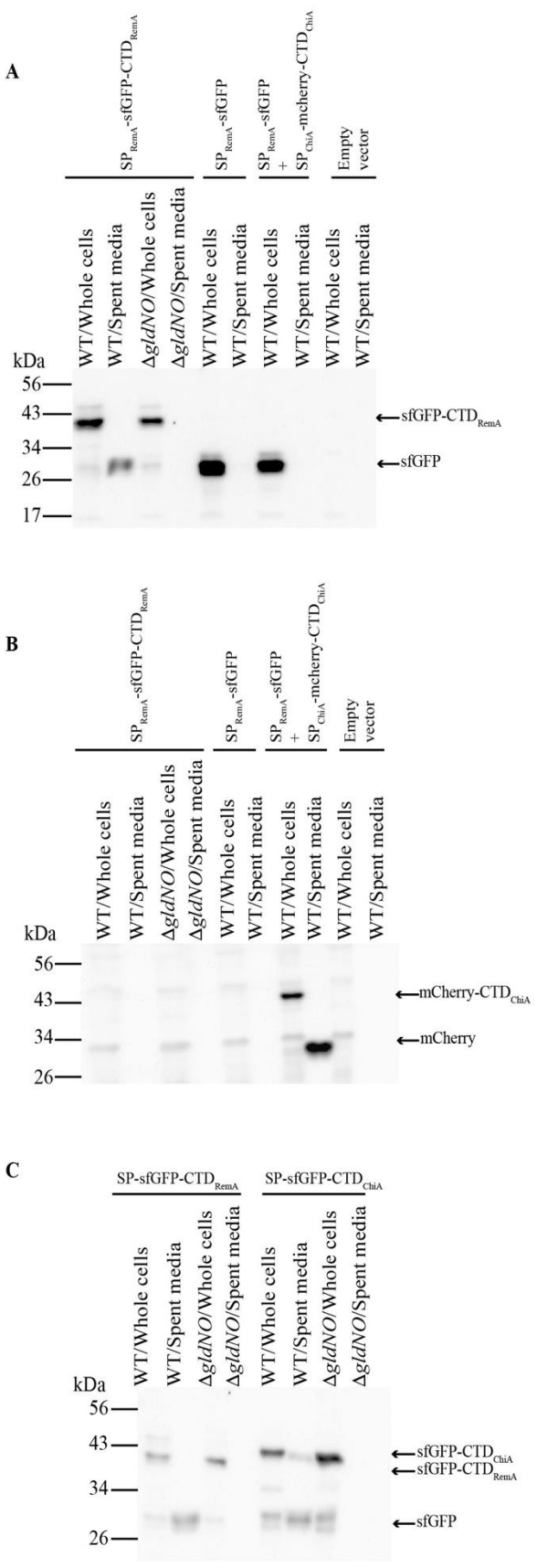

Fig. 5. Analysis of secreted proteins to determine if overexpression of CTD causes cell lysis or periplasmic leakage**.** Cultures of wild type cells or of the T9SS mutant Δ*gldNO* expressing SP_{RemA}-sfGFP-CTD_{RemA} (pSK30), SP_{RemA}-sfGFP (pSK37), or SP_{RemA}-sfGFP (pSK96) and SP_{ChiA}-mCherry-CTD_{ChiA} (pSSK52) were incubated in CYE at 25°C with shaking. 'Empty vector' refers to pCP23. Samples were processed as in Fig. 3 analyzed by SDS-PAGE, followed by western blot analysis using (A) anti-GFP antibodies and (B) anti-mCherry antibodies. Identical samples were used in panels A and B. (C) To estimate protein expression from the *remA* and *chiA* promoters, cultures of wild type cells or of the T9SS mutant Δ*gldNO* expressing SP_{RemA}-sfGFP-CTD_{RemA} (pSK30; Pr_{remA}), or SP_{ChiA}-sfGFP-CTD_{ChiA} (pCB3; Pr_{chiA}) were incubated in CYE at 25°C with shaking. The culture supernatant (spent medium) and intact cells were analyzed by SDS-PAGE, followed by western blot analysis with anti-GFP antibodies. For all panels cell samples corresponded to 10 µg protein per lane and samples from spent media corresponded to the volume of spent medium that contained 10 µg cell protein before the cells were removed.

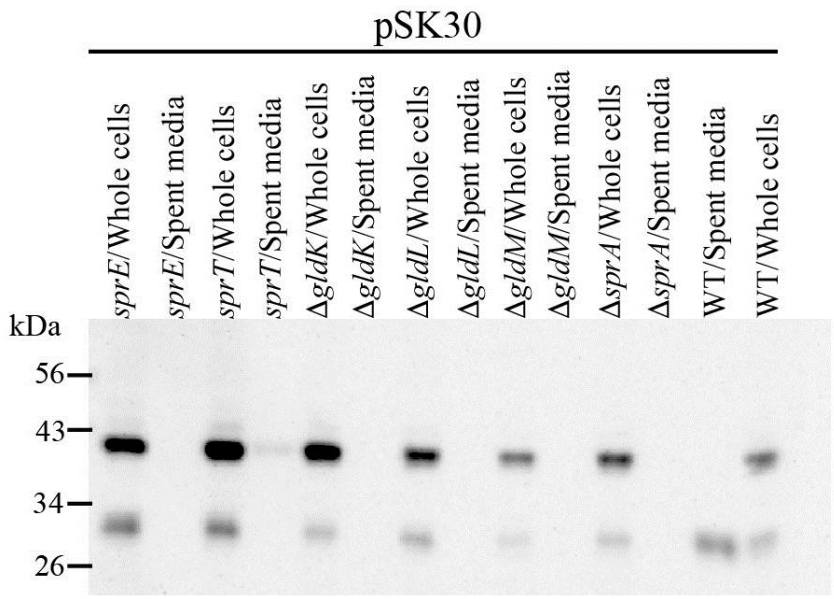

Fig. 6. Components of T9SS required for secretion of SP-sfGFP-CTD$_{RemA}$. Cultures of wild type cells (WT) or of the T9SS mutants were incubated in CYE at 25°C with shaking and harvested in stationary phase (22 h). 1 ml samples were centrifuged at 22,000 x g for 15 min. The culture supernatant (spent medium) and intact cells were analyzed for sfGFP by western blot. Cells carried pSK30, which expresses SP-sfGFP fused to the 97-amino acid CTD of RemA (SP-sfGFP-CTD$_{RemA}$). Whole cell samples corresponded to 10 μg protein per lane and samples from spent media corresponded to the volume of spent medium that contained 10 μg cell protein before the cells were removed. Samples were separated by SDS-PAGE, and sfGFP was detected using anti-serum against GFP.

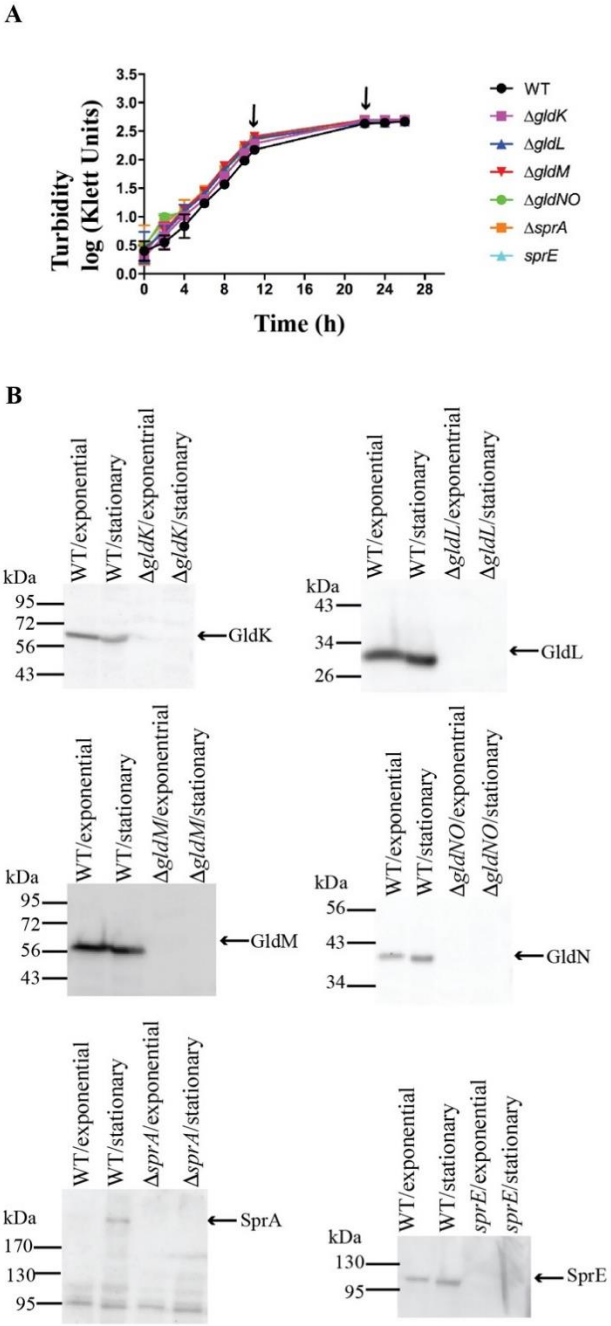

Fig. 7. Levels of T9SS proteins in cells grown to exponential and stationary phase. (A) Cultures of wild type cells or of cells of the T9SS mutants Δ*gldK*, Δ*gldL*, Δ*gldM*, Δ*gldNO*, Δ*sprA*, and of the *sprE* mutant FJ149 were incubated in CYE at 25°C with shaking and harvested in late exponential phase (11 h) and stationary phase (22 h) as indicated by the arrows. Growth experiments were performed in triplicates and error bars indicate standard deviations. (B) Cells were analyzed for T9SS proteins by western blot. Equal amounts (10 µg cell protein) were loaded per lane, separated by SDS-PAGE, and antibodies were used to detect the respective proteins.

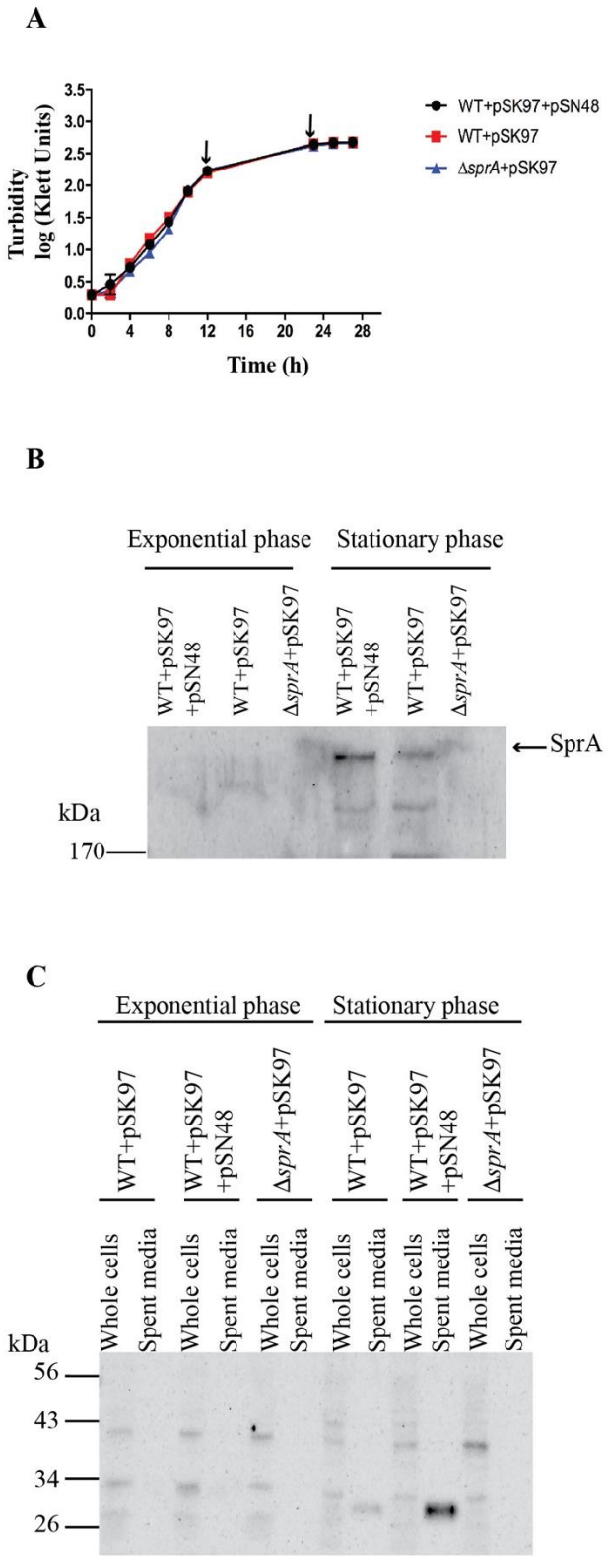

Fig. 8. Overexpression of SprA results in increased sfGFP secretion in stationary phase. Cultures of wild type cells or of cells of the T9SS mutant Δ*sprA* were incubated in CYE at 25°C with shaking and harvested in late exponential phase and stationary phase as indicated in Panel (A) Cells carried pSK97 which expresses SP-sfGFP-CTD_{RemA} (97 AA CTD). Where indicated cells also carried pSN48 which expresses SprA. Cells were analyzed by western blot using anti-SprA antibodies (B) or anti-GFP antibodies (C). For Panel B, equal amounts (10 μg whole cell protein) were loaded per lane. For panel C, whole cell samples corresponded to 10 μg protein per lane and samples from spent media corresponded to the volume of spent medium that contained 10 μg cell protein before the cells were removed. Samples were separated by SDS-PAGE, and antibodies were used to detect the respective proteins. Growth experiments were performed in triplicates and error bars indicate standard deviations.

Identification of regions of the CTDs of *F. johnsoniae* RemA, AmyB, and ChiA that allow secretion of the foreign protein sfGFP. To elucidate the features of type A CTDs that are important for secretion we examined the *F. johnsoniae* proteins RemA and AmyB (Fjoh_1208). Different lengths of CTD_{RemA} were attached to sfGFP to determine the minimal region needed for secretion. C-terminal regions of 97 and 87 amino acids facilitated secretion of sfGFP, whereas a region spanning the final 62 amino acids of RemA did not (Fig. 9A). sfGFP carrying the 62-amino acid CTD appeared to be unstable, since decreased amounts of sfGFP were detected. In contrast, sfGFP carrying no CTD attachment was stable (Fig. 3B). We do not know the reason for the instability of sfGFP carrying the final 62 amino acids of RemA. It might be due to improper folding of the fusion protein resulting in degradation by periplasmic proteases, although other explanations are possible.

In wild type cells, RemA is found both on the cell surface and in soluble form in the spent medium (18). We examined cells expressing sfGFP fused to various regions of RemA to determine if sfGFP was targeted to the cell surface. This was done by incubating cells with Protein-G-coated latex spheres carrying antibodies against GFP and determining the percentage of cells to which spheres attached. Whereas full length RemA fused to sfGFP (RemA C-terminal region of 1386 amino acids) resulted in attachment of spheres indicating surface localization, none of the other fusion proteins containing C-terminal regions of 97, 87, or 62 amino acids did (Fig. 9B).

The constructs described above each had the N-terminal signal peptide from RemA. In order to determine if this region had any role in secretion by the T9SS beyond targeting to the Sec system for initial export across the cytoplasmic membrane, we replaced it with the N-terminal signal peptide from the predicted *F. johnsoniae* periplasmic cytochrome C, Fjoh_1634. The

cytochrome C signal peptide allowed secretion of SP_{Fjoh_1634}-sfGFP-CTD_{RemA} (carrying 97 amino acids of the RemA CTD) by the T9SS (Fig. 10), suggesting that any cleavable N-terminal signal peptide that facilitates export across the cytoplasmic membrane may be sufficient to allow secretion across the outer membrane by the T9SS provided that an appropriate CTD is present. Such secretion was not observed in the Δ*gldNO* mutant, indicating that the T9SS was required.

The extreme C-terminus of CTD_{RemA} was explored in more detail. Attachment of amino acids 1339-1424 of RemA (97 AA CTD minus the C-terminal 12 AAs) failed to support secretion of SP_{RemA}-sfGFP (Fig. 11A). The final 12 amino acids have a lysine residue that is highly conserved among type A CTDs including those from *F. johnsoniae* (Fig. 1). Replacement of this lysine with alanine resulted in failure to secrete SP_{RemA}-sfGFP suggesting that it may be important in secretion by the *F. johnsoniae* T9SS (Fig. 11B). Similar results have previously been reported for the distantly related bacteroidete *P. gingivalis*. Deletion of the C-terminal 13 amino acids or 2 amino acids of gingipain RgpB resulted in accumulation of the truncated protein in the periplasm (20, 24), and conversion of the conserved lysine 732 to alanine also resulted in decreased secretion (20). Amino acids near the C-terminus appear to be important components of the *Bacteroidetes* T9SS secretion signal.

PorU and PorV are involved in the secretion of some but not all proteins that are targeted to the T9SS (18, 23, 45). PorU has been suggested to function as a C-terminal signal peptidase, whereas the function of PorV is less certain. RemA requires PorV but not PorU for secretion by the T9SS (18). A *porV* deletion mutant failed to secrete SP_{RemA}-sfGFP carrying the 97-amino acid CTD_{RemA} (Fig. 12). Cells of a *porU* deletion mutant secreted some SP_{RemA}-sfGFP-CTD_{RemA}, but

less than did wild type cells. *F. johnsoniae* PorU is not essential for secretion but appears to allow more efficient secretion.

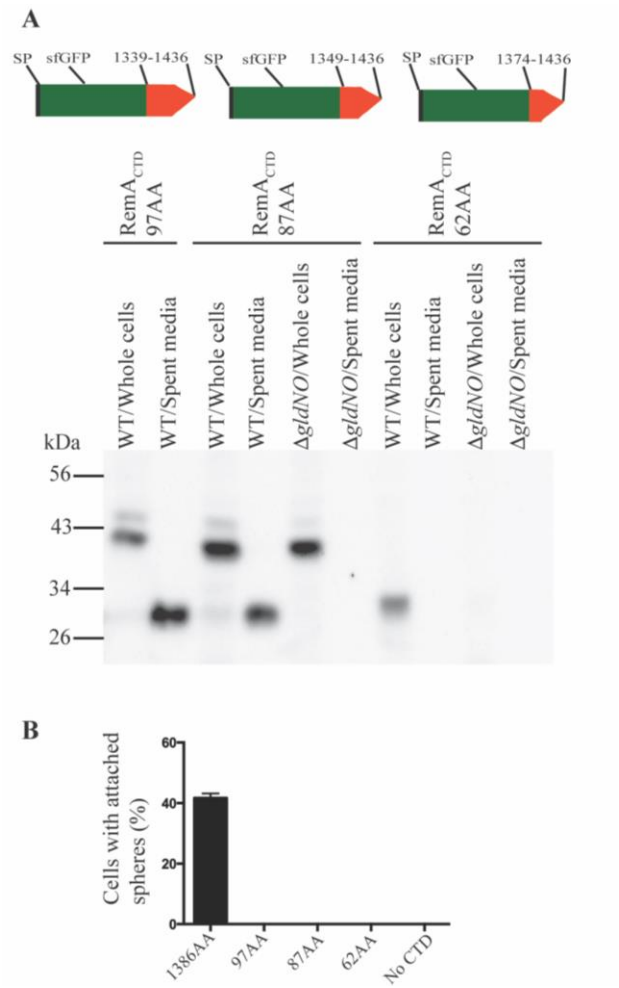

Fig. 9. Determination of the region of RemA needed to allow secretion of sfGFP. (A) The minimum region necessary for sfGFP secretion was determined by examining cells carrying plasmids that expressed SP_{RemA}-sfGFP-CTD_{RemA} with CTD regions of 62 amino acids (pSK81), 87 amino acids (pSK71) and 97 amino acids (pSK30). Cartoons at the top indicate the plasmid-encoded proteins carrying CTDs extending from amino acids 1339-1436, 1349-1436, and 1374-1436 of RemA. Cell free spent media and whole cells were examined for sfGFP by SDS-PAGE followed by western blotting using anti-serum against sfGFP. Whole cell samples corresponded to 10 μg protein per lane and samples from spent media corresponded to the volume of spent medium that contained 10 μg cell protein before the cells were removed. (B) Attachment of sfGFP on the cell surface was determined by examining cells carrying plasmids that expressed SP_{RemA}-sfGFP-CTD_{RemA} with CTD regions of 62 amino acids, 87 amino acids, 97 amino acids, or 1386 amino acids (pYT180). Anti-GFP antiserum and 0.5-μm-diameter protein G-coated polystyrene spheres were added to cells as described in Materials and Methods. Samples were introduced into a tunnel slide, incubated for 3 minutes at 25°C, and examined using a phase-contrast microscope. Images were recorded for 30s, and 100 randomly selected cells were examined for the presence of spheres that remained attached to the cells during this time. Error bars indicate standard deviations from three measurements.

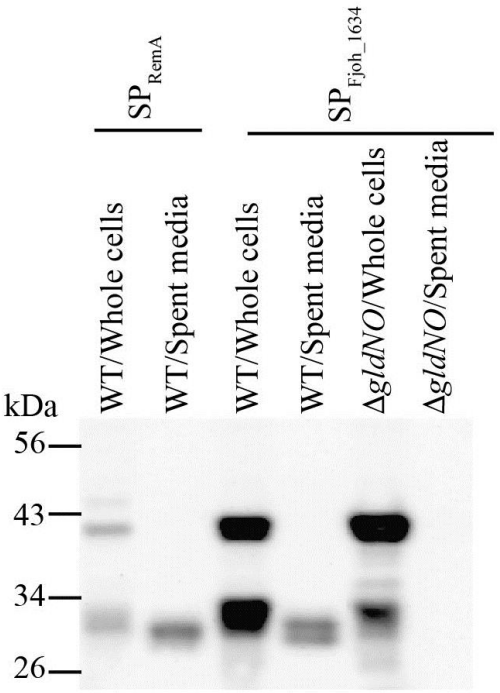

Fig. 10. The N –terminal signal peptide from a periplasmic protein allows secretion of SP-sfGFP-CTD_{RemA}. Cell free spent media and whole cells were analyzed for wild-type (WT) cells carrying fusion plasmid pSK30, which expresses SP_{RemA}-sfGFP-CTD_{RemA}, or carrying pSK84, which expresses SP_{Fjoh_1634}-sfGFP-CTD_{RemA}. Both fusion proteins had 97 amino acid C-terminal regions of RemA. Cells and spent media from cultures of the T9SS mutant Δ*gldNO* carrying pSK84 were also analyzed. Whole cell samples corresponded to 10 μg protein per lane and samples from spent media corresponded to the volume of spent medium that contained 10 μg cell protein before the cells were removed. Samples were separated by SDS-PAGE, and sfGFP was detected using anti-serum against GFP.

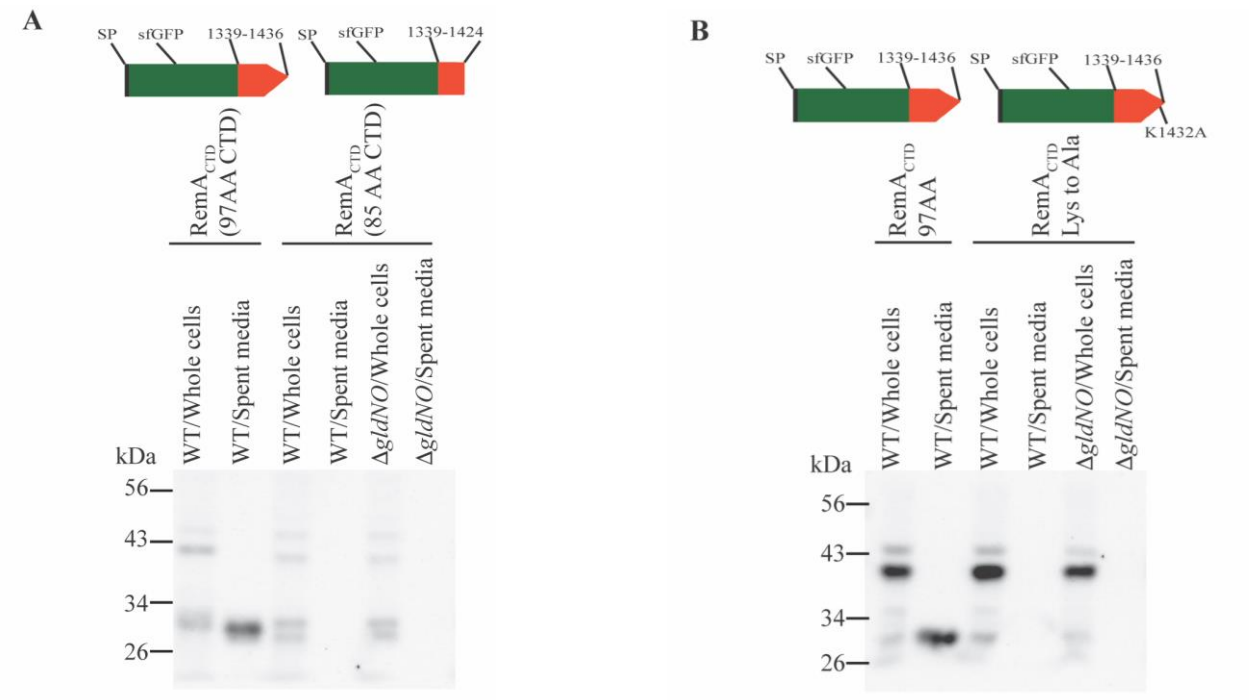

Fig. 11. The C-terminal 12 amino acids of RemA are critical for secretion. Cells carrying pSK30 which expresses SP_{RemA}-sfGFP fused to the C-terminal 97 amino acids of RemA (amino acids 1339-1436), pSK79 which expresses SP_{RemA}-sfGFP fused to amino acids 1339 to 1424 of RemA but lacking the C-terminal 12 amino acids, and pSK91 which expresses SP_{RemA}-sfGFP fused to the C-terminal 97 amino acids of RemA but with the conserved lysine 1432 (Fig. 1) replaced by alanine, were examined for sfGFP in intact cells and in cell-free spent media by Western blot analysis. Cultures of wild-type (WT) and T9SS mutant (Δ*gldNO*) carrying the plasmids were analyzed. Whole cell samples corresponded to 10 μg protein per lane and samples from spent media corresponded to the volume of spent medium that contained 10 μg cell protein before the cells were removed. (A) Effect of deletion of the C-terminal 12 amino acids of SP_{RemA}-sfGFP-CTD_{RemA} on secretion. (B) Effect on secretion of replacement of conserved lysine 1432 of CTD_{RemA} with alanine.

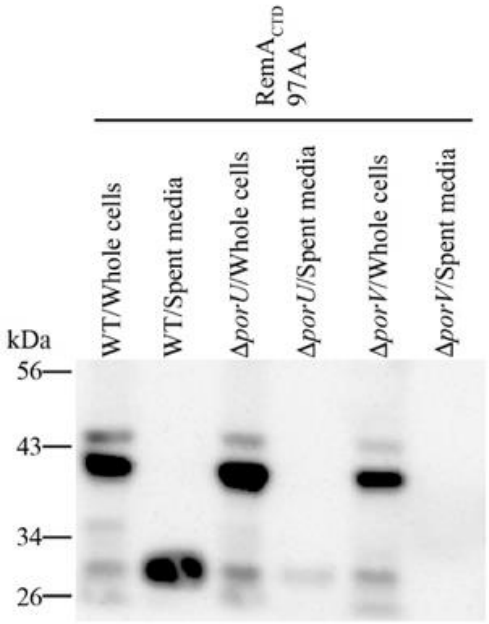

Fig. 12. Effect of deletion of *porU* and *porV* on secretion of SP_{RemA}-sfGFP-CTD_{RemA}. Cell free spent medium and whole cells were analyzed for cells of wild-type (WT), *porU* deletion mutant (Δ*porU*), and *porV* deletion mutant (Δ*porV*), each carrying pSK30. pSK30 expresses SP_{RemA}-sfGFP-CTD_{RemA}, where CTD_{RemA} refers to the 97 amino acid C-terminal region of RemA. Samples were separated by SDS-PAGE, and sfGFP was detected using anti-serum against GFP. Whole cell samples corresponded to 10 μg protein per lane and samples from spent media corresponded to the volume of spent medium that contained 10 μg cell protein before the cells were removed.

The results above indicate that the C-terminal 87 amino acid region of RemA is sufficient to target a foreign protein carrying an N-terminal signal peptide for secretion by the T9SS. Similar regions of the CTDs of *F. johnsoniae* AmyB (Fjoh_1208) and ChiA were examined for their ability to facilitate secretion of SP_{RemA}-sfGFP. The CTD of AmyB is a member of the protein domain family TIGR04183 (type A CTD) and is much closer to the consensus type A CTD sequence than is the CTD of RemA (Fig. 1). The 99-amino acid CTD_{AmyB} facilitated efficient secretion of SP_{RemA}-sfGFP, whereas 73-amino acid CTD_{AmyB} did not (Fig. 13A). In contrast the ChiA CTD is not similar in sequence to members of this or any other protein domain family (21). For ChiA, 105-amino acid CTD_{ChiA} and 79-amino acid CTD_{ChiA} both facilitated secretion of SP_{RemA}-sfGFP by the T9SS (Fig. 13B). Attachment of the 79-amino acid region of ChiA had a negative effect on the accumulation of sfGFP in whole cells. In contrast a 62-amino acid CTD_{ChiA} failed to support secretion but allowed accumulation of sfGFP within the cells. The 79-amino acid CTD_{ChiA} may have interacted with components of the T9SS in a way that destabilized the attached sfGFP, perhaps making it susceptible to proteolysis, although other explanations are possible.

Some type A CTDs from other *Bacteroidetes* facilitate secretion of sfGFP by the *F. johnsoniae* T9SS. T9SSs, and proteins with type A CTDs are common among members of the phylum *Bacteroidetes* (1). *F. johnsoniae* (Class *Flavobacteriia*) has 40 proteins with type A CTDs, the marine bacterium *C. algicola* DSM 14237 (Class *Flavobacteriia*) has 13, *C. hutchinsonii* ATCC 33406 (Class *Cytophagia*) has 118, and *P. gingivalis* ATCC 33277 (Class *Bacteroidia*) has 17 (Table 3). We examined a single representative type A CTD from *C. algicola*, from *C. hutchinsonii*, and from *P. gingivalis* to determine if they support secretion of SP_{RemA}-sfGFP by the *F. johnsoniae* T9SS. Plasmids expressing sfGFP carrying the N-terminal RemA signal peptide and C-terminal regions from *C. algicola* Celal_2532 (AmyA), *C. hutchinsonii* Cel9B (46), and *P.*

gingivalis RgpB (26) were introduced into *F. johnsoniae.* The CTD regions from *C. algicola* AmyA and *C. hutchinsonii* Cel9B functioned in *F. johnsoniae* resulting in secretion of sfGFP from wild type cells (Fig. 14). They failed to support secretion in the Δ*gldNO* mutant, indicating that a functional T9SS was required. The results indicate that T9SS CTDs are not species specific. In contrast to the results described above, the CTD from *P. gingivalis* RgpB did not support secretion of SP_{RemA}-sfGFP (Fig. 14), suggesting that some foreign T9SS CTDs may not function properly with T9SSs from distantly related bacteria. The CTD of RgpB lacks the consensus 'YPNP' sequence that is found in most type A CTD proteins at approximately 80 amino acids from the C-terminus (Fig. 1) which may explain its inability to function with the *F. johnsoniae* T9SS.

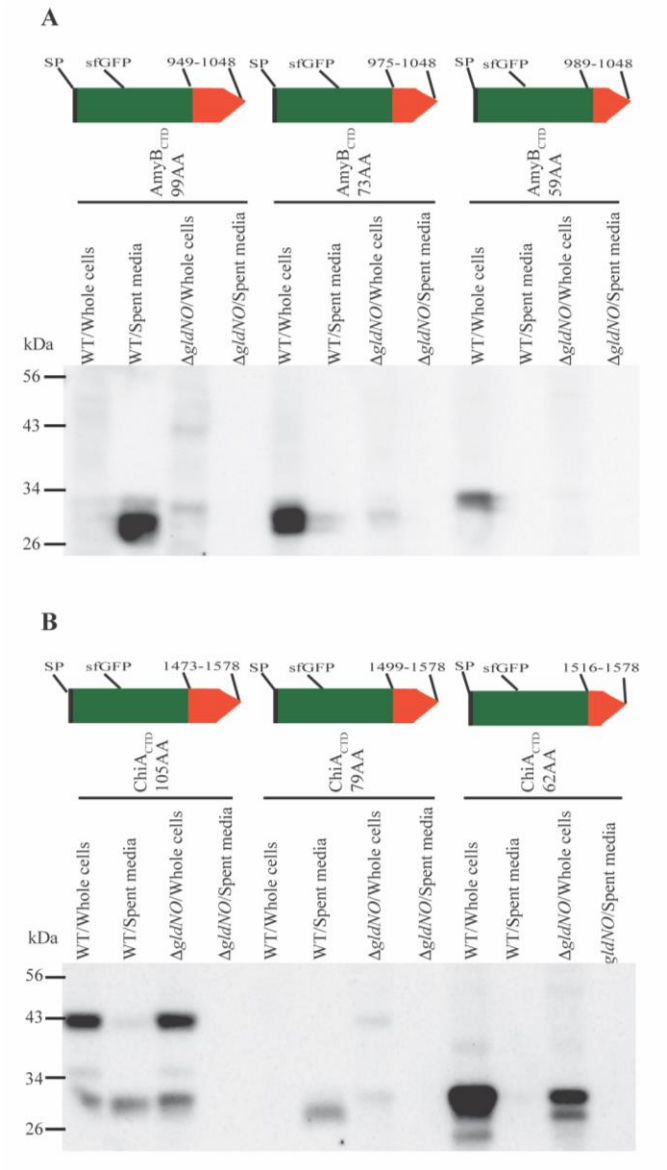

Fig. 13. T9SS-mediated secretion of SP_{RemA}-sfGFP-CTD_{AmyB}, and SP_{RemA}-sfGFP-CTD_{ChiA}. Cell free spent media and whole cells were analyzed for wild-type (WT) cells and for cells of the T9SS mutant Δ*gldNO*. Cells carried plasmids expressing either SP_{RemA}-sfGFP-CTD_{AmyB} (A), or SP_{RemA}-sfGFP-CTD_{ChiA} (B) with CTD regions of various lengths. Proteins produced by pSK82, pSK85, and pSK86 produced SP_{RemA}-sfGFP-CTD_{AmyB} with CTD regions of 99, 73, and 59 amino acids respectively as indicated by the cartoons (A). Proteins produced by pCB3, pSK89, and pCB4 produced SP_{RemA}-sfGFP-CTD_{ChiA} with CTD regions of 105, 79, and 62 amino acids respectively as indicated by the cartoons (B). Samples were separated by SDS-PAGE, and sfGFP was detected using anti-serum against GFP. Whole cell samples corresponded to 10 μg protein per lane and samples from spent media corresponded to the volume of spent medium that contained 10 μg cell protein before the cells were removed.

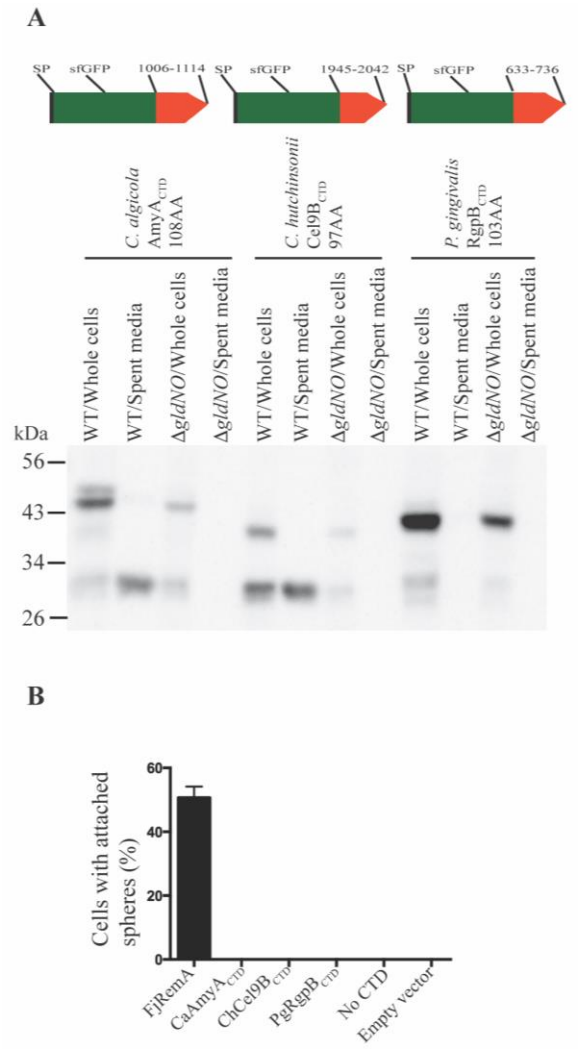

Fig. 14. C-terminal regions from *Cellulophaga algicola* AmyA and *Cytophaga hutchinsonii* Cel9B target sfGFP for secretion by the *F. johnsoniae* T9SS. (A) Cell free spent media and whole cells were analyzed for wild-type (WT) cells and for cells of the T9SS mutant Δ*gldNO*. Cells carried pSK65, pSK76, or pSK75 expressing SP_{RemA}-sfGFP fused to C-terminal regions of *C. algicola* AmyA (108 amino acids), *C. hutchinsonii* Cel9B (97 amino acids) or *P. gingivalis* RgpB (103 amino acids) respectively. Samples were separated by SDS-PAGE, and sfGFP was detected using anti-serum against GFP. Cell samples corresponded to 10 μg protein per lane and samples from spent media corresponded to the volume of spent medium that contained 10 μg cell protein before the cells were removed. (B) To determine if any sfGFP was attached on the cell surface, anti-GFP antiserum and 0.5-μm-diameter protein G-coated polystyrene spheres were added to cells as described in Materials and Methods. Samples were introduced into a tunnel slide and examined using a phase-contrast microscope for the presence of spheres attached to the cells. Error bars indicate standard deviations from three measurements. FjRemA is full length RemA with sfGFP inserted immediately after the N-terminal signal peptide and was produced from pYT180. FjRemA was used as a positive control. 'No CTD' refers to cells expressing SP_{RemA}-sfGFP without a CTD, and 'Empty vector' refers to cells carrying pCP23.

The CTD of SprB is required for secretion but is not closely related to type A CTDs or to CTD_{ChiA}. SprB has a type B CTD and requires the T9SS for its secretion to the cell surface (3, 8, 13). Previous results suggested that this region might be involved in secretion because SprB and six other *F. johnsoniae* proteins that had type B CTDs were secreted by wild type cells but not by cells of the T9SS mutant Δ*gldNO* (18). The genome analyses presented above also linked type B CTDs to the T9SS (Table 3). Finally, a role for CTD_{SprB} in secretion is also suggested by the properties of mutant FJ117, which has a *HimarEm2* transposon inserted 101 nucleotides upstream of the stop codon of *sprB* (16). FJ117 produces truncated SprB lacking the C-terminal 34 amino acids (Fig. 15A). The cell-associated truncated SprB protein was apparently not present on the cell surface (data not shown) suggesting that it was instead trapped inside of the cells. *sprF*, which lies immediately downstream of and is cotranscribed with *sprB*, is required for SprB secretion (41). SprF protein was detected in wild type cells and in cells of the FJ117 mutant (Fig. 15B) indicating that the failure to secrete SprB was not caused by a polar effect of the transposon on *sprF*. *HimarEm2* has an internal promoter that was predicted to allow expression of *sprF* (41), which explains the presence of SprF protein in the mutant. Since the defect in secretion of SprB was not caused by lack of SprF it is likely that the absence of the C-terminal 34 amino acids of SprB were responsible for the defect, lending further support for a role for this type B CTD in T9SS-mediated secretion.

C-terminal regions of SprB and of Fjoh_3952 fail to support secretion of sfGFP. The highly conserved regions of type B CTDs extend approximately 80 to 100 amino acids from the C-termini (Fig. 2). Plasmids were constructed that encode SP_{RemA}-sfGFP with various lengths (99 to 1182 amino acids) of the C-terminal region of SprB. None of these facilitated secretion of sfGFP from wild type cells (Fig. 16). sfGFP was not detected in the cell-free spent medium, and was also

not detected on intact cells using latex spheres coated with anti-sfGFP. In addition, the sfGFP fusion proteins in intact cells were not susceptible to proteinase K, further indicating that they were not secreted and attached on the cell surface (Fig. 17). We also examined the type B CTD of Fjoh_3952, which is secreted by wild type cells but not by cells of a T9SS mutant (18). SP_{RemA}-sfGFP carrying the C-terminal 228 AAs of Fjoh_3952 failed to be secreted by wild type cells (Fig. 17). The results indicate that whereas short (97 amino acid or less) type A CTDs were sufficient to target a foreign protein for secretion by the T9SS, even much longer regions of type B CTDs were not. Type B CTDs may require additional regions of the secreted protein to interact productively with the T9SS.

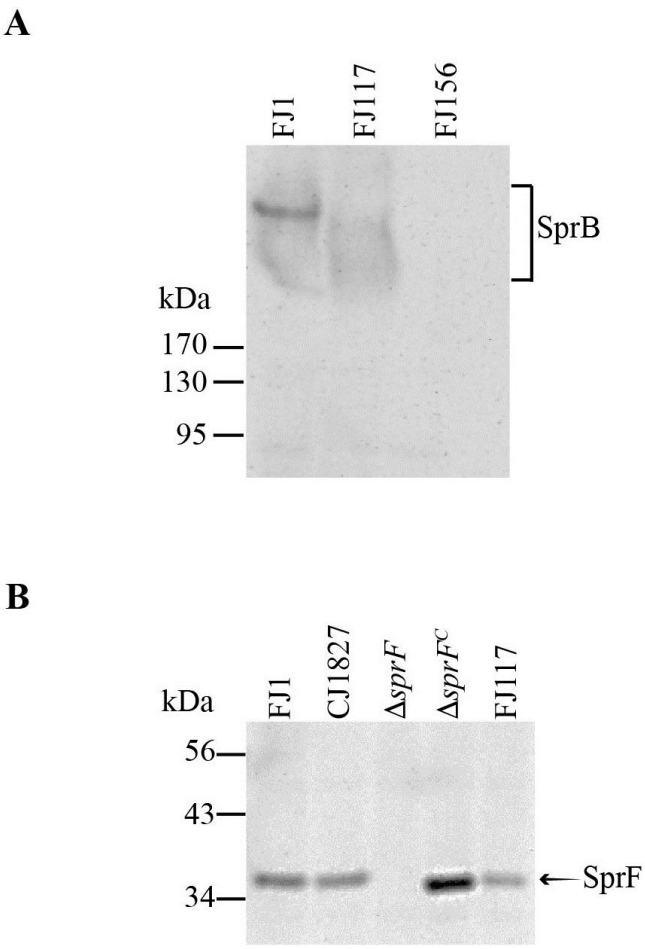

Fig. 15. The C-terminal region of SprB is required for secretion. (A) Immunodetection of SprB from wild type (WT) cells and from cells of the *sprB* transposon mutants FJ117 and FJ156 that should produce truncated SprB proteins lacking the C-terminal 34 and 5880 amino acids respectively. Total cell extracts were prepared by boiling in SDS loading buffer and samples corresponding to 25 μg protein per lane were analyzed for SprB by SDS-PAGE and Western blot analysis. (B) The transposon insertion in FJ117 is not polar on the downstream gene *sprF*. Cells were examined for SprF by SDS-PAGE and western blot analysis using antiserum against SprF. Wild-type *F. johnsoniae* CJ1827 and FJ1, Δ*sprF*, Δ*sprF* complemented with pRR48 (Δ*sprF*^{c}), and the transposon mutant FJ117 were examined. Equal amount (10 μg protein) of each sample were loaded in each lane.

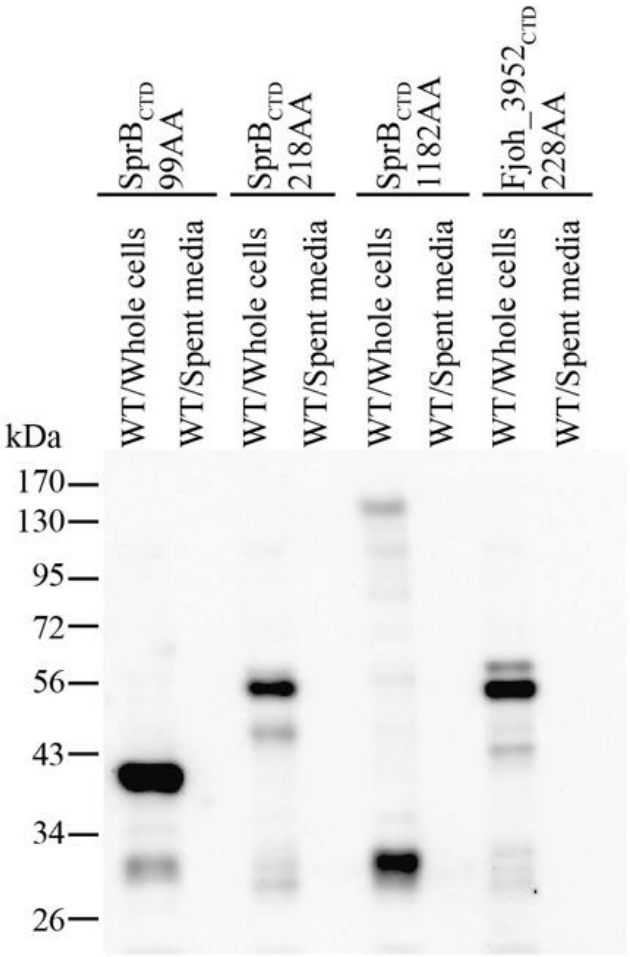

Fig. 16. C-terminal regions of SprB fused to SP_{RemA}-sfGFP failed to result in secretion of sfGFP. (A) Cell free spent media and whole cells were analyzed for cultures of wild-type cells carrying fusion plasmid pSK93, pSK56 and pSK62, which express SP_{RemA}-sfGFP with 99, 218, and 1182-amino acid C-terminal regions of SprB, respectively. Cells carrying fusion plasmid pSK58, which expresses SP_{RemA}-sfGFP with the 228-amino acid C-terminal region of the SprB-like protein Fjoh_3952 were also analyzed. Samples were separated by SDS-PAGE, and sfGFP was detected using anti-serum against GFP. Whole cell samples corresponded to 10 μg protein per lane, and samples from spent media corresponded to the volume of spent medium that contained 10 μg cell protein before the cells were removed.

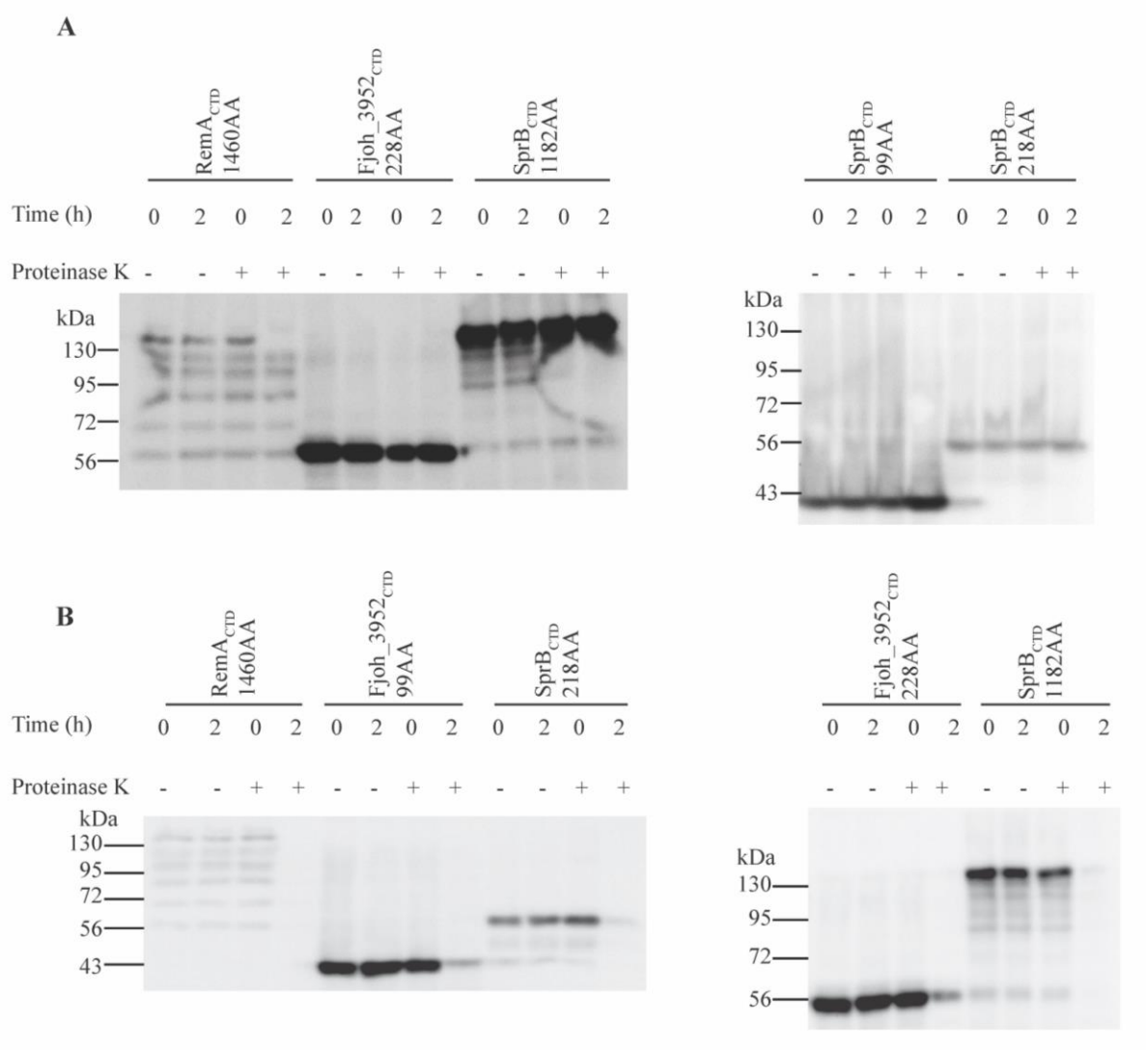

Fig. 17. Proteinase K treatment to determine if SP_{RemA}-sfGFP-CTD_{SprB} localizes to the cell surface. Strains expressing sfGFP fusion proteins with $SprB_{CTDs}$ of 99 amino acids (pSK93), 218 amino acids (pSK56), 1182 amino acids (pSK62), and with Fjoh_3952_{CTD} of 228 amino acids (pSK58) were analyzed. Strain expressing SP_{RemA}-sfGFP-CTD_{RemA} with 1386 amino acids of $RemA_{CTD}$, which is cell surface localized, was used as a positive control. Proteinase K was added at a final concentration of 1 mg/ml to intact cells (A) and to cell extracts prepared by French pressure cell treatment (B), and cells and extracts were incubated at 25˚C. Samples were removed at 0 h and 2 h for immunoblot analyses. Samples were separated by SDS-PAGE, and sfGFP was detected using antiserum against GFP. Samples not exposed to proteinase K (-) were also included.

Discussion

T9SSs are common in members of the phylum *Bacteroidetes* but have only recently been recognized and studied. Many secreted proteins of *P. gingivalis* have conserved CTDs (4, 20, 24) and it is now clear that this extends to the many other members of the phylum that have T9SSs (1, 2, 18). There is considerable diversity among these CTDs, but most belong to protein domain families TIGR04183 (type A CTDs) or TIGR04131 (type B CTDs). There have been only a few functional studies of the CTDs of secreted proteins, most of which were conducted on *P. gingivalis* (24, 26, 47). These studies focused on *P. gingivalis* RgpB and HBP35, which have type A CTDs. The only other functional studies involved *F. johnsoniae* ChiA, which has a unique CTD that is apparently unrelated to either type A or type B CTDs (21). The full spectrum of CTDs that facilitate secretion by T9SSs has not yet been thoroughly explored. Here we examined the characteristics of several *F. johnsoniae* T9SS CTDs including two type A CTDs, two type B CTDs, and the novel CTD of the chitinase ChiA.

The results confirm findings from *P. gingivalis* indicating that type A CTDs of less than 100 amino acids in length are sufficient to target foreign proteins for secretion by T9SSs. The length of the two *F. johnsoniae* type A CTDs required for efficient secretion of sfGFP were between 80 and 100 amino acids, and similar results were obtained for the ChiA CTD. Even shorter CTDs allowed secretion of *P. gingivalis* RgpB and HBP35 (25). The results also confirm previous findings from *P. gingivalis* regarding the importance of the extreme C-terminal amino acids. Deletion of the final 12 amino acids of an otherwise functional CTD_{RemA} eliminated secretion of the foreign protein sfGFP to which it was attached. Conversion of a conserved lysine within this terminal region to alanine had a similar effect. The similarity of these results to those reported for

the *P. gingivalis* T9SS CTDs suggests that targeting of proteins for secretion by type A CTDs may be broadly similar throughout the phylum *Bacteroidetes*.

The components of T9SSs are conserved across the many members of the phylum *Bacteroidetes* that use this system (Table 3). Recognizable type A CTDs are found in all members of the phylum that have the core components of the T9SS, but they are generally absent in the few species that lack T9SSs. The T9SS components from diverse members of the phylum, although conserved, also exhibit considerable divergence in sequence. For example, *F. johnsoniae* GldK, GldL, GldM, GldN, SprA, SprE, and SprT exhibit only 14-33 % amino acid identity with their orthologs from *C. hutchinsonii* (Table 5).

Table 5. Amino acid sequence identities of *F. johnsoniae* T9SS components with orthologs from other members of the phylum *Bacteroidetes*[a].

F. johnsoniae	*C. algicola*	*C. hutchinsonii*	*P. gingivalis*
GldK (464 AA)	67% over 467 AA	33% over 477 AA	34% over 502 AA
GldL (215 AA)	58% over 219 AA	27% over 273 AA	19% over 313 AA
GldM (513 AA)	41% over 526 AA	20% over 546 AA	24% over 539 AA
GldN (329 AA)	52% over 334 AA	14% over 353 AA	16% over 409 AA
SprA (2403 AA)	52% over 2460 AA	32% over 2537 AA	32% over 2622 AA
SprE (870 AA)	40% over 887 AA	21% over 902 AA	15% over 1191 AA
SprT (237 AA)	48% over 240 AA	25% over 244 AA	22% over 254 AA
PorU (1278 AA)	No ortholog	33% over 1332 AA	23% over 1335 AA
PorV (402 AA)	57% over 404 AA	34% over 417 AA	42% over 413 AA

[a]*F. johnsoniae* T9SS components were aligned with orthologs from *Cellulophaga algicola* (Class *Flavobacteriia*), *Cytophaga hutchinsonii* (Class *Cytophagia*) and *Porphyromonas gingivalis* (Class *Bacteroidia*) using MUSCLE. In each case percent amino acid (AA) identity over the region of similarity is listed. Note that *C. algicola*, which has a functional T9SS, lacks a PorU ortholog.

This is perhaps not surprising since *F. johnsoniae* (Class *Flavobacteriia*) and *C. hutchinsonii* (Class *Cytophagia*) are only distantly related (48). In spite of the divergence in sequence between their T9SS components, the *C. hutchinsonii* Cel9B CTD functioned with the *F. johnsoniae* T9SS to allow secretion of the foreign protein sfGFP. While not all CTDs functioned in this way, the results indicate that individual CTDs can function with the secretion system from a distantly related member of the phylum. The structure of one CTD was recently solved (49). It is likely that the 3-dimensional structures of the CTDs and of the components of the T9SS, rather than the primary sequences, are most important in directing proteins for secretion by the T9SS. Determination of the structures of additional T9SS CTDs may help to determine if the diversity of primary sequences obscures a common fold that allows recognition of diverse CTDs by T9SSs. Similarly, determination of the structure of the secretion apparatus and its components may help determine how secreted proteins interact with this machine. Electron microscopic analyses recently revealed structural features of a complex comprised of *P. gingivalis* PorK and PorN (orthologs of GldK and GldN) suggesting that they form a large ring on the periplasmic side of the outer membrane (50). Evidence for a cell envelope-spanning complex consisting of *P. gingivalis* PorK, PorL, PorM, PorN, and PorP was also recently presented (51). Further studies will undoubtedly reveal additional structural features of the T9SS. Regardless, it is clear that CTDs play a role in secretion of proteins by the T9SS. The ability to predictably target proteins for secretion by addition of a type A CTD allows engineering of *F. johnsoniae* or other members of the *Bacteroidetes* to secrete proteins of interest.

Given the ability of type A CTDs to target foreign proteins for secretion, the inability of type B CTDs to function similarly was somewhat surprising. All of the *F. johnsoniae* proteins with type B CTDs are large. SprB (6497 AAs in length) has a type B CTD that appears to be required

for its secretion. Secretion of such large proteins may require additional secretion system proteins to be involved and additional regions of the secreted protein to interact with the secretion system. For example, *sprF*, which lies immediately downstream of *sprB*, is required for secretion of SprB but not for secretion of RemA or of ChiA (14, 21, 41). Most genes encoding secreted proteins with type B CTDs have *sprF*-like genes immediately downstream, and it has been suggested that these may be involved in secretion of their cognate proteins (41). Additional experiments are needed to determine the features of type B CTD-containing proteins that are necessary for secretion by the T9SS. This may be challenging given the large size of these proteins.

Genome analyses indicate that T9SSs are found in most but not all members of the phylum *Bacteroidetes* (1) (Table 3). Our results suggest that the function of type A CTDs in secretion may be similar across diverse members of the phylum. The co-occurrence of type A CTDs and SprA across the entire phylum *Bacteroidetes* and extending to the phyla *Rhodothermaeota*, *Chlorobi*, *Fibrobacteres*, and *Ignavibacteriae* suggests the possibility that type A CTDs may interact directly or indirectly with SprA. Our results also highlight the diversity of CTDs involved in secretion and suggest that type A CTDs and type B CTDs may interact differently with the T9SS to facilitate secretion of specific sets of proteins.

Our results also identified the growth phase dependence of secretion. The *F. johnsoniae* T9SS appeared to function poorly in cells growing exponentially in rich media but it supported secretion in cells that were in stationary phase of growth. The exponentially growing cells had little if any SprA protein which may explain the secretion defect. The *F. johnsoniae* T9SS is needed for secretion of numerous proteases, polysaccharide digesting enzymes and motility proteins. Polymer digestion and cell movement may only be needed when cells are nutrient limited and the

T9SS may be regulated so that it is only fully expressed under these conditions. Little is known regarding regulation of the genes encoding T9SS components. Further research is needed to explore this topic and to fully understand the structure and function of the T9SS apparatus in the many diverse members of the phylum *Bacteroidetes* that rely on these secretion machines.

References

1. McBride MJ, Zhu Y. 2013. Gliding motility and Por secretion system genes are widespread among members of the phylum *Bacteroidetes*. J Bacteriol 195:270-278.
2. Veith PD, Nor Muhammad NA, Dashper SG, Likic VA, Gorasia DG, Chen D, Byrne SJ, Catmull DV, Reynolds EC. 2013. Protein substrates of a novel secretion system are numerous in the *Bacteroidetes* phylum and have in common a cleavable C-terminal secretion signal, extensive post-translational modification and cell surface attachment. J Proteome Res 12:4449-4461.
3. Sato K, Naito M, Yukitake H, Hirakawa H, Shoji M, McBride MJ, Rhodes RG, Nakayama K. 2010. A protein secretion system linked to bacteroidete gliding motility and pathogenesis. Proc Natl Acad Sci USA 107:276-281.
4. Sato K, Sakai E, Veith PD, Shoji M, Kikuchi Y, Yukitake H, Ohara N, Naito M, Okamoto K, Reynolds EC, Nakayama K. 2005. Identification of a new membrane-associated protein that influences transport/maturation of gingipains and adhesins of *Porphyromonas gingivalis* J Biol Chem 280:8668-8677.
5. Kita D, Shibata S, Kikuchi Y, Kokubu E, Nakayama K, Saito A, Ishihara K. 2016. Involvement of the Type IX Secretion System in *Capnocytophaga ochracea* Gliding Motility and Biofilm Formation. Appl Environ Microbiol 82:1756-66.
6. Tomek MB, Neumann L, Nimeth I, Koerdt A, Andesner P, Messner P, Mach L, Potempa JS, Schaffer C. 2014. The S-layer proteins of *Tannerella forsythia* are secreted via a type IX secretion system that is decoupled from protein O-glycosylation. Mol Oral Microbiol 29:307-20.
7. Zhu Y, McBride MJ. 2014. Deletion of the *Cytophaga hutchinsonii* type IX secretion system gene *sprP* results in defects in gliding motility and cellulose utilization. Appl Microbiol Biotechnol 98:763-775.
8. Rhodes RG, Samarasam MN, Shrivastava A, van Baaren JM, Pochiraju S, Bollampalli S, McBride MJ. 2010. *Flavobacterium johnsoniae gldN* and *gldO* are partially redundant genes required for gliding motility and surface localization of SprB. J Bacteriol 192:1201-1211.
9. Lasica AM, Goulas T, Mizgalska D, Zhou X, de Diego I, Ksiazek M, Madej M, Guo Y, Guevara T, Nowak M, Potempa B, Goel A, Sztukowska M, Prabhakar AT, Bzowska M, Widziolek M, Thogersen IB, Enghild JJ, Simonian M, Kulczyk AW, Nguyen KA, Potempa J, Gomis-Ruth FX. 2016. Structural and functional probing of PorZ, an essential bacterial surface component of the type-IX secretion system of human oral-microbiomic *Porphyromonas gingivalis*. Sci Rep 6:37708.
10. Braun TF, Khubbar MK, Saffarini DA, McBride MJ. 2005. *Flavobacterium johnsoniae* gliding motility genes identified by mariner mutagenesis. J Bacteriol 187:6943-6952.
11. Nelson SS, Glocka PP, Agarwal S, Grimm DP, McBride MJ. 2007. *Flavobacterium johnsoniae* SprA is a cell-surface protein involved in gliding motility. J Bacteriol 189:7145-7150.
12. Saiki K, Konishi K. 2007. Identification of a *Porphyromonas gingivalis* novel protein Sov required for the secretion of gingipains. Microbiol Immunol 51:483-491.

13. Shrivastava A, Johnston JJ, van Baaren JM, McBride MJ. 2013. *Flavobacterium johnsoniae* GldK, GldL, GldM, and SprA are required for secretion of the cell-surface gliding motility adhesins SprB and RemA. Journal of Bacteriology 195:3201-3212.
14. Shrivastava A, Rhodes RG, Pochiraju S, Nakane D, McBride MJ. 2012. *Flavobacterium johnsoniae* RemA is a mobile cell-surface lectin involved in gliding. J Bacteriol 194:3678-3688.
15. Nakane D, Sato K, Wada H, McBride MJ, Nakayama K. 2013. Helical flow of surface protein required for bacterial gliding motility. Proc Natl Acad Sci USA 110:11145-11150.
16. Nelson SS, Bollampalli S, McBride MJ. 2008. SprB is a cell surface component of the *Flavobacterium johnsoniae* gliding motility machinery. J Bacteriol 190:2851-2857.
17. Shrivastava A, Lele PP, Berg HC. 2015. A rotary motor drives *Flavobacterium* gliding. Curr Biol 25:338-341.
18. Kharade SS, McBride MJ. 2015. *Flavobacterium johnsoniae* PorV is required for secretion of a subset of proteins targeted to the type IX secretion system. J Bacteriol 197:147-158.
19. Sato K, Yukitake H, Narita Y, Shoji M, Naito M, Nakayama K. 2013. Identification of *Porphyromonas gingivalis* proteins secreted by the Por secretion system. FEMS microbiology letters 338:68-76.
20. Nguyen KA, Travis J, Potempa J. 2007. Does the importance of the C-terminal residues in the maturation of RgpB from *Porphyromonas gingivalis* reveal a novel mechanism for protein export in a subgroup of Gram-Negative bacteria? J Bacteriol 189:833-843.
21. Kharade SS, McBride MJ. 2014. The *Flavobacterium johnsoniae* chitinase ChiA is required for chitin utilization and is secreted by the type IX secretion system. J Bacteriol 196:961-970.
22. Gorasia DG, Veith PD, Chen D, Seers CA, Mitchell HA, Chen YY, Glew MD, Dashper SG, Reynolds EC. 2015. *Porphyromonas gingivalis* Type IX Secretion Substrates Are Cleaved and Modified by a Sortase-Like Mechanism. PLoS Pathog 11:e1005152.
23. Glew MD, Veith PD, Peng B, Chen YY, Gorasia DG, Yang Q, Slakeski N, Chen D, Moore C, Crawford S, Reynolds E. 2012. PG0026 is the C-terminal signal peptidase of a novel secretion system of *Porphyromonas gingivalis*. J Biol Chem 287:24605-24617.
24. Seers CA, Slakeski N, Veith PD, Nikolof T, Chen YY, Dashper SG, Reynolds EC. 2006. The RgpB C-terminal domain has a role in attachment of RgpB to the outer membrane and belongs to a novel C-terminal-domain family found in *Porphyromonas gingivalis*. J Bacteriol 188:6376-6386.
25. Shoji M, Sato K, Yukitake H, Kondo Y, Narita Y, Kadowaki T, Naito M, Nakayama K. 2011. Por secretion system-dependent secretion and glycosylation of *Porphyromonas gingivalis* hemin-binding protein 35. PLOS One 6:e21372.
26. Slakeski N, Seers CA, Ng K, Moore C, Cleal SM, Veith PD, Lo AW, Reynolds EC. 2011. C-terminal domain residues important for secretion and attachment of RgpB in *Porphyromonas gingivalis*. J Bacteriol 193:132-142.
27. Chang LYE, Pate JL, Betzig RJ. 1984. Isolation and characterization of nonspreading mutants of the gliding bacterium *Cytophaga johnsonae*. J Bacteriol 159:26-35.
28. McBride MJ, Braun TF. 2004. GldI is a lipoprotein that is required for *Flavobacterium johnsoniae* gliding motility and chitin utilization. J Bacteriol 186:2295-2302.
29. McBride MJ, Xie G, Martens EC, Lapidus A, Henrissat B, Rhodes RG, Goltsman E, Wang W, Xu J, Hunnicutt DW, Staroscik AM, Hoover TR, Cheng YQ, Stein JL. 2009. Novel

features of the polysaccharide-digesting gliding bacterium *Flavobacterium johnsoniae* as revealed by genome sequence analysis. Appl Environ Microbiol 75:6864-6875.
30. McBride MJ, Kempf MJ. 1996. Development of techniques for the genetic manipulation of the gliding bacterium *Cytophaga johnsonae*. J Bacteriol 178:583-590.
31. Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
32. Liu J, McBride MJ, Subramaniam S. 2007. Cell-surface filaments of the gliding bacterium *Flavobacterium johnsoniae* revealed by cryo-electron tomography. J Bacteriol 189:7503-7506.
33. Rhodes RG, Samarasam MN, Van Groll EJ, McBride MJ. 2011. Mutations in *Flavobacterium johnsoniae sprE* result in defects in gliding motility and protein secretion. Journal of Bacteriology 193:5322-7.
34. Haft DH, Selengut JD, Richter RA, Harkins D, Basu MK, Beck E. 2013. TIGRFAMs and Genome Properties in 2013. Nucleic Acids Res 41:D387-95.
35. Bolivar F, Backman K. 1979. Plasmids of *Escherichia coli* as cloning vectors. Methods Enzymol 68:245-267.
36. Figurski DH, Helinski DR. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci USA 76:1648-1652.
37. Rhodes RG, Pucker HG, McBride MJ. 2011. Development and use of a gene deletion strategy for *Flavobacterium johnsoniae* to identify the redundant motility genes *remF*, *remG*, *remH*, and *remI*. J Bacteriol 193:2418-2428.
38. Zhu Y, Thomas F, Larocque R, Li N, Duffieux D, Cladiere L, Souchaud F, Michel G, McBride MJ. submitted. Novel genetic tools unravel the crucial role of a laterally acquired alginate lyase for brown algal biomass degradation by *Zobellia galactanivorans*
39. Agarwal S, Hunnicutt DW, McBride MJ. 1997. Cloning and characterization of the *Flavobacterium johnsoniae* (*Cytophaga johnsonae*) gliding motility gene, *gldA*. Proc Natl Acad Sci USA 94:12139-12144.
40. Mally M, Cornelis GR. 2008. Genetic tools for studying *Capnocytophaga canimorsus*. Applied and environmental microbiology 74:6369-77.
41. Rhodes RG, Nelson SS, Pochiraju S, McBride MJ. 2011. *Flavobacterium johnsoniae sprB* is part of an operon spanning the additional gliding motility genes *sprC*, *sprD*, and *sprF*. J Bacteriol 193:599-610.
42. Uehara T, Dinh T, Bernhardt TG. 2009. LytM-domain factors are required for daughter cell separation and rapid ampicillin-induced lysis in *Escherichia coli*. Journal of Bacteriology 191:5094-5107.
43. Coyne MJ, Roelofs KG, Comstock LE. 2016. Type VI secretion systems of human gut Bacteroidales segregate into three genetic architectures, two of which are contained on mobile genetic elements. BMC Genomics 17:58.
44. Wilson MM, Anderson DE, Bernstein HD. 2015. Analysis of the outer membrane proteome and secretome of Bacteroides fragilis reveals a multiplicity of secretion mechanisms. PLoS One 10:e0117732.
45. Ishiguro I, Saiki K, Konishi K. 2009. PG27 is a novel membrane protein essential for a *Porphyromonas gingivalis* protease secretion system. FEMS Microbiol Lett 292:261-267.

46. Zhu Y, Han L, Hefferon KL, Silvaggi NR, Wilson DB, McBride MJ. 2016. Periplasmic *Cytophaga hutchinsonii* endoglucanases are required for use of crystalline cellulose as the sole source of carbon and energy. Appl Environ Microbiol 82:4835-45.
47. Shoji M, Ratnayake DB, Shi Y, Kadowaki T, Yamamoto K, Yoshimura F, Akamine A, Curtis MA, Nakayama K. 2002. Construction and characterization of a nonpigmented mutant of *Porphyromonas gingivalis*: cell surface polysaccharide as an anchorage for gingipains. Microbiol 148:1183-1191.
48. Krieg NR, Ludwig W, Euzeby J, Whitman WB. 2011. Phylum XIV. *Bacteroidetes* phyl. nov., p 25-469. *In* Krieg NR, Staley JT, Brown DR, Hedlund BR, Paster BJ, Ward NL, Ludwig W, Whitman WB (ed), Bergey's Manual of Systematic Bacteriology, vol 4. Springer.
49. de Diego I, Ksiazek M, Mizgalska D, Koneru L, Golik P, Szmigielski B, Nowak M, Nowakowska Z, Potempa B, Houston JA, Enghild JJ, Thogersen IB, Gao J, Kwan AH, Trewhella J, Dubin G, Gomis-Ruth FX, Nguyen KA, Potempa J. 2016. The outer-membrane export signal of *Porphyromonas gingivalis* type IX secretion system (T9SS) is a conserved C-terminal beta-sandwich domain. Sci Rep 6:23123.
50. Gorasia DG, Veith PD, Hanssen EG, Glew MD, Sato K, Yukitake H, Nakayama K, Reynolds EC. 2016. Structural Insights into the PorK and PorN Components of the *Porphyromonas gingivalis* Type IX Secretion System. PLoS Pathog 12:e1005820.
51. Vincent MS, Canestrari MJ, Leone P, Stathopulos J, Ize B, Zoued A, Cambillau C, Kellenberger C, Roussel A, Cascales E. 2017. Characterization of the *Porphyromonas gingivalis* Type IX Secretion Trans-envelope PorKLMNP Core Complex. J Biol Chem 292:3252-3261.

Chapter 3. Secretion and cell-surface localization of proteins carrying C-terminal regions of the *Flavobacterium johnsoniae* motility protein SprB

This chapter is a manuscript in preparation and will be submitted to a journal for publication after completion.

Abstract

The *Flavobacterium johnsoniae* adhesin SprB is propelled rapidly along the cell surface resulting in gliding motility. Secretion of SprB requires the type IX secretion system (T9SS). Proteins secreted across the outer membrane by the T9SS typically have conserved C-terminal domains (CTDs) that belong to protein domain families TIGR04183 (type A CTDs) or TIGR04131 (type B CTDs). They also have amino-terminal signal peptides (SPs) that facilitate export across the cytoplasmic membrane by the Sec system. Attachment of 80 to 100 amino acid (AA) regions of type A CTDs to a foreign protein such as sfGFP allow its secretion across the outer membrane. In contrast, similar regions of type B CTDs fail to result in secretion. Type B CTDs are common in the *Bacteroidetes* but little is known regarding their roles in secretion. Here the secretion of the foreign protein sfGFP fused to an N-terminal SP and to C-terminal regions of SprB (SP-sfGFP-CTD_{SprB}) was analyzed. CTDs of 218 AAs or longer resulted in secretion whereas a CTD of 149 AAs did not. *sprF*, which lies downstream of *sprB*, is known to be required for SprB secretion. SP-sfGFP-CTD_{SprB} also required SprF for secretion. Efficient secretion only occurred when SP-sfGFP-CTD_{SprB} and SprF were expressed together. Under these conditions CTDs of 218 AAs and 448 AAs resulted in secretion of soluble sfGFP, whereas longer CTDs (663 and 1182 AAs) resulted in attachment of sfGFP to the cell surface. Most *F. johnsoniae* genes encoding proteins with type B CTDs lie immediately upstream of *sprF*-like genes. The CTD from one such protein,

Fjoh_3952, facilitated secretion of sfGFP only when it was coexpressed with its cognate SprF-like protein, Fjoh_3951. Secretion did not occur when SP-sfGFP-CTD_{Fjoh_3952} was expressed with SprF, or when SP-sfGFP-CTD_{SprB} was expressed with Fjoh_3951. The results highlight the need for extended regions of type B CTDs for secretion and cell-surface localization, and the requirement for the cognate SprF-like protein for secretion. Since type B CTD-containing proteins and associated SprF-like proteins are common among members of the phylum *Bacteroidetes* the unique features required for secretion of these proteins may have broad implications.

Introduction

Protein secretion systems are key players in regulating interactions of Gram-negative bacteria with their environment (1). Secreted proteins may be anchored to the cell-surface or released into the extra-cellular milieu (2). Type I, III, IV, and VI secretion systems transport proteins directly from the cytoplasm to the outside. In contrast, Type II, V, VII, VIII and IX secretion systems deliver proteins across the outer membrane from the periplasm, and rely on the Sec or Tat systems for the initial export across the cytoplasmic membrane. The type IX secretion system (T9SS) is common in, but limited, to members of the phylum *Bacteroidetes* (3). It was first studied in the non-motile oral pathogen *Porphryomonas gingivalis* and in the gliding bacterium *Flavobacterium johnsoniae* (4, 5). The core proteins of the *F. johnsoniae* T9SS include GldK, GldL, GldM, GldN, SprA, SprE and SprT (4, 6-9), which correspond to *P. gingivalis* PorK, PorL, PorM, PorN, sov, PorW and PorT respectively (4). The *F. johnsoniae* T9SS is required for secretion of dozens of proteins, including the cell surface motility proteins SprB and RemA and the soluble extracellular enzymes ChiA and AmyB (8, 10). The *P. gingivalis* T9SS is involved in secretion of virulence factors such as gingipain proteases and adhesins (4). The secreted proteins

have signal peptides that allow export across the cytoplasmic membrane via the Sec machinery, and conserved C-terminal domains (CTDs) that are required for T9SS-mediated secretion and are typically cleaved during or after secretion (11-14).

Proteins secreted by the *F. johnsoniae* T9SS have CTDs that belong to either TIGR04183 (type A CTDs), or TIGR04131 (type B CTDs). The roles of type A CTDs in secretion of *F. johnsoniae* and *P. gingivalis* proteins have been studied. Type A CTD regions of 80 to 100 AA are usually sufficient to target a foreign protein such as sfGFP for secretion from the periplasm across the outer membrane by the T9SS (12, 14, 15). Type B CTDs of similar lengths however fail to result in secretion (15). Here we demonstrate that regions of type B CTDs longer than 149 AA are needed for secretion, and regions longer than 448 AA are needed for attachment of the secreted protein to the cell surface. An additional protein, SprF, is required for secretion of SprB but is not needed for secretion of other proteins targeted to the T9SS. *sprF* and *sprB* are adjacent on the chromosome and are co-transcribed (16). Here we characterize the role of type B CTDs, and of SprF and SprF-like proteins in secretion by the T9SS.

Materials and Methods

Bacterial strains, plasmids and growth conditions. *F. johnsoniae* ATCC 17061 (UW101) was the wild-type strain used in this study (17-19). *F. johnsoniae* strains were grown in Casitone-yeast extract (CYE) medium at 30°C, as previously described (20). *Escherichia coli* strains were grown in Luria-Bertani medium (LB) at 37°C (21). Strains and plasmids used in this study are listed in Table 1, and primers are Table 2. Antibiotics were used at the following final concentrations when needed: ampicillin, 100 μg/ml; kanamycin, 30 μg/ml; streptomycin, 100 μg/ml; and tetracycline, 20 μg/ml.

Generation of plasmids that express sfGFP with signal peptides at the N-terminus and with regions of SprB CTDs at the C-terminus. Plasmids that express sfGFP fused to regions of the CTD of SprB were constructed. Regions of DNA encoding the C-terminus of SprB (CTD_{SprB}) were introduced into plasmid pSK179 that expressed the N-terminal signal peptide of RemA fused to sfGFP (SP-sfGFP) (15), resulting in plasmids that produce SP-sfGFP-CTD_{SprB}. These include pSK93 (expresses SP-sfGFP-$CTD_{SprB99AA}$), pSK56 (expresses SP-sfGFP-$CTD_{SprB218AA)}$ and pSK62 (expresses SP-sfGFP-$CTD_{SprB1182AA}$) that have been previously described (15). Additional plasmids expressing SP-sfGFP-$CTD_{SprB149AA}$ (pSK60), SP-sfGFP-$CTD_{SprB368AA}$ (pSK53), SP-sfGFP-$CTD_{SprB448AA}$ (pSK54), and SP-sfGFP-$CTD_{SprB663AA}$ (pSK50), were constructed in a similar way using the primers listed in Table 2. In each case a region that encodes the C-terminus of SprB was amplified and inserted into the XbaI and SphI sites of pYT179, to generate a construct encoding SP-sfGFP-CTD_{SprB}. Plasmids that also included *sprF* downstream from each of the SP-sfGFP-CTD_{SprB}-encoding constructs were also prepared. For example, a 657-bp fragment encoding 218 amino acids of CTD_{SprB}, 17-bp intergenic region, and 1294–bp fragment encoding SprF was amplified by PCR using Phusion DNA polymerase (New England Biolabs, Ipswich, MA) and primers 1843 (engineered XbaI site) and 955 (engineered SphI site). This fragment was introduced into XbaI and SphI digested pYT179 to generate pSK55. Plasmids encoding SP-sfGFP-CTD_{SprB} with 99, 149, 368, 448, 663, and 1182 amino acids from the C-terminus of SprB followed by and coexpressed with *sprF* (pSK41, pSK59, pSK51, pSK52, pSK45, and pSK61 respectively) were constructed similarly using the primers listed in Table 2. A 3537-bp fragment encoding 1170 amino acids near the C-terminus but lacking the C-terminal 12 amino acids was also cloned into pYT179 using primers 1935 (engineered XbaI site) and 1880 (engineered SphI site), generating plasmid pSK78. A fragment encoding SprF was amplified using

primers 1936 (engineered SphI site) and 1937 (engineered SphI site) and cloned into the SphI site of pSK78 to generate pSK83 and confirmed by sequencing.

Generation of plasmids that express SP-sfGFP fused to Fjoh_3952 and Fjoh_1123 CTDs. A 687-bp fragment encoding 228 amino acids of the CTD of the SprB-like protein Fjoh_3952 and the entire Fjoh_3951 (*sprF*-like) gene was amplified and cloned into pYT179 using primers 1868 (engineered XbaI site) and 1869 (engineered SphI site) to generate plasmid pSK57. Plasmids were constructed that encoded SP-sfGFP-CTD_{SprB} and Fjoh_3951 (pSK69), and that encoded SP-sfGFP-CTD_{Fjoh_3952} and SprF (pSK68). Fjoh_3951 was amplified using primers 1892 (engineered SphI site) and 1969 (engineered SphI site) and cloned into the SphI site of pSK56, generating pSK69. Similarly, *sprF* was amplified using primers 1883 (engineered SphI site) and 955 (engineered SphI site), and cloned into the SphI site of pSK58 to generate pSK68. Plasmids were confirmed by sequencing. A region spanning 762-bp of $Fjoh\_1123_{CTD}$ was also cloned into pYT179 using primers 1881 (engineered XbaI site) and 1182 (engineered SphI site), generating plasmid pSK64 which encodes SP-sfGFP-CTD_{Fjoh_1123}.

Microscopic observation of cells. The movement of *F. johnsoniae* cells on glass was examined by phase-contrast microscopy at 25˚C. Cells were grown in MM at 25˚C without shaking. Motility on glass was analyzed using liquid filled tunnel slides prepared as described previously (22), using Nichiban NW-5 double sided tape (Nichiban Co, Tokyo, Japan) to hold a glass coverslip over a glass slide. Cells suspended in CYE medium were introduced into tunnel slides and incubated for 3 min. Cell movements were observed using an Olympus BH-2 phase-contrast microscope. Images were recorded using a Photometrics Cool-$SNAP_{cf}^2$ camera and analyzed using Metamorph software.

Binding of protein G-coated polystyrene spheres. Anti-GFP (1 µl of 0.5 mg per ml; GeneScript), 0.5-µm-diameter protein G-coated polystyrene spheres (1 µl of a 0.1% stock preparation; Sphereotech Inc., Libertyville, IL), and bovine serum albumin (BSA) (1 µl of 1% solution) were spotted on a glass slide, covered with a glass coverslip, and images were recorded and analyzed using Metamorph software.

Western blot analyses. *F. johnsoniae* cells were grown to early stationary phase in CYE at 25°C with shaking. Cells were pelleted by centrifugation at 22,000 x g for 15 min, and the culture supernatant (spent medium) was separated. For whole-cell samples, the cells were suspended in the original culture volume of phosphate-buffered saline consisting of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, and 2 mM KH_2PO_4 (pH 7.4). Equal amounts of spent media and whole cells were boiled in SDS-PAGE loading buffer for 10 min. Proteins were separated by SDS-PAGE, and Western blot analyses were performed as previously described (23). Equal amounts of each sample based on the starting material were loaded in each lane. For cell extracts this corresponded to 10 µg protein, whereas for spent medium this corresponded to the equivalent volume of spent medium that contained 10 µg cell protein before the cells were removed. Anti-GFP (0.5 mg per ml) was used at a dilution of 1: 3,000 to detect sfGFP in Western blots. Polyclonal antibodies against SprF peptides were produced by Biomatik Corporation (Cambridge, Ontario, Canada) and were used at a dilution of 1:3,000.

Proteinase K treatment of cells to determine the localization of SprF. Cells of *F. johnsoniae* were grown in CYE at 25°C with shaking. Cells were collected, washed and suspended in 20 mM sodium phosphate-10 mM $MgCl_2$ (pH 7.5) and diluted to an OD_{600} of 1.5. To examine SprF, proteinase K was added to the intact cells to a final concentration of 1 mg/ml and incubated

at 25°C with gentle mixing. In each case an identical sample was lysed using a French pressure cell, unbroken cells and debris were removed by centrifugation and proteinase K was added as above. At 0 and 2 h, 150 µl of cells or lysed cells were sampled, 10 mM phenylmethylsulfonyl fluoride was added and the samples were boiled for 1 min to stop digestion. SDS-PAGE loading buffer was added and the samples were boiled for another 7 min. Control samples that were not exposed to proteinase K were also included. Equal volumes were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes, and proteins were detected with anti-serum against SprF.

Table 1. Strains and plasmids used in this study.

Strain	Description[a]	Source or reference
***E. coli* strains**		
DH5αmcr	Strain used for general cloning	Life Technologies (Grand Island, NY, USA)
HB101	Strain used with pRK2013 for triparental conjugation	(24, 25)
***F. johnsoniae* strains**		
CJ1827	*rpsL2*; Sm^r 'wild-type' *F. johnsoniae* strain used in construction of deletion mutants	(26)
CJ2122	Δ*gldK*	(27)
CJ1922	Δ*sprB*	(26)
CJ2518	Δ*sprF*	(28)

Plasmid	Description	Source or reference
pCP11	*E.coli- F. johnsoniae* shuttle plasmid; Ap^r (Em^r)	(29)
pCP23	*E.coli- F. johnsoniae* shuttle plasmid; Ap^r (Tc^r)	(30)
pSK37	sfGFP with stop codon cloned into pYT40; Ap^r (Tc^r)	(15)
pSK41	300-bp region encoding 99 amino acids of CTD_{SprB}, 16-bp intergenic region and 1178-bp region encoding 333 amino acids of SprF inserted into pYT179; Ap^r (Tc^r)	This study

pSK45	1992-bp region encoding 663 amino acids of CTD_{SprB}, 16-bp intergenic region and 1178-bp region encoding 333 amino acids of SprF inserted into pYT179; Ap^r (Tc^r)	This study
pSK50	1992-bp region encoding 663 amino acids of CTD_{SprB} inserted into pYT179; Ap^r (Tc^r)	This study
pSK51	1107-bp region encoding 368 amino acids of CTD_{SprB}, 16-bp intergenic region and 1178-bp region encoding 333 amino acids of SprF inserted into pYT179; Ap^r (Tc^r)	This study
pSK52	1347-bp region encoding 448 amino acids of CTD_{SprB}, 16-bp intergenic region and 1178-bp region encoding 333 amino acids of SprF inserted into pYT179; Ap^r (Tc^r)	This study
pSK53	1107-bp region encoding 368 amino acids of CTD_{SprB} inserted into pYT179; Ap^r (Tc^r)	This study
pSK54	1347-bp region encoding 448 amino acids of CTD_{SprB} inserted into pYT179; Ap^r (Tc^r)	This study
pSK55	657-bp region encoding 218 amino acids of CTD_{SprB}, 16-bp intergenic region and 1178-bp region encoding 333 amino acids of SprF inserted into pYT179; Ap^r (Tc^r)	This study
pSK56	657-bp region encoding 218 amino acids of CTD_{SprB} inserted into pYT179; Ap^r (Tc^r)	(15)
pSK57	687-bp region encoding 228 amino acids of CTD_{SprB}, 16-bp intergenic region and 1032-bp region encoding 303 amino acids of Fjoh_3951 inserted into pYT179; Ap^r (Tc^r)	This study
pSK58	687-bp region encoding 228 amino acids of CTD_{Fjoh_3952} inserted into pYT179; Ap^r (Tc^r)	(15)
pSK59	450-bp region encoding 149 amino acids of CTD_{SprB}, 16-bp intergenic region and 1178-bp region encoding 333 amino acids of SprF inserted into pYT179; Ap^r (Tc^r)	This study
pSK60	450-bp region encoding 149 amino acids of CTD_{SprB} inserted into pYT179; Ap^r (Tc^r)	This study

pSK61	3549-bp region encoding 1182 amino acids of $CTD_{SprB,}$ 16-bp intergenic region and 1178-bp region encoding 333 amino acids of SprF inserted into pYT179; Ap^r (Tc^r)	This study
pSK62	3549-bp region encoding 1182 amino acids of CTD_{SprB} inserted into pYT179; Ap^r (Tc^r)	(15)
pSK64	762-bp region encoding 228 amino acids of Fjoh_1123 inserted into pYT179; Ap^r (Tc^r)	This study
pSK68	1294-bp region encoding 333 amino acids of SprF inserted into pSK58; Ap^r (Tc^r)	This study
pSK69	1032-bp region encoding 303 amino acids of Fjoh_3951 inserted into pSK56; Ap^r (Tc^r)	This study
pSK78	3513-bp region encoding 1170 amino acids near the C-terminus of SprB but lacking the C-terminal 12 amino acids inserted into pYT179; Ap^r (Tc^r)	This study
pSK83	3513-bp region encoding 1170 amino acids near the C-terminus of SprB but lacking the C-terminal 12 amino acids, and 1178-bp region encoding 333 amino acids of SprF inserted into pYT179 inserted into pYT179; Ap^r (Tc^r)	This study
pSK93	300-bp region encoding 99 amino acids of CTD_{SprB} inserted into pYT179; Ap^r (Tc^r)	(15)
pSK98	1294-bp region encoding 333 amino acids SprF inserted into pCP11; Ap^r (Em^r)	This study
pTB263	Plasmid expressing fluorescent protein sfGFP; Ap^r	(31)
pYT40	511-bp fragment spanning the *remA* promoter, start codon, and the N-terminal signal peptide-encoding region inserted into pCP23; Ap^r (Tc^r)	(15)
pYT179	735-bp sfGFP amplified without stop codon and cloned into pYT140; Ap^r (Tc^r)	(15)

[a]Antibiotic resistance phenotypes are as follows: ampicillin, Ap^r; streptomycin, Sm^r; tetracycline, Tc^r. The antibiotic resistance phenotypes given in parentheses are those expressed in *F. johnsoniae* but not in *E. coli*. The antibiotic resistance phenotypes without parentheses are those expressed in *E. coli* but not in *F. johnsoniae*.

Table 2. Primers used in this study

954	5' GCTAGTCTAGAATGGCGAGGAATTACCTTCTGGTGA 3'; forward primer used in construction of pSK42, pSK44, pSK45, pSK51, pSK52, pSK55; XbaI site underlined
955	5' GCTAGGCATGCGGACATTTCGGCTTGTGTTAAATTCG 3'; reverse primer used in construction of pSK42, pSK59, pSK61, pSK68; SphI site underlined
1399	5' GCTAGTCTAGAACAGATACGAAAGATTATTACATCGAG 3'; forward primer used in construction of pSK41, pSK93; XbaI site underlined
1400	5' GCTAGGCATGCTTATCTGTATAAAGTGAAATGTCCAAC 3'; reverse primer used in construction of pSK50, pSK53, pSK54, pSK56, pSK60, pSK62, pSK93; SphI site underlined
1694	5' GCTAGTCTAGACCGGATCCAATTACATTTACAGCAG 3'; forward primer used in construction of pSK45, pSK50; XbaI site underlined
1695	5' GCTAGTCTAGACCAAATGGTGATGGAGTTAACG 3'; forward primer used in construction of pSK44; XbaI site underlined
1828	5' GCTAGTCTAGA ACAGCTTACGAAGTACCAGGATCTATG 3'; forward primer used in construction of pSK51, pSK53; XbaI site underlined
1829	5' GCTAGTCTAGAGCAGGTACAGAAATTAGACCGGCA 3'; forward primer used in construction of pSK52, pSK54; XbaI site underlined
1843	5' GCTAGTCTAGAGTGGTGATTACAATTGATCCAAGC 3'; forward primer used in construction of pSK55, pSK56; XbaI site underlined
1868	5' GCTAGTCTAGAGTCGAAGTGCCATCGATTACAGTA 3'; forward primer used in construction of pSK57; XbaI site underlined
1869	5' GCTAGGCATGCAACTGCTTTTTGTGCTATTGCGTT 3'; reverse primer used in construction of pSK58, pSK69; SphI site underlined
1879	5' GCTAGTCTAGAGGTGTTTGGAACGTAATTACAGCT 3'; forward primer used in construction of pSK59, pSK60; XbaI site underlined
1880	5' GCTAGTCTAGACGTTCTGAAATTACGCTTACTCCG 3'; forward primer used in construction of pSK61, pSK62, pSK78; XbaI site underlined
1881	5' GCTAGTCTAGATTCGTAAATGATCTGCCAACAGTA 3'; forward primer used in construction of pSK64; XbaI site underlined

1882 5' GCTAGGCATGCATAAATGTTTGAATGCCATCTCCT 3'; reverse primer used in construction of pSK64; SphI site underlined

1883 5' GCTAGGCATGCTGGCGAGGAATTACCTTCTGGTGA 3'; reverse primer used in construction of pSK68; SphI site underlined

1892 5' GCTAGGCATGCAGTCCAAATCAATAAAATGGCTTA 3'; reverse primer used in construction of pSK69; SphI site underlined

1935 5' GCTAGGCATGCTTAATCATTCTCGTCATTTAGTTTAAGAAC 3'; reverse primer used in construction of pSK78; SphI site underlined

1936 5' GCTAGGCATGCGCCCCTATGATGTTATCTAAAAAAATT 3'; reverse primer used in construction of pSK83; SphI site underlined

1937 5' GCTAGGCATGCTTAATCGTGAACCGGGCTTTG 3'; reverse primer used in construction of pSK83; SphI site underlined

2074 5' GCTAGGTCGACTGGCGAGGAATTACCTTCTGGTGA 3'; reverse primer used in construction of pSK98; SalI site underlined

2075 5' GCTAGTCTAGAGGACATTTCGGCTTGTGTTAAATTCG 3'; reverse primer used in construction of pSK98; SphI site underlined

Results

Efficient secretion of sfGFP depends on SprF. Many proteins secreted by T9SSs have type A CTDs, and the involvement of these in secretion has been demonstrated (10, 12, 14, 15, 32). Type B CTDs, such as the SprB CTD, have been less well studied. A truncated form of SprB lacking the C-terminal 34 AAs is not secreted (33), and fusion of C-terminal regions of SprB ranging from 99 to 1182 AA in length to the foreign protein sfGFP that also carried an N-terminal signal peptide (SP-sfGFP-CTD$_{SprB}$) failed to result in secretion of sfGFP from wild type cells (15). Here we examined the requirements for secretion of sfGFP in greater detail. One possibility for failure to secrete sfGFP is that a component of the secretion system was fully occupied with secretion of the large and abundant protein SprB. For this reason, we examined secretion of sfGFP by Δ*sprB* mutant cells. Expression of SP-sfGFP-CTD$_{SprB(218AA)}$ in Δ*sprB* cells resulted in a small amount of soluble secreted sfGFP, whereas expression of the same construct in wild type cells did not result in detectable secretion (Fig. 1A). *sprB* and *sprF* are cotranscribed, and SprF is required for secretion of SprB, but not for other proteins that are targeted to the T9SS (16). We previously suggested that SprF may play an adapter or chaperone-like function that facilitates secretion of SprB (16). The coexpression of SP-sfGFP-CTD$_{SprB}$ and SprF from the same plasmid resulted in efficient secretion of sfGFP from both wild type and Δ*sprB* mutant cells (Fig. 1B). Secretion did not occur in Δ*sprB* Δ*gldK* mutant cells carrying the same plasmid, indicating that a functional T9SS was required (Fig. 1B). The results suggest that SprF may interact with the C-terminal region of SprB and facilitate secretion. A shorter region of the C-terminus of SprB (149 AA) failed to result in sfGFP secretion even when coexpressed with SprF (Fig. 2).

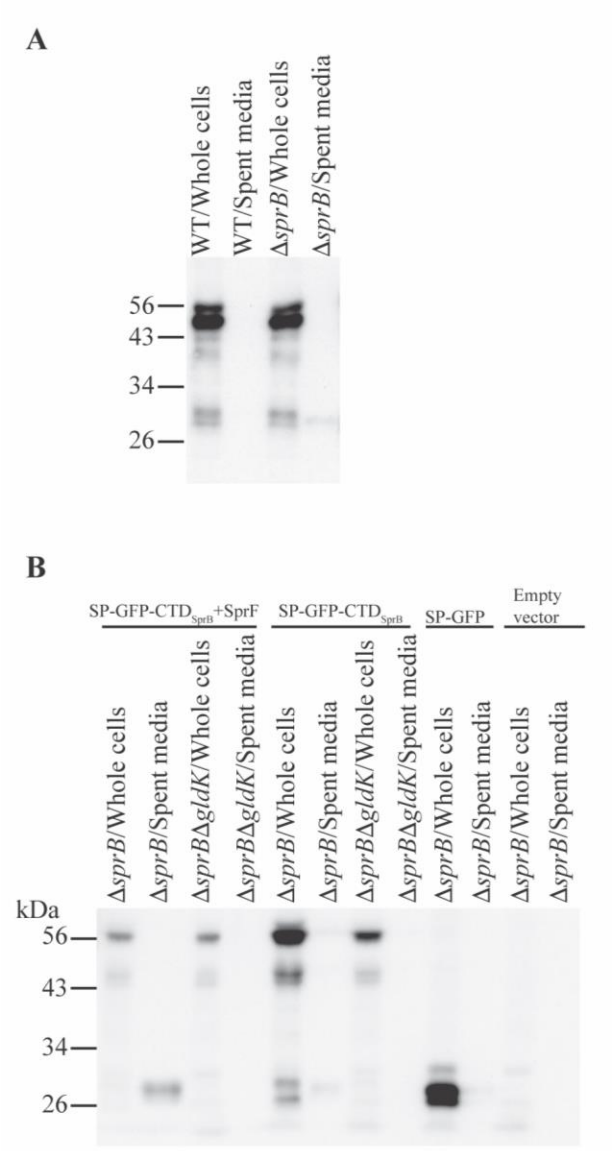

Fig. 1. Efficient secretion of sfGFP depends on CTD_{SprB} and SprF. (A) Wild-type and Δ*sprB* cells carrying pSK55, which expresses SP-sfGFP fused to the 218-amino acid CTD of SprB (SP-sfGFP-CTD_{SprB}) were analyzed. (B) Cultures of Δ*sprB* cells and of T9SS mutant Δ*sprB* Δ*gldK* were incubated in CYE at 25°C with shaking. 1ml samples were centrifuged at 22,000 x g for 15 min. The culture supernatant (spent medium) and intact cells were analyzed for sfGFP by western blot. Cells carried either pCP23 (Empty vector), pSK37, which expresses sfGFP with the N-terminal signal peptide from RemA (SP-sfGFP), pSK55, which expresses SP-sfGFP fused to the 218 amino acid CTD of SprB (SP-sfGFP-CTD_{SprB}) or pSK56, which expresses SP-sfGFP fused to the 218 amino acid CTD of SprB (SP-sfGFP-CTD_{SprB}) and SprF. For both panels, whole cell samples corresponded to 10 µg protein per lane and samples from spent media corresponded to the volume of spent medium that contained 10 µg protein before the cells were removed. Samples were separated by SDS-PAGE, and sfGFP was detected using anti-serum against GFP.

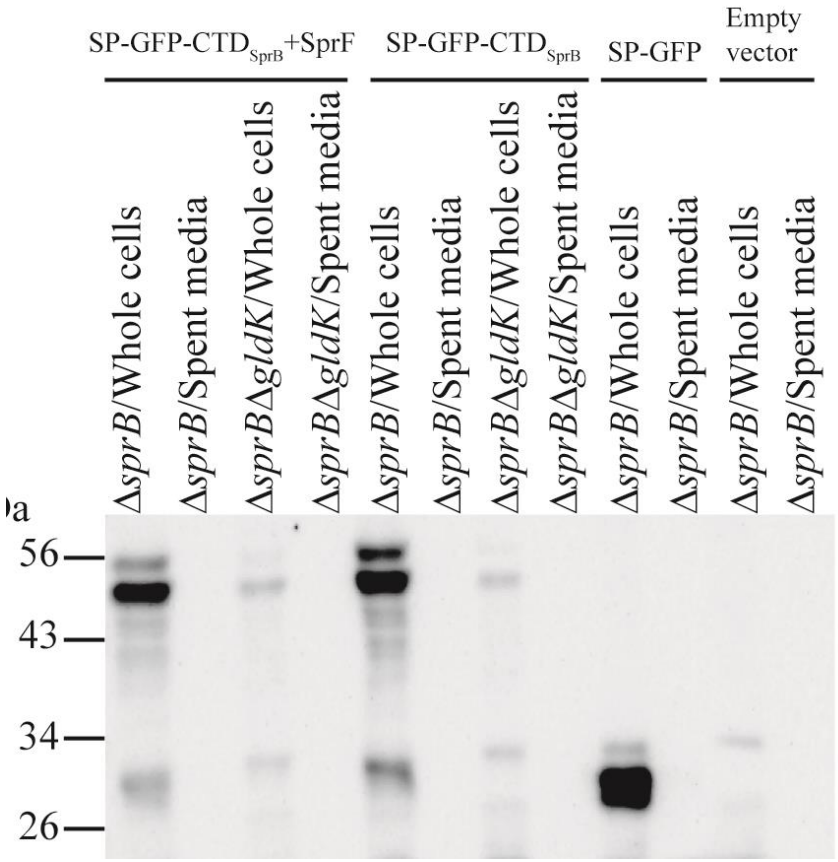

Fig. 2. Determination of the region of the CTD of SprB needed to allow secretion of sfGFP. The minimum region necessary for sfGFP secretion was determined by examining cells of Δ*sprB* and of T9SS mutant Δ*sprB* Δ*gldK*, carrying plasmids that expressed SP-sfGFP-CTD$_{SprB}$ with CTD region of 149 AA with SprF (pSK59) and without SprF (pSK60). Cell free spent media and whole cells were examined for sfGFP by SDS-PAGE followed by western blotting using anti-serum against sfGFP. Whole cell samples corresponded to 10 μg protein per lane and samples from spent media corresponded to the volume of spent medium that contained 10 μg protein before the cells were removed.

Longer regions of SprB$_{CTD}$ are needed for cell surface attachment. In wild type cells, SprB is attached to the cell surface after secretion by the T9SS. Regions of the C-terminus of SprB ranging from 368 to 1182 AAs were fused to sfGFP and examined for their ability to localize sfGFP to the cell surface. *sprF* was co-expressed on the same plasmids. Western immunoblot analyses of the strains expressing the fusion proteins show the accumulation of sfGFP in the spent medium when *sprF* was co-expressed (Fig. 3). The apparent molecular mass of the sfGFP band did not change when the length of the CTD fused to sfGFP was increased. This suggests processing of the fusion protein during or after secretion, with accumulation of stably folded sfGFP in the medium. *F. johnsoniae* produces many secreted proteases that could contribute to this partial digestion of the soluble secreted protein (34).

We examined if the cell-associated sfGFP was surface exposed using anti-GFP-coated polystyrene spheres. Spheres attached to cells that expressed sfGFP fused to CTDs of 663 and 1182 amino acids in length, but not to cells expressing sfGFP with smaller CTD regions (Table 3). This indicates that regions of SprB between 5315 and 5834 AA may be important for attachment to the cell surface. Cell surface proteins secreted by the *P. gingivalis* T9SS are modified by glycosylation and/or lipidation (32, 35, 36). This may also occur in *F. johnsoniae*, although it has not been well studied. Full length cell-associated SprB protein migrates as a ladder-like smear during PAGE (33) which could be the result of such modifications. Similar ladder-like banding patterns were observed in our studies. The extreme C-terminal region of SprB was explored in more detail. When the C-terminal 12 amino acids of SP-sfGFP-CTD$_{SprB218AA}$ were deleted sfGFP was not secreted indicating that the C-terminal 12 amino acids may be important for secretion (Fig. 4).

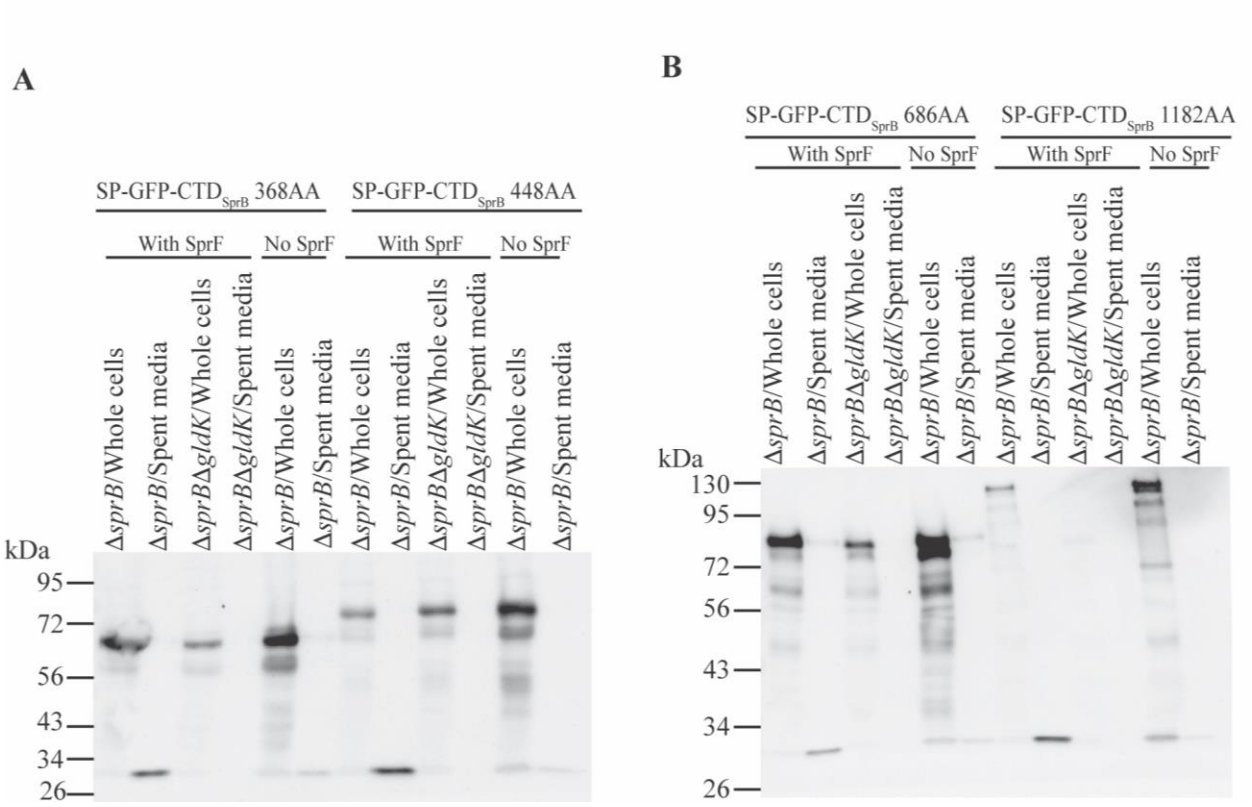

Fig. 3. Longer regions of CTD_{SprB} allow sfGFP secretion. Cultures of Δ*sprB* cells and of T9SS mutant Δ*sprB* Δ*gldK* were incubated in CYE at 25°C with shaking. 1 ml samples were centrifuged at 22,000 x g for 15 min. The culture supernatant (spent medium) and intact cells were analyzed for sfGFP by western blot. (A) Cells carried either pSK51, which expresses SP-sfGFP fused to the 368-amino acid CTD of SprB with SprF (SP-sfGFP-CTD_{SprB}) and SprF) or pSK53, which expresses 368-amino acid CTD of SprB without SprF (SP-sfGFP-CTD_{SprB}), and pSK52, which expresses SP-sfGFP fused to the 448-amino acid CTD of SprB with SprF (SP-sfGFP-CTD_{SprB}) and SprF) or pSK54, which expresses 448-amino acid CTD of SprB without SprF (SP-sfGFP-CTD_{SprB}). (B) Cells carried either pSK45, which expresses SP-sfGFP fused to the 663-amino acid CTD of SprB with SprF (SP-sfGFP-CTD_{SprB}) and SprF) or pSK50, which expresses 663-amino acid CTD of SprB without SprF (SP-sfGFP-CTD_{SprB}), and pSK61, which expresses SP-sfGFP fused to the 1182-amino acid CTD of SprB with SprF (SP-sfGFP-CTD_{SprB}) and SprF) or pSK62, which expresses 1182-amino acid CTD of SprB without SprF (SP-sfGFP-CTD_{SprB}). For all panels, whole cell samples corresponded to 10 μg protein per lane and samples from spent media corresponded to the volume of spent medium that contained 10 μg protein before the cells were removed.

Strains	Protein encoded by plasmid	Antibody used	Avg (SD) % of cells with spheres attached
CJ1922 (Δ*sprB*)	NA	No antibody	0.0 (0.0)
CJ1922 (Δ*sprB*)	NA	GFP	0.0 (0.0)
CJ1922 (Δ*sprB*)/pSK59	SP-sfGFP-CTD$_{SprB}$149 AA + SprF	GFP	0.0 (0.0)
CJ1922 (Δ*sprB*)/pSK56	SP-sfGFP-CTD$_{SprB}$218AA + SprF	GFP	0.0 (0.0)
CJ1922 (Δ*sprB*)/pSK51	SP-sfGFP-CTD$_{SprB}$368 AA + SprF	GFP	0.0 (0.0)
CJ1922 (Δ*sprB*)/pSK52	SP-sfGFP-CTD$_{SprB}$448 AA + SprF	GFP	0.0 (0.0)
CJ1922 (Δ*sprB*)/pSK45	SP-sfGFP-CTD$_{SprB}$663 AA + SprF	GFP	14.67 (1.15)
CJ1922 (Δ*sprB*)/pSK61	SP-sfGFP-CTD$_{SprB}$1182 AA + SprF	GFP	24.67 (2.52)
CJ1922 (Δ*sprB*)/pSK37	SP-sfGFP	GFP	0.0 (0.0)

Table 3. Longer regions of more than 448 AA are needed for cell surface attachment. Attachment of sfGFP on the cell surface was determined by examining cells carrying plasmids that expressed SP-sfGFP-CTD$_{SprB}$ and SprF with CTD regions of 218, 368, 448, 663, and 1182 amino acids. Anti-GFP antiserum and 0.5- μm-diameter protein G-coated polystyrene spheres were added to cells as described in the materials and methods. Samples were introduced into a tunnel slide, incubated for 3 minutes at 25°C, and examined using a phase-contrast microscope. Images were recorded for 30s, and 100 randomly selected cells were examined for the presence of spheres that remained attached to the cells, during this time. The numbers in the parenthesis are standard deviations calculated from three measurements.

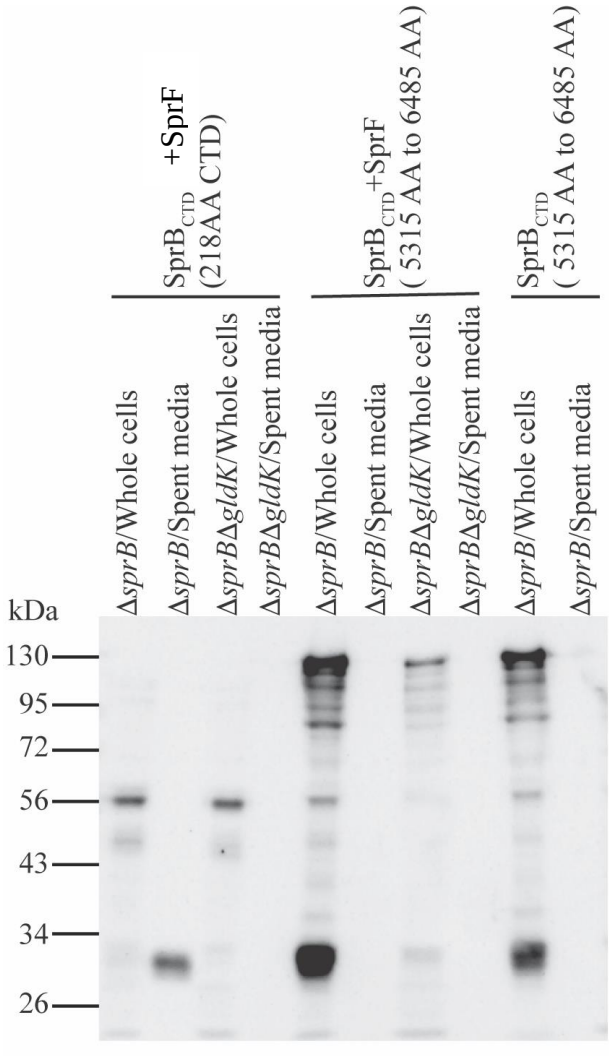

Fig. 4. The C-terminal 12 amino acids of SprB are critical for secretion. Cells carrying plasmids which express SP-sfGFP fused to amino acids 5315 to 6485 of SprB (lacking the C-terminal 12 amino acids) with SprF (pSK83) and without SprF (pSK78) were examined for sfGFP in intact cells and in cell-free spent media by Western blot analysis. Cultures of Δ*sprB* cells and of T9SS mutant Δ*sprB* Δ*gldK* carrying the plasmids were analyzed. Whole cell samples corresponded to 10 μg protein per lane and samples from spent media corresponded to the volume of spent medium that contained 10 μg protein before the cells were removed. sfGFP was detected using anti-serum against GFP.

SprF and the SprF-like protein Fjoh_3951 exhibit specificity for their cognate secreted proteins. Additional proteins with SprB-like CTDs were studied to determine if they behave as CTD_{SprB}. For this purpose, 228 amino acids of the type B CTD of Fjoh_3952 were fused to SP-sfGFP and the secretion was monitored with or without coexpression of its cognate *sprF*-like gene, Fjoh_3951. When SP-sfGFP-CTD_{Fjoh_3952} and Fjoh_3951 were coexpressed from the same plasmid, sfGFP accumulated in the spent medium of the Δ*sprB* cells but not in the spent medium of the Δ*sprB* Δ*gldK* cells. The SprF-like protein Fjoh_3951 was required for this secretion (Fig. 5A). To determine if SprF and the SprF-like protein Fjoh_3951 are interchangeable, we constructed a plasmid that expressed SP-sfGFP-CTD_{Fjoh_3952} and SprF, and another plasmid that expressed SP-sfGFP-CTD_{SprB} and Fjoh_3951. In both cases the cells failed to secrete sfGFP (Fig. 5B), suggesting that the cognate SprF-like proteins were required for secretion, and expression of the paralog could not satisfy that requirement.

Type B CTD with no SprF-like protein. Fjoh_1123 encodes a protein with a type B CTD that is expressed and appears to be secreted by the T9SS (10). Unlike most other *F. johnsoniae* genes encoding proteins with type B CTDs, Fjoh_1123 is not located near an *sprF*-like gene. For this reason, we suspected that secretion of Fjoh_1123 might not require an SprF-like protein. To test this, we constructed a plasmid that expressed SP-sfGFP fused to the C-terminal 238 amino acids of Fjoh_1123 (SP-sfGFP-CTD_{Fjoh_1123}). Western blot analysis demonstrated that sfGFP was not secreted by cells expressing this fusion protein (Fig. 6). This suggests the possibility that one of the 10 SprF-like proteins predicted to be encoded by the genome may facilitate secretion of Fjoh_1123. The 'orphan' *sprF*-like gene, Fjoh_1677, is a possible candidate, although any of them could possibly perform this function.

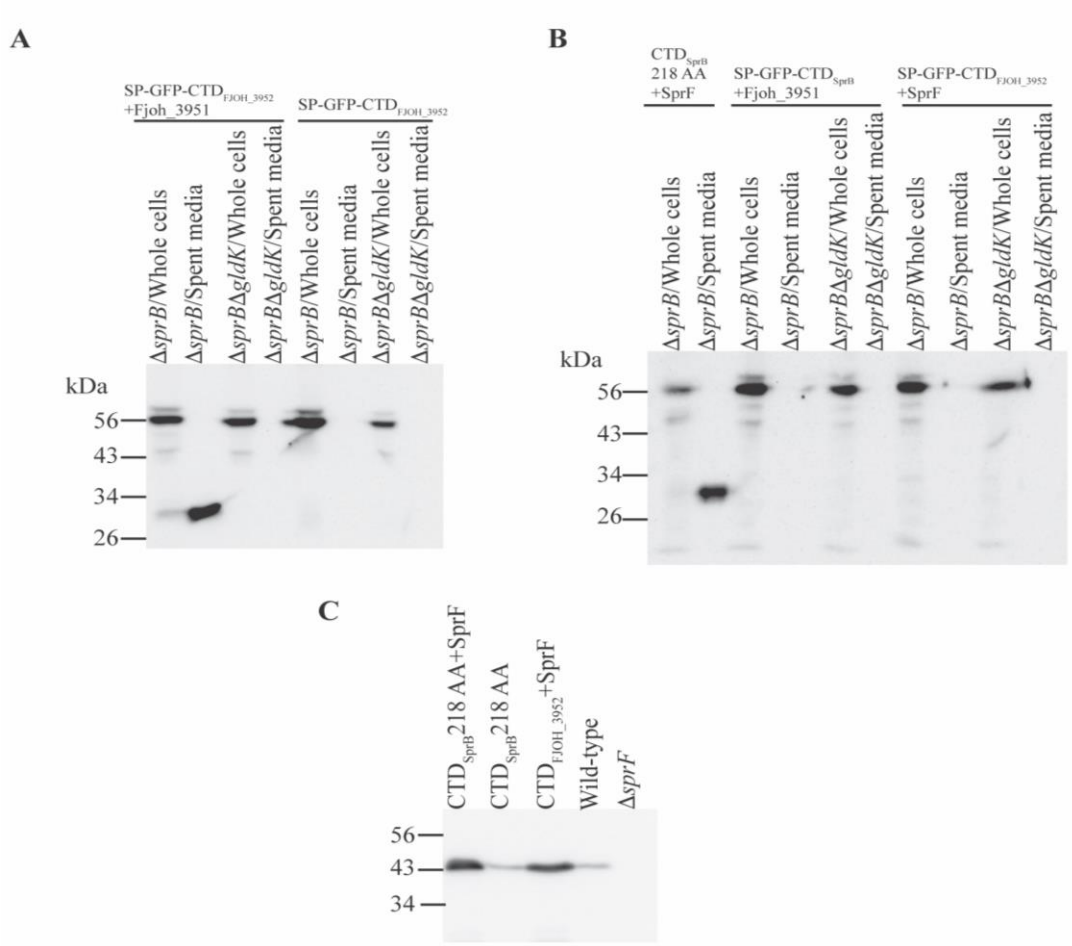

Fig. 5. Efficient secretion of SP-sfGFP fused to CTD_{SprB} or to CTD_{Fjoh_3952} requires coexpression with the cognate SprF-like protein (A) Fusion of the type B CTD encoded by Fjoh_3952 to SP-sfGFP and coexpression with the *sprF*-like gene Fjoh_3951 results in T9SS-mediated secretion. Cells of Δ*sprB* and of T9SS mutant Δ*sprB* Δ*gldK* carrying either pSK58 expressing SP-sfGFP-$CTD_{Fjoh\_3952(228\ AA)}$, or pSK57 expressing both SP-sfGFP-$CTD_{Fjoh\_3952(228\ AA)}$ and the SprF-like protein encoded by Fjoh_3951 were examined. Whole cells and cell-free spent media were examined for sfGFP by SDS-PAGE and western blotting with anti-GFP antiserum. Whole cell samples corresponded to 10 μg protein per lane and samples from spent media corresponded to the volume of spent medium that contained 10 μg protein before the cells were removed. (B) Secretion of SP-sf-GFP was examined as described above except that cells carried either pSK69, which expresses both SP-sfGFP-CTD_{SprB} and the SprF-like protein encoded by Fjoh_3951, or pSK68 which expresses both SP-sfGFP-CTD_{Fjoh_3952} and SprF. Control cells that secreted sfGFP carried pSK56 expressing SP-sfGFP-CTD_{SprB} and SprF. (C) SprF levels were examined in wild-type and Δ*sprF* mutant cells, and in cells of the Δ*sprB* mutant carrying either pSK56 expressing SP-sfGFP-$CTD_{SprB(218\ AA)}$ and SprF, pSK55 expressing SP-sfGFP-CTD_{SprB}, or pSK68 expressing SP-sfGFP-CTD_{Fjoh_3952} and SprF. Equal amount (10 μg protein) of each sample were loaded in each lane and western blot analysis was performed using anti-SprF antibodies.

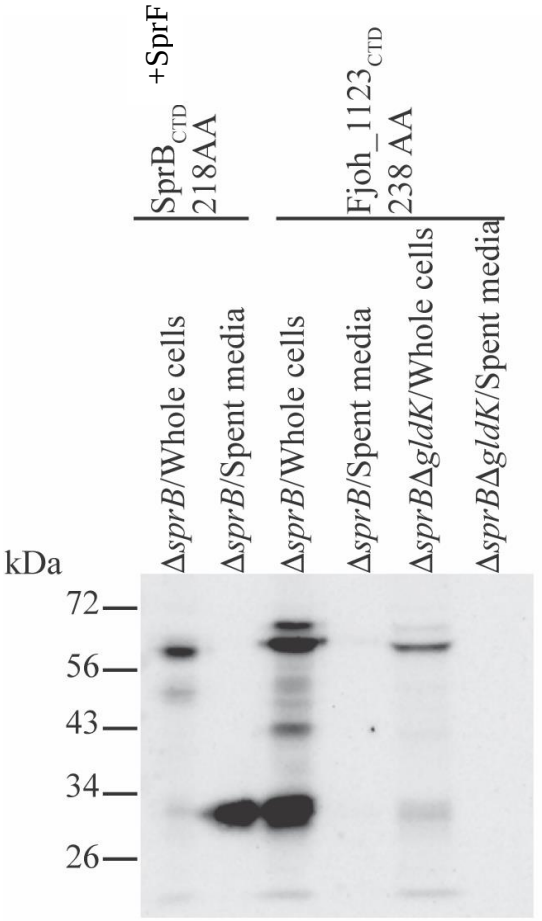

Fig. 6. A lone type B CTD by itself does not support sfGFP secretion. To determine of Fjoh_1123 CTD can target SP-sfGFP for secretion, cells of Δ*sprB* and of T9SS mutant Δ*sprB* Δ*gldK*, carrying plasmids that expressed SP-sfGFP fused to 238 AA of Fjoh_1123 (pSK64) were analyzed. The culture supernatant (spent medium) and intact cells were analyzed for sfGFP by western blot using anti-GFP antiserum. Whole cell samples corresponded to 10 μg protein per lane and samples from spent media corresponded to the volume of spent medium that contained 10 μg protein before the cells were removed.

SprF outer membrane localization. SprF is needed for SprB localization to the cell surface. *sprB, sprC, sprD* and *sprF* are part of an operon (16). SprF might be an adaptor or chaperone that interacts with SprB to aid in its secretion to the cell surface. To characterize SprF further, the protein was localized. SprF was not detected in the cell free spent medium or on the surface of intact cells using latex spheres coated with anti-SprF. SprF was not susceptible to proteinase K in the wild-type cells but was partially digested in Δ*sprB* cells (Fig. 7). This suggests that it may be protected from extracellular proteases by SprB in wild-type cells. Further studies need to be done to understand the interactions between CTD_{SprB} and SprF.

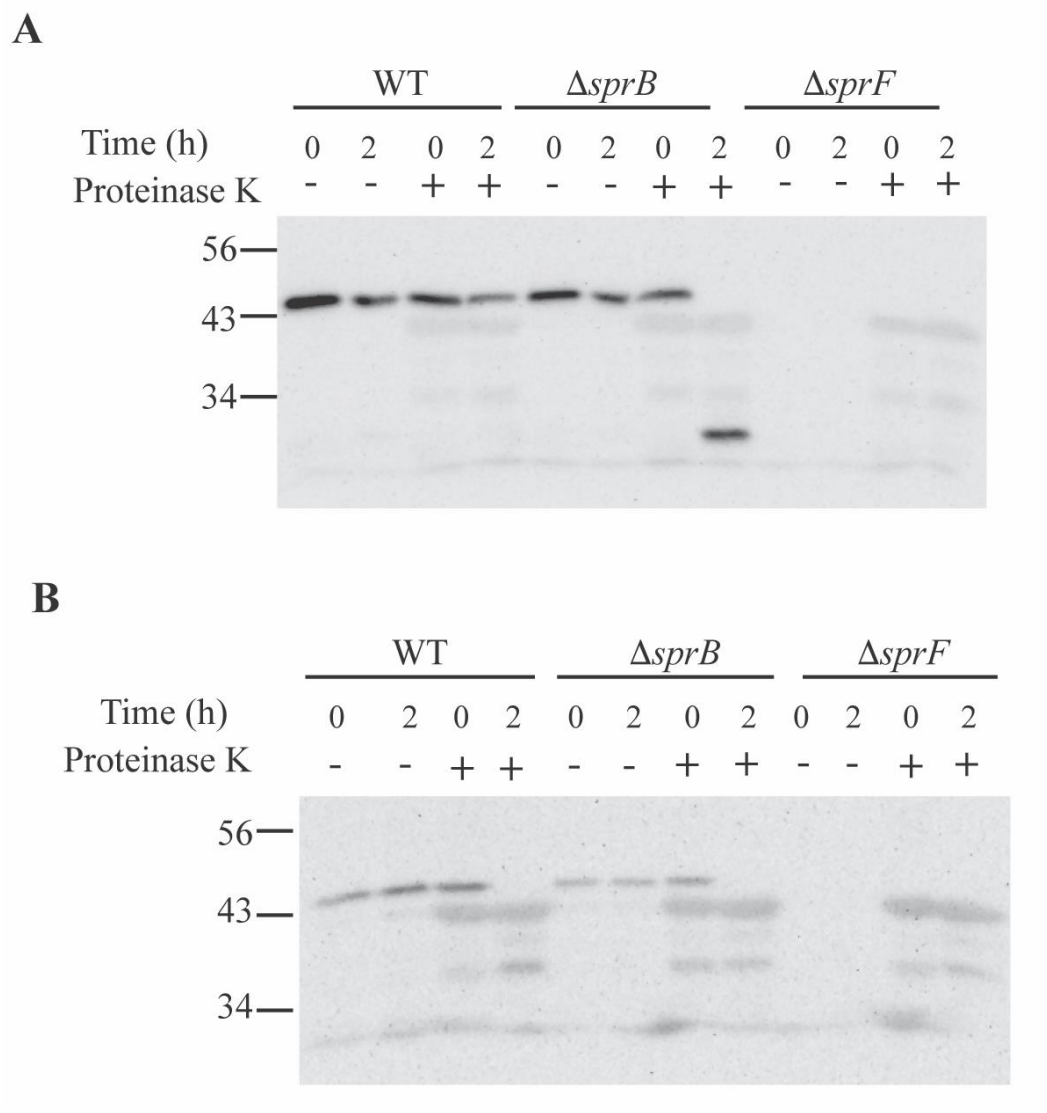

Fig. 7. Proteinase K treatment to determine if SprF localizes to the cell surface. Wild-type, Δ*sprB* and Δ*sprF* strains were analyzed. Proteinase K was added at a final concentration of 1 mg/ml to intact cells (A) and to cells extracts prepared by French pressure cell treatment (B), and cells and extracts were incubated at 25ºC. Samples were removed at 0 h and 2 h for immunoblot analyses. Samples were separated by SDS-PAGE and SprF was detected using antiserum against SprF. Samples not exposed to proteinase K (-) were also included.

Discussion

T9SSs are prevalent in members of the phylum *Bacteroidetes*. Proteins secreted by T9SSs have N-terminal SPs, and rely on the Sec system for export across the cytoplasmic membrane (2, 5, 10). They also have conserved CTDs that target them for secretion across the outer membrane by the T9SS (5, 8, 10, 32, 33). Most T9SS CTDs belong to one of two protein domain families TIGR04183 (type A CTDs) and TIGR04131 (type B CTDs) (10). The features of type A CTDs have been functionally studied in *F. johnsonia*e and *P. gingivialis* (12, 14, 15). Type A CTDs typically extend less than 100 AAs from the C-terminus. Truncated proteins lacking their type A CTD are not secreted and instead accumulate in the cell, presumably in the periplasm (14). In addition, fusion of type A CTDs to foreign proteins such as GFP results in secretion across the outer membrane (12). CTDs are typically cleaved during or after secretion. PorU is the predicted peptidase that cleaves the CTD. Mutation of *porU* results in secretion of *P. gingivalis* proteins such as RgpB to the cell surface but without CTD removal (37). Deletion of core T9SS genes eliminates secretion of proteins carrying type A CTDs (8, 10, 15), indicating that the T9SS is required for this secretion. Unlike type-A CTDs, the potential roles and features of type B CTDs have not been studied. The results presented here demonstrate that type B CTDs can target a foreign protein for secretion by the T9SS. They also demonstrate the requirement for the appropriate cognate SprF-like protein for efficient secretion.

Type B CTDs are not similar in sequence to type A CTDs (15, 38). They also appear to differ functionally from type A CTDs. Type B CTDs of more than 149 amino acids were required to target sfGFP for secretion, and coexpression with cognate SprF-like proteins was required for efficient secretion. SprF was already known to be required for secretion of the type B CTD-

containing protein SprB (16). In the current study we demonstrate that wild type levels of SprF were not sufficient to facilitate efficient secretion of SP-sfGFP-CTD$_{SprB}$ expressed from a plasmid. Efficient secretion only occurred when SP-sfGFP-CTD$_{SprB}$ and SprF were coexpressed from the same plasmid. *F. johnsoniae* encodes nine SprF-like proteins in addition to SprF. One of these, encoded by Fjoh_3951, was shown to be required for efficient secretion of sfGFP fused to the type B CTD of the protein encoded by the upstream gene, Fjoh_3952. SprF and Fjoh_3951 were not interchangeable. Expression of SprF did not facilitate secretion of SP-sfGFP-CTD$_{Fjoh_3952}$, and expression of Fjoh_3951 did not facilitate secretion of SP-sfGFP-CTD$_{SprB}$. It appears that proteins carrying each of these CTDs require coexpression of their appropriate cognate SprF-like proteins for secretion.

SprF is predicted to be an outer membrane protein, and it may remain associated with SprB on the cell surface. This is suggested by the observation that SprF in wild type cells, but not in Δ*sprB* cells, was protected from proteinase K digestion. The exact role of SprF and other SprF-like proteins in secretion is not known. The apparent requirement for coexpression suggests that SprF may function as a chaperone for SprB. These proteins may travel to the T9SS and the cell surface together, and may remain associated with each other in their mature forms.

P. gingivalis has a single SprF-like protein, PorP. *porP* lies immediately upstream of the core T9SS genes, *porK*, *porL*, *porM*, and *porN* (39), which are orthologs of *F. johnsoniae gldK*, *gldL*, *gldM*, and *gldN* respectively. Deletion of *P. gingivalis porP* results in lack of secretion of proteins targeted to the T9SS, many of which have type A CTDs (4). *P. gingivalis* encodes a single protein with a type B CTD, and this protein has not yet been studied. The presence of a single type

B CTD-containing protein may explain why *P. gingivalis* has only one *sprF*-like gene (*porP*) whereas *F. johnsoniae* has ten.

The *F. johnsoniae* ortholog of *porP* has not been identified, but it is not adjacent to *gldK*. It is possible that one of the *sprF*-like genes, perhaps the 'orphan' *sprf*-like gene (Fjoh_1677), performs this role and is essential for secretion of proteins with type A CTDs. It is also possible that the SprF-like proteins exhibit redundancy for secretion of type A CTD containing proteins, or that an *sprF*-like gene is not needed for secretion of these proteins in *F. johnsoniae*. Additional experiments are needed to address these questions. We do not know why SprB and other proteins with type B CTDs require specific SprF-like proteins for their secretion. Many of the proteins with type B CTDs are very large. SprB for example is about 669 kDa in size. Perhaps efficient secretion of such large proteins drove the evolution of specific SprF-like proteins that are best adapted to assist in this process. It should be noted however that SprF was also needed for the secretion of SP-sfGFP-CTD$_{SprB}$, which is much smaller than SprB.

While the C-terminal 218 AA region of SprB facilitated secretion of SP-sfGFP-CTD$_{SprB}$, it did not result in cell-surface localization of the protein. Rather, sfGFP was released in soluble form. However, longer regions near the C-terminus of SprB (663 and 1182 amino acids) did result in attachment to the cell surface. We do not know how SprB interacts with the cell surface, although SprF and other outer membrane motility proteins (SprC, SprD) are candidates that may interact with SprB to facilitate this interaction. C-terminal deletions and site-directed mutagenesis of the *P. gingivalis* RgpB protein indicated that the terminal 13 amino acid residues are important for proper processing and glycosylation of the protein (13). In our studies, deletion of the C-terminal 12 amino acids abolished sfGFP secretion and cell surface localization. This study

highlights the roles of type B CTDs and SprF-like proteins in T9SS-mediated secretion. Additional studies are required to understand the interactions between the type B CTDs and SprF-like proteins to fully understand the mechanisms by which proteins are targeted to the T9SS.

References:

1. Filloux A. 2010. Secretion signal and protein targeting in bacteria: a biological puzzle. J Bacteriol 192:3847-9.
2. Chagnot C, Zorgani MA, Astruc T, Desvaux M. 2013. Proteinaceous determinants of surface colonization in bacteria: bacterial adhesion and biofilm formation from a protein secretion perspective. Front Microbiol 4:303.
3. McBride M, and Zhu Y. 2013. Gliding motility and Por secretion system genes are widespread among members of the phylum *Bacteroidetes*. J Bacteriol 195:170-8.
4. Sato K, Naito M, Yukitake H, Hirakawa H, Shoji M, McBride MJ, Rhodes RG, Nakayama K. 2010. A protein secretion system linked to bacteroidete gliding motility and pathogenesis. Proc Natl Acad Sci U S A 107:276-81.
5. Sato K, Yukitake H, Narita Y, Shoji M, Naito M, Nakayama K. 2013. Identification of *Porphyromonas gingivalis* proteins secreted by the Por secretion system. FEMS Microbiol Lett 338:68-76.
6. Braun TF, Khubbar MK, Saffarini DA, McBride MJ. 2005. *Flavobacterium johnsoniae* gliding motility genes identified by mariner mutagenesis. J Bacteriol 187:6943-52.
7. Nelson SS, Glocka PP, Agarwal S, Grimm DP, McBride MJ. 2007. *Flavobacterium johnsoniae* SprA is a cell surface protein involved in gliding motility. J Bacteriol 189:7145-50.
8. Shrivastava A JJ, Barren JM, McBride MJ. 2012. *Flavobacterium johnsoniae* GldK, GldL, GldM, and SprA are required for secretion of the cell suraface gliding motility adhesins SprB and RemA. J Bacteriol 195:3201-12.
9. Rhodes RG, Samarasam MN, Van Groll EJ, McBride MJ. 2011. Mutations in *Flavobacterium johnsoniae sprE* result in defects in gliding motility and protein secretion. J Bacteriol 193:5322-7.
10. Kharade SS, McBride MJ. 2015. *Flavobacterium johnsoniae* PorV is required for secretion of a subset of proteins targeted to the type IX secretion system. J Bacteriol 197:147-58.
11. Slakeski N, Seers CA, Ng K, Moore C, Cleal SM, Veith PD, Lo AW, Reynolds EC. 2011. C-terminal domain residues important for secretion and attachment of RgpB in *Porphyromonas gingivalis*. J Bacteriol 193:132-42.
12. Shoji M, Sato K, Yukitake H, Kondo Y, Narita Y, Kadowaki T, Naito M, Nakayama K. 2011. Por secretion system-dependent secretion and glycosylation of *Porphyromonas gingivalis* hemin-binding protein 35. PLoS One 6:e21372.
13. Nguyen KA, Travis J, Potempa J. 2007. Does the importance of the C-terminal residues in the maturation of RgpB from *Porphyromonas gingivalis* reveal a novel mechanism for protein export in a subgroup of Gram-Negative bacteria? J Bacteriol 189:833-43.
14. Seers CA, Slakeski N, Veith PD, Nikolof T, Chen YY, Dashper SG, Reynolds EC. 2006. The RgpB C-terminal domain has a role in attachment of RgpB to the outer membrane and belongs to a novel C-terminal-domain family found in *Porphyromonas gingivalis*. J Bacteriol 188:6376-86.
15. Kulkarni SS, Zhu Y, Brendel CJ, McBride MJ. 2017. Diverse C-terminal sequences involved in *Flavobacterium johnsoniae* protein secretion. J Bacteriol (in press; https://doi.org/10.1128/JB.00884-16).

16. Rhodes RG, Nelson SS, Pochiraju S, McBride MJ. 2011. *Flavobacterium johnsoniae* sprB is part of an operon spanning the additional gliding motility genes sprC, sprD, and sprF. J Bacteriol 193:599-610.
17. Chang LYE, Pate JL, Betzig RJ. 1984. Isolation and characterization of nonspreading mutants of the gliding bacterium *Cytophaga johnsonae*. J Bacteriol 159:26-35.
18. McBride MJ, Braun TF. 2004. GldI is a lipoprotein that is required for *Flavobacterium johnsoniae* gliding motility and chitin utilization. J Bacteriol 186:2295-2302.
19. McBride MJ, Xie G, Martens EC, Lapidus A, Henrissat B, Rhodes RG, Goltsman E, Wang W, Xu J, Hunnicutt DW, Staroscik AM, Hoover TR, Cheng YQ, Stein JL. 2009. Novel features of the polysaccharide-digesting gliding bacterium *Flavobacterium johnsoniae* as revealed by genome sequence analysis. Appl Environ Microbiol 75:6864-6875.
20. McBride MJ, Kempf MJ. 1996. Development of techniques for the genetic manipulation of the gliding bacterium *Cytophaga johnsonae*. J Bacteriol 178:583-590.
21. Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
22. Shrivastava A, Rhodes RG, Pochiraju S, Nakane D, McBride MJ. 2012. *Flavobacterium johnsoniae* RemA is a mobile cell surface lectin involved in gliding. J Bacteriol 194:3678-88.
23. Rhodes RG, Samarasam MN, Shrivastava A, van Baaren JM, Pochiraju S, Bollampalli S, McBride MJ. 2010. *Flavobacterium johnsoniae gldN* and *gldO* are partially redundant genes required for gliding motility and surface localization of SprB. J Bacteriol 192:1201-11.
24. Bolivar F, Backman K. 1979. Plasmids of *Escherichia coli* as cloning vectors. Methods Enzymol 68:245-267.
25. Figurski DH, Helinski DR. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci USA 76:1648-1652.
26. Rhodes RG, Pucker HG, McBride MJ. 2011. Development and use of a gene deletion strategy for *Flavobacterium johnsoniae* to identify the redundant motility genes *remF*, *remG*, *remH*, and *remI*. J Bacteriol 193:2418-2428.
27. Shrivastava A, Johnston JJ, van Baaren JM, McBride MJ. 2013. *Flavobacterium johnsoniae* GldK, GldL, GldM, and SprA are required for secretion of the cell-surface gliding motility adhesins SprB and RemA. Journal of Bacteriology 195:3201-3212.
28. Zhu Y, Thomas F, Larocque R, Li N, Duffieux D, Cladiere L, Souchaud F, Michel G, McBride MJ. 2017. Genetic analyses unravel the crucial role of a horizontally acquired alginate lyase for brown algal biomass degradation by *Zobellia galactanivorans*. Environ Microbiol doi:10.1111/1462-2920.13699.
29. McBride MJ BS. 1996. Development of techniques to genetically manipulate members of the genera Cytophaga, Flavobacterium, Flexibacter, and Sporocytophaga. Appl Environ Microbiol 62:3017-22.
30. Agarwal SD HD, McBride MJ. 1997. Cloning and characterization of the *Flavobacterium johnsoniae* (*Cytophaga johnsonae*) gliding motility gene, *gldA*. Proc Natl Acad Sci U S A 94:12139-12144.

31. Uehara T, Dinh T, Bernhardt TG. 2009. LytM-domain factors are required for daughter cell separation and rapid ampicillin-induced lysis in *Escherichia coli*. Journal of Bacteriology 191:5094-5107.
32. Veith PD, Nor Muhammad NA, Dashper SG, Likic VA, Gorasia DG, Chen D, Byrne SJ, Catmull DV, Reynolds EC. 2013. Protein substrates of a novel secretion system are numerous in the *Bacteroidetes* phylum and have in common a cleavable C-terminal secretion signal, extensive post-translational modification, and cell-surface attachment. J Proteome Res 12:4449-61.
33. Nelson SS, Bollampalli S, McBride MJ. 2008. SprB is a cell surface component of the *Flavobacterium johnsoniae* gliding motility machinery. J Bacteriol 190:2851-7.
34. McBride MJ, Xie G, Martens EC, Lapidus A, Henrissat B, Rhodes RG, Goltsman E, Wang W, Xu J, Hunnicutt DW, Staroscik AM, Hoover TR, Cheng YQ, Stein JL. 2009. Novel features of the polysaccharide-digesting gliding bacterium *Flavobacterium johnsoniae* as revealed by genome sequence analysis. Appl Environ Microbiol 75:6864-75.
35. Shoji M RD, Shi Y, Kadowaki T, Yamamoto K, Yoshimura F, Akamine A, Curtis M, Nakayama K. 2002. Construction and characterization of a nonpigmented mutant of *Porphyromonas gingivalis*: Cell surface polysaccharide as an anchorage for gingipanis. Microbiology 148:1183-1191.
36. Rangarajan M, Aduse-Opoku J, Paramonov N, Hashim A, Bostanci N, Fraser OP, Tarelli E, Curtis MA. 2008. Identification of a second lipopolysaccharide in *Porphyromonas gingivalis* W50. J Bacteriol 190:2920-32.
37. Glew MD, Veith PD, Peng B, Chen YY, Gorasia DG, Yang Q, Slakeski N, Chen D, Moore C, Crawford S, Reynolds EC. 2012. PG0026 is the C-terminal signal peptidase of a novel secretion system of *Porphyromonas gingivalis*. J Biol Chem 287:24605-17.
38. Kharade SS MM. 2014. The *Flavobacterium johnsoniae* chitinae ChiA is required for chitin utilization and is secreted by the type IX secretion system. J Bacteriol 196:961-970.
39. Vincent MS, Canestrari MJ, Leone P, Stathopulos J, Ize B, Zoued A, Cambillau C, Kellenberger C, Roussel A, Cascales E. 2017. Characterization of the *Porphyromonas gingivalis* Type IX Secretion Trans-envelope PorKLMNP Core Complex. J Biol Chem 292:3252-3261.

Chapter 4. Summary

Flavobacterium johnsoniae is a gliding bacterium that belongs to the phylum *Bacteroidetes*. It has a novel protein secretion system called the type IX secretion system (T9SS), that secretes cell surface adhesins SprB and RemA to the cell surface and secretes extra-cellular enzymes such as the chitinase ChiA and the amylase AmyB to the extra-cellular milieu. These proteins secreted by the T9SS have N-terminal signal peptides for export across the cytoplasmic membrane into the periplasmic space. Based on the results in this thesis, a model for protein targeting to the T9SS is hypothesized (Fig. 1). The T9SS secreted proteins have conserved Carboxy-terminal domains (CTDs) that appear to target them to the T9SS. The CTDs in *F. johnsoniae* belong to at least two distinct protein domain families. This thesis focused on understanding the diversity of these CTDs, the features needed for secretion and cell surface attachment, and the interaction of CTDs with some of the components of the T9SS. Chapter 2 explored the features of type-A CTDs which belong to TIGR04183. Three *F. johnsoniae* proteins were studied in chapter 2: cell surface adhesin RemA, and extra-cellular enzymes ChiA and AmyB. About 80 to 100 AA of the CTDs of these proteins were sufficient and necessary to target the heterologous protein sfGFP to the T9SS for secretion. It was also found that secretion is growth-phase dependent with substantial secretion seen in stationary phase cells and little secretion observed for cells in the exponential phase of growth. Chapter 3 focused on the CTD of SprB, which belongs to TIGR04131. It appears that regions longer regions than 149 AAs of SprB CTD are needed to target sfGFP for secretion. In addition, coexpresion with SprF was needed for secretion of the fusion protein. *sprF* and *sprB* are a part of an operon, and SprF is required for secretion of SprB but not for secretion of other proteins targeted to the T9SS. Additionally, while

the C-terminal 218 AA of SprB facilitated secretion of sfGFP, longer regions (greater than 448 AAs) were needed for cell surface localization of sfGFP. These requirements for targeting proteins for secretion and surface localization in *F. johnsoniae* are likely to have broad significance given the prevalence of T9SSs, and the large number of proteins secreted by these systems, in members of the large and diverse phylum *Bacteroidetes*.

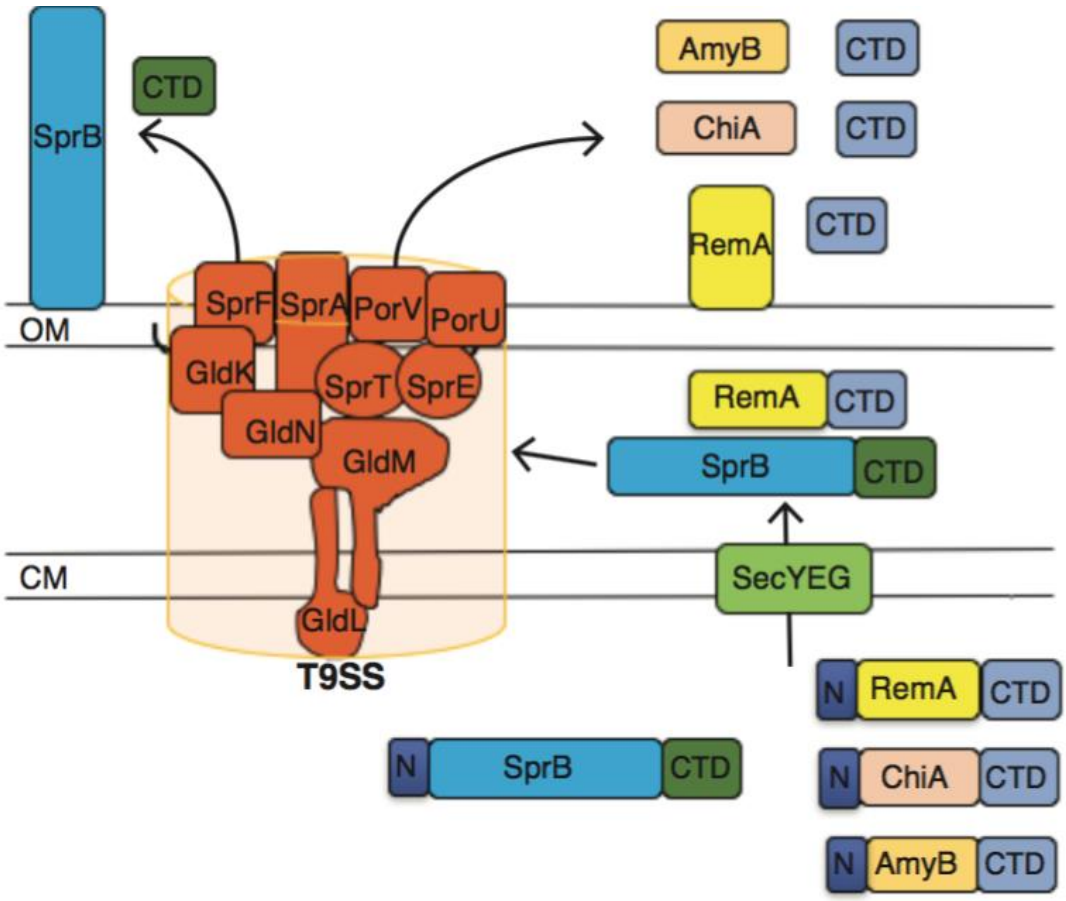

Fig. 1. Members of the genus *Flavobacterium*, and many related bacteria, secrete proteins across the outer membrane using the type IX secretion system (T9SS core proteins in orange). Proteins secreted by T9SSs have amino-terminal signal peptides (N) for export across the cytoplasmic membrane by the Sec system, and carboxy-terminal domains (CTDs) targeting them for secretion across the outer membrane by the T9SS. Most T9SS CTDs belong to either family TIGR04183 (type A CTDs; blue) or TIGR04131 (type B CTDs; dark green). The CTDs are cleaved off during or after secretion of the effector proteins.

Appendix 1. *F. johnsoniae* ChiA$_{CTD}$ is recognized as a targeting signal by the *Flavobacterium columnare* T9SS

To test if the *F. johnsoniae* ChiA T9SS CTD is recognized as a 'targeting signal' by the T9SSs of another member of the phylum *Bacteroidetes*, the plasmid pSSK52 expressing the fusion protein SP_{ChiA}-mCherry-CTD_{ChiA} was introduced into *Flavobacterium columnare* wild-type and *gldN* mutant cells. pSSK52 expresses mCherry that has an N-terminal signal peptide to allow export across the cytoplasmic membrane by the Sec system, and is fused to the C-terminal 105 amino acids of *F. johnsoniae* ChiA (1). *F. columnare* is a common fish pathogen that causes columnaris disease and is a major issue faced by aquaculture facilities worldwide (2). *F. columnare* genomovar 2 strain C#2 (3, 4) was the wild-type strain used in this study. *F. columnare* strains were grown in Shieh medium (5) at 30°C. Tetracycline was used at a concentration of 10 μg/ml. To detect secretion of recombinant mCherry, *F. columnare* cells were grown overnight in Shieh medium at 30°C with shaking. Cells were pelleted by centrifugation at 22,000 x g for 15 min at 4°C, and the culture supernatant (spent medium) was separated. Supernatant was ultracentrifuged at 352,900 x g for 30 mins at 4°C. For whole-cell samples, the cells were suspended in the original culture volume of phosphate-buffered saline consisting of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, and 2 mM KH_2PO_4 (pH 7.4). Equal amounts of spent media and whole cells were boiled in SDS-PAGE loading buffer for 10 minutes. Proteins were separated by SDS-PAGE, and Western blot analyses were performed as previously described (6). Equal amounts of each sample based on the starting material were loaded in each lane. For cell extracts this corresponded to 10 μg protein, whereas for spent medium this corresponded to the equivalent volume of spent medium that contained 10 μg cell protein before the cells were removed. Commercially available antibodies against mCherry (0.5 mg per ml; BioVision Incorporated, Milpitas, CA) were used at dilution of

1:5,000 to detect mCherry. The CTD of *F. johnsoniae* ChiA functioned in *F. columnare* strain C#2, as demonstrated by the accumulation of mCherry in the spent culture fluid of wild type cells but not of the *gldN* mutant (Fig. 1). In contrast, mCherry accumulated in whole cells of the *gldN* mutant. Together the results demonstrate that the CTD signal from *F. johnsoniae* was recognized by the T9SS of another member of the phylum *Bacteroidetes*, *F. columnare*. This was especially impressive because CTD_{ChiA} is not similar to members of the type A or type B families of CTDs, and because *F. columnare* does not appear to produce any proteins with CTDs similar in sequence to $ChiA_{CTD}$. The results also indicated that the *F. columnare* T9SS component GldN was needed for secretion of proteins targeted to the T9SS.

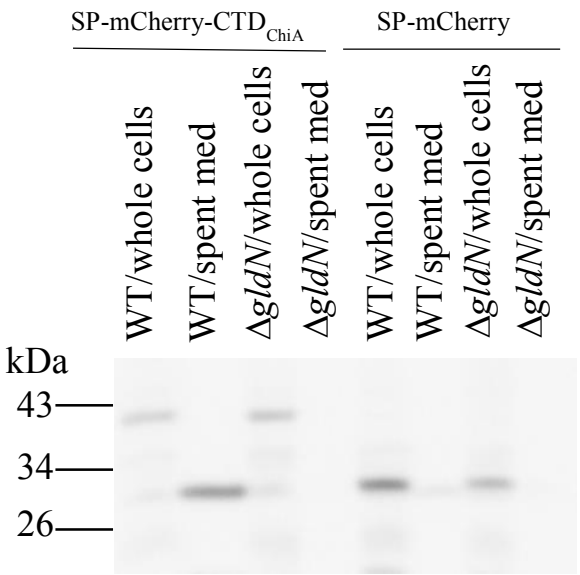

Fig. 1. Cultures of Wild-type (WT) cells and of T9SS mutant Δ*gldN* were incubated in Shieh medium at 30°C with shaking. 1 ml samples were centrifuged at 22,000 x g for 15 min and the supernatant was ultracentrifuged at 352,900 x g for 30 min at 4°C. The culture supernatant (spent medium) and intact cells were analyzed for mCherry by western blot. Cells carried either pSSK52, which expresses mCherry with the N-terminal signal peptide from ChiA fused to the 105-amino acid CTD of ChiA (SP-mCherry-CTD_{ChiA}) or pSSK54, which expresses SP-mCherry (no CTD control). Samples were separated by SDS-PAGE, and sfGFP was detected using anti-serum against mCherry.

References:

1. Kharade SS MM. 2014. The *Flavobacterium johnsoniae chitinae* ChiA is required for chitin utilization and is secreted by the type IX secretion system. J Bacteriol 196:961-970.
2. Declercq AM HF, Van den Broeck W, Bossier P, Decostere A. 2013. Columnaris disease in fish: a review with emphasis on bacterium-host interactions. Vet Res 44.
3. Bartelme RP, Newton RJ, Zhu Y, Li N, LaFrentz BR, McBride MJ. 2016. Complete Genome Sequence of the Fish Pathogen *Flavobacterium columnare* Strain C#2. Genome Announc 4.
4. Thomas-Jinu S, Goodwin AE. 2004. Morphological and genetic characteristics of *Flavobacterium columnare* isolates: correlations with virulence in fish. Journal of Fish Diseases 27:29-35.
5. Decostere A HF, Devriese LA. 1997. Shieh medium supplemented with tobramycin for selective isolation of *Flavobacterium columnare* (*Flexibacter columnaris*) from diseased fish. . J Clin Microbiol 35:322-324.
6. Rhodes RG, Samarasam MN, Shrivastava A, van Baaren JM, Pochiraju S, Bollampalli S, McBride MJ. 2010. *Flavobacterium johnsoniae gldN* and *gldO* are partially redundant genes required for gliding motility and surface localization of SprB. J Bacteriol 192:1201-11.

Appendix 2. Deletion of *F. johnsoniae* orthologs of *E. coli* chemotaxis genes *cheR* and *cheB* have no apparent effect on motility behavior

Gliding motility is common in members of the phylum *Bacteroidetes*, and other forms of motility are rare or nonexistent. For example, analysis of completed genomes revealed no members of the phylum that harbor genes for flagellar motility or for type IV pilus mediated twitching motility (Table 1). Studies of *Flavobacterium johnsoniae* gliding have revealed some aspects of the mechanism of cell movement (1), but nothing is currently known regarding how the motility machinery is controlled to result in directed movement. The chemotactic responses of gliding members of the phylum *Bacteroidetes* have not been extensively studied, but several reports suggest the presence of chemotactic responses (2, 3). Other motile bacteria that have been well studied have chemotaxis systems related to the canonical ones studied in the flagellated bacteria *Escherichia coli* and *Salmonella enterica* (4, 5). These include CheA, CheB, CheR, CheW, CheY, methyl-accepting chemotaxis proteins (MCPs), and sometimes additional proteins. In *E. coli* and other diverse flagellated bacteria belonging to many phyla these proteins control the functioning of the flagellar motor. Similar chemotaxis systems have been shown to control other types of motility machineries, including Type IV pili (twitching motility) (6) and the *M. xanthus* gliding motility apparatus (7). Gliding motility and gliding motility genes are widespread among members of the phylum *Bacteroidetes*, but gliding of these bacteria is apparently not related to myxobacterial gliding. Analyses of the genomes of gliding members of the phylum *Bacteroidetes* revealed the absence of genes encoding proteins with similarity to the core chemotaxis proteins CheA, CheW, and MCPs (Table 1 and (8)).

F. johnsoniae, and some other members of the phylum did have homologs of *cheR* and *cheB* (8). In *E. coli* CheR and CheB add and remove methyl groups from MCPs. Since CheA, CheW and predicted MCPs are lacking in all sequenced members of the *Bacteroidetes*, and since *cheB* and *cheR* homologs were lacking from some of the gliding members of the phylum *Bacteroidetes* analyzed (Table 1), a role for these genes in chemotaxis seemed unlikely.

To probe the function of *F. johnsoniae cheB* and *cheR* genes, strains with in-frame deletions were generated using a gene deletion strategy described previously (9). Briefly, approximately 2-kbp regions upstream and downstream of *cheR* were amplified by PCR using primer pairs 1287/1288 and 1289/1290 respectively and ligated into pRR51 to generate pSK03. Plasmid pSK03 was introduced into the streptomycin-resistant wild-type *F. johnsoniae* strain CJ1827 by triparental conjugation, and the *cheR* deletion mutant was isolated as previously described (9). Deletion of *cheR* was confirmed by PCR amplification using primers 1242/1243, which flank the gene. *F. johnsoniae cheB* was deleted in a similar way using the plasmids and primers listed in Table 2.

F. johnsoniae Δ*cheB* and Δ*cheR* strains were analyzed for their ability to move on agar and glass. Δ*cheB* and Δ*cheR* formed spreading colonies on PY2 agar similar to wild-type *F. johnsoniae* (Fig. 1). Colonies on PY2 agar were observed using an Olympus BH-2 phase-contrast microscope and images were recorded using a Photometrics Cool-SNAP$_{cf}^2$ camera, and analyzed using MetaMorph software (Molecular Devices, Downingtown, PA). Cells of Δ*cheR* and Δ*cheB* strains attached to and moved on glass similar to wild-type cells (data not shown). Wild-type and mutant cells were grown overnight in motility medium at 25°C without shaking and examined on glass. Tunnel slides were prepared to analyze motility on glass by attaching a glass cover slip to a glass

slide with strips of double stick tape as previously described to make a chamber to which cells suspended in growth media were added (10). Cells near the edge of the cover slip (within 1 mm) or near the tape (within 1 mm) were observed to avoid loss of motility as a result of depletion of O_2.

These observations suggest that the *cheR* and *cheB* homologs are unlikely to have critical roles in controlling motility. This is in contrast to the results observed for similar mutations in the swimming bacterium *E. coli* and the gliding proteobacterium *M. xanthus*. Mutations in *E. coli cheR* result in suppression of tumbling and decreased spreading in swim agar plates (11). Similarly, mutations in the *cheR* homolog of *M. xanthus*, *frzF*, result in inhibition of cell reversals and formation of multicellular donut-shaped 'frizzy' swirls (12). Cells of *F. johnsoniae* exhibit cell reversals, and move in swarms, and it is likely that a sensory system controls these behaviors, but the canonical *E. coli* type of chemotactic signal transduction system does not appear to be involved. This suggests the presence of novel chemotaxis machinery in *F. johnsoniae*. Further studies are needed to identify the proteins that control gliding motility of *F. johnsoniae* and of the many other gliding members of the phylum *Bacteroidetes*.

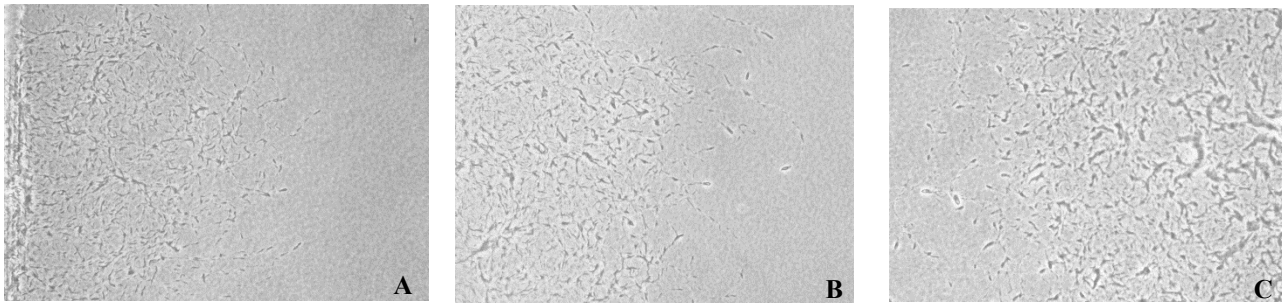

Fig. 1. Deletion of *cheB* and *cheR* homologs in *F. johnsoniae* has no effect on colony morphology. Colonies were grown for 19 h at 25°C on PY2 agar medium. Photomicrographs were taken with a Photometrics CoolSNAP$_{cf}^{2}$ camera mounted on an Olympus IMT-2 phase contrast microscope. (A) Wild-type CJ1827 (B) *cheB* deletion mutant CJ2352 (C) *cheR* deletion mutant CJ2249.

Table 1. Motility and chemotaxis genes present in members of the phylum *Bacteroidetes*[a].

Strains	Gliding motility genes[b]	Flagellar motility genes[c]	Twitching Motility Genes[d]	MCP(s)[e]	*cheA*[e]	*cheB*[e]	*cheR*[e]	*cheW*[e]	*cheZ*[e]
Class *Flavobacteriia*									
Capnocytophaga ochracea DSM 7271[T]	+	-	-	-	-	-	-	-	-
Cellulophaga algicola DSM 14237[T]	+	-	-	-	-	-	-	-	-
Cellulophaga lytica DSM 7489[T]	+	-	-	-	-	-	-	-	-
Croceibacter atlanticus HTCC2559[T]	+	-	-	-	-	+	+	-	-
Flavobacterium johnsoniae ATCC 17061[T]	+	-	-	-	-	+	+	-	-
Flavobacterium psychrophilum JIP02/86	+	-	-	-	-	-	-	-	-
'Gramella forsetii' KT0803	+	-	-	-	-	+	+	-	-
Maribacter sp. HTCC2170	+	-	-	-	-	-	-	-	-
Riemerella anatipestifer DSM 15868[T]	+	-	-	-	-	-	-	-	-
Robiginitalea biformata HTCC2501[T]	+	-	-	-	-	-	-	-	-
Weeksella virosa DSM 16922[T]	+	-	-	-	-	-	-	-	-
Zunongwangia profunda SM-A87[T]	+	-	-	-	-	+	+	-	-

Class *Cytophagia* (next page) **Class *Cytophagia***									
Cytophaga hutchinsonii ATCC 33406 [T]	+	-	-	-	-	+	+	-	-
Dyadobacter fermentans DSM 18053 [T]	+	-	-	-	-	+	+	-	-
Leadbetterella byssophila DSM 17132 [T]	+	-	-	-	-	-	-	-	-
Marivirga tractuosa DSM 4126 [T]	+	-	-	-	-	+	+	-	-
Spirosoma linguale DSM 74 [T]	+	-	-	-	-	+	+	-	-
Class *Sphingobacteriia*									
Chitinophaga pinensis DSM 2588 [T]	+	-	-	-	-	+	+	-	-
Pedobacter heparinus DSM 2366 [T]	+	-	-	-	-	+	+	-	-
Pedobacter saltans DSM 12145 [T]	+	-	-	-	-	+	+	-	-
Class *Bacteroidia*									
Alistipes shahii WAL 8301 [T]	-	-	-	-	-	-	-	-	-
Bacteroides fragilis NCTC 9343 [T]	-	-	-	-	-	-	-	-	-
Bacteroides helcogenes P 36-108 [T]	-	-	-	-	-	-	-	-	-
Bacteroides salanitronis BL78 [T]	-	-	-	-	-	-	-	-	-

Bacteroides thetaiotaomicron VPI-5482 ^{T}	-	-	-	-	-	-	-	-	-
Bacteroides vulgatus ATCC 8482 ^{T}	-	-	-	-	-	-	-	-	-
Bacteroides xylanisolvens XB1A ^{T}	-	-	-	-	-	-	-	-	-
Odoribacter splanchnicus DSM 20712 ^{T}	-	-	-	-	-	-	-	-	-
Paludibacter propionicigenes WB4 ^{T}	+	-	-	-	-	-	+	-	-
Parabacteroides distasonis ATCC 8503 ^{T}	-	-	-	-	-	-	-	-	-
Porphyromonas gingivalis ATCC 33277 ^{T}	-	-	-	-	-	-	-	-	-
Prevotella melaninogenica ATCC 25845 ^{T}	-	-	-	-	-	-	-	-	-
Prevotella ruminicola 23	-	-	-	-	-	-	-	-	-

[a] Except for gliding motility genes, all motility and chemotaxis genes were identified by searching each genome for matches to specific COGs, PFAMs, or TIGRFAMs corresponding to key components of each system as indicated below using the IMG v 3.5 Function Profile tool. For COGs, rpsblast was used in identification of hits, with maximum E-value of 1e-2. For PFAMs, HMM specific cutoffs (gathering thresholds) were used as assigned by the PFAM curator when the family was built, to eliminate false positives. For TIGRFAMs, HMM specific noise cutoffs were used as assigned by TIGRFAM to eliminate false positives.

[b] Gliding motility genes were identified by BLASTP analysis as described in Table 1. '+' indicates the presence of homologs for all of the core gliding motility genes, *gldB*, *gldD*, *gldH*, *gldJ*, *gldK*, *gldL*, *gldM*, *gldN*, *sprA*, *sprE*, and *sprT*. '-' indicates that some of the core gliding motility genes were missing.

[c] Flagellar motility genes were identified using the following: pfam00460 (flagella basal body rod protein); pfam00669 (flagellin, N-terminal region); pfam00700 (flagellin, C-terminal region); pfam01706 (FliG); pfam02049 (FliE); pfam02050 (FliJ); pfam02107 (FlgH); pfam02108 (FliH); pfam02119 (FlgI); pfam02154 (FliM); pfam02465 (FliD, N-terminal region); pfam02561 (FliS); pfam03748 (FliL); pfam03963 (FlgD); pfam06429 (flagellar basal body rod and hook proteins); pfam07195 (FliD, C-terminal region); pfam07559 (FlaE); pfam08345 (FliF); and COG1291 (MotA).). '+' indicates that genes predicted to encode each of the proteins listed above were present. '-' indicates that none of these genes were present.

Note that a previous study indicated that two members of the phylum *Bacteroidetes* (*Salinibacter ruber* and *Rhodothermus marinus*) have flagellar genes (13). However, recent data indicate that these bacteria are not members of the phylum *Bacteroidetes* but rather belong to the new phylum *Rhodothermaeota* (14, 15).

[d] Twitching motility genes were identified using the following: tigr01420 (PilT); COG2804 (PilB/PulE). '+' indicates the presence of both motor proteins (PilT and PilB). '-' indicates the absence of a gene encoding PilT. In each case where *pilT* was present, it was located near *pilB*, and near other pilus associated genes.

[e] Chemotaxis genes were identified using the following: pfam00015 (MCP); pfam01339 (CheB); pfam01739 (CheR); pfam03705 (CheR N-terminal region); pfam04344 (CheZ); pfam01584 (CheW); pfam02895 (CheA); COG0643 (CheA). '+' indicates that genes predicted to encode MCPs, CheA, CheB, CheR, CheW, and CheZ, were present. '-' indicates that these genes were not present.

Table 2. Strains and plasmids used in this study

Strain	Description[a]	Source or reference
***E. coli* strains**		
DH5αmcr	Strain used for general cloning	Life Technologies (Grand Island, NY, USA)
HB101	Strain used with pRK2013 for triparental conjugation	(16, 17)
***F. johnsoniae* strains**		
CJ1827	*rpsL2*; Sm^r 'wild-type' *F. johnsoniae* strain used in construction of deletion mutants	(9)

Plasmid	Description	Source or reference
pRR51	*rpsL*-containing suicide vector; Ap^r (Em^r)	(9)
pSK01	2-kbp fragment downstream of Fjoh_3352 (*cheR*) amplified with primers 1289 and 1290 and inserted in XbaI and SphI sites of pRR51; Ap^r (Em^r)	This study
pSK02	2-kbp fragment downstream of Fjoh_3351 (*cheB*) amplified with primers 1285 and 1286 and inserted in XbaI and SphI sites of pRR51; Ap^r (Em^r)	This study
pSK03	2-kbp fragment upstream of Fjoh_3352 (*cheR*) amplified with primers 1287 and 1288 and inserted in XbaI and SphI sites of pSK01; Ap^r (Em^r)	This study

pSK23	2-kbp fragment upstream of Fjoh_3351 (*cheB*) amplified with primers 1318 and 1405 and inserted in XbaI and SphI sites of pSK02; Ap^r (Em^r)	This study

[a]Antibiotic resistance phenotypes are as follows: ampicillin, Ap^r; erythromycin, Em^r; streptomycin, Sm^r; tetracycline, Tc^r. The antibiotic resistance phenotypes given in parentheses are those expressed in *F. johnsoniae* but not in *E. coli*. The antibiotic resistance phenotypes without parentheses are those expressed in *E. coli* but not in *F. johnsoniae*.

References:

1. Nakane D. SK, Wada H., McBride M. J., Nakayama K. . 2013. Helical flow of surface protein required for bacterial gliding motility. Proc Natl Acad Sci USA 110:11145–11150.
2. Liu ZX F. 1996. Negative chemotaxis in *Cytophaga johnsonae*. Can J, Microbiol 42:515-518.
3. Klesius PH, Shoemaker CA, Evans JJ. 2008. *Flavobacterium columnare* chemotaxis to channel catfish mucus. FEMS Microbiol Lett 288:216-20.
4. Armitage JP. 1999. Bacterial tactic responses. Advances in Microbial Physiology 41:229-289.
5. Manson MD. 1992. Bacterial motility and chemotaxis. Advances in Microbial Physiology 33:277-346.
6. Mattick JS. 2002. Type IV pili and twitching motility. Annu Rev Microbiol 56:289-314.
7. Zusman DR, Scott AE, Yang Z, Kirby JR. 2007. Chemosensory pathways, motility and development in *Myxococcus xanthus*. Nat Rev Microbiol 5:862-872.
8. McBride MJ, Xie G, Martens EC, Lapidus A, Henrissat B, Rhodes RG, Goltsman E, Wang W, Xu J, Hunnicutt DW, Staroscik AM, Hoover TR, Cheng YQ, Stein JL. 2009. Novel features of the polysaccharide-digesting gliding bacterium *Flavobacterium johnsoniae* as revealed by genome sequence analysis. Appl Environ Microbiol 75:6864-75.
9. Rhodes RG, Pucker HG, McBride MJ. 2011. Development and use of a gene deletion strategy for *Flavobacterium johnsoniae* to identify the redundant gliding motility genes remF, remG, remH, and remI. J Bacteriol 193:2418-28.
10. Shrivastava A, Rhodes RG, Pochiraju S, Nakane D, McBride MJ. 2012. *Flavobacterium johnsoniae* RemA is a mobile cell surface lectin involved in gliding. J Bacteriol 194:3678-88.
11. Parkinson JS, Houts SE. 1982. Isolation and behavior of *Escherichia coli* deletion mutants lacking chemotaxis functions. J Bacteriol 151:106-113.
12. McCleary WR, McBride MJ, Zusman DR. 1990. Developmental sensory transduction in *Myxococcus xanthus* involves methylation and demethylation of FrzCD. J Bacteriol 172:4877-4887.
13. McBride M, and Zhu Y. 2013. Gliding motility and Por secretion system genes are widespread among members of the phylum *Bacteroidetes*. J Bacteriol 195:170-8.
14. Hahnke RL, Meier-Kolthoff JP, Garcia-Lopez M, Mukherjee S, Huntemann M, Ivanova NN, Woyke T, Kyrpides NC, Klenk HP, Goker M. 2016. Genome-Based Taxonomic Classification of *Bacteroidetes*. Front Microbiol 7:2003.
15. Munoz R, Rossello-Mora R, Amann R. 2016. Revised phylogeny of *Bacteroidetes* and proposal of sixteen new taxa and two new combinations including *Rhodothermaeota* phyl. nov. Syst Appl Microbiol 39:281-96.
16. Bolivar F, Backman K. 1979. Plasmids of *Escherichia coli* as cloning vectors. Methods Enzymol 68:245-267.
17. Figurski DH, Helinski DR. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci USA 76:1648-1652.

Appendix 3. Transposon mutagenesis to isolate novel motility mutants

HimarEm1 mutagenesis was used to isolate mutants with partial defects in motility to identify novel genes involved in *F. johnsoniae* gliding motility and secretion. pHimarEm1 was introduced into wild-type cells by conjugation from *E. coli* S17-1 λ *pir* as previously described (1). In previous *F. johnsoniae Himar* mutagenesis experiments, colonies were screened to obtain those that failed to spread on agar. This resulted in the identification of many motility genes (1). The proteins encoded by these genes are thought to comprise components of the motility machinery and of the type IX secretion system (T9SS) that is involved in assembly of the motility apparatus (2). Genes involved in regulation of expression of the motility apparatus, and genes involved in control of the motility apparatus to result in chemotaxis, have eluded detection. Here we ignored nonspreading colonies and instead screened for colonies that exhibited some spreading but less than that exhibited by the wild type. Eleven such 'poor spreading' colonies were identified. Cells of the wild-type UW-101 and of the transposon mutants were grown on PY2 agar for 24 h at 25°C to examine single colonies for spreading by phase contrast microscopy. The transposon mutants had reduced or ‘poor’ spreading phenotypes as compared to the wild-type strains. Identification of the *HimarEm*1 insertions was performed as described previously by cloning the disrupted region and determining the DNA sequence near the site of insertion (1). A list of these mutants, gene loci and phenotypes has been compiled in Table 1.

Four of the eleven 'poor spreading' mutants had insertions in genes predicted to be involved in polysaccharide synthesis and/or transport. Three of these had insertions in genes encoding glycosyl transferase enzymes. The fourth predicted polysaccharide synthesis mutant had an insertion in *degT*, which is similar to *Porphryomonas gingivalis porR*. *P. gingivalis* PorR is

involved in biosynthesis of aminoglycoside sugars of LPS (3). Polysaccharides have previously been implicated in *F. johnsoniae* gliding motility (4-7). The exact roles played by polysaccharides in gliding are not known, but it has been suggested that cells make different polysaccharides to coat the substratum. At least one of these polysaccharides appears to interact with the mobile cell surface motility adhesin, RemA (5). The polysaccharides may function as 'roads' and facilitate interaction of the motility adhesins with the substratum, thus providing the traction needed for cell movement.

One mutant isolated from this screen had a transposon insertion in *sprE*. Previous studies have shown that *sprE* insertion mutants form non-spreading colonies but that individual cells exhibit slight gliding movements on glass (8). *sprE* mutants are also defective for T9SS-mediated protein secretion. The *sprE* mutant described here (CJ2215) exhibited a slightly different phenotype. Isolated colonies of CJ2215 failed to spread, but colonies in close proximity to each other exhibited slight spreading. CJ2215 produces 589 AA out of the 870 AA of SprE. This explains the poor spreading phenotype rather than non spreading phenotype previously (8) observed for SprE mutant that produce shorter truncated version of SprE protein.

Mutant CJ2160 has transposon inserted in Fjoh_3155, which is predicted to encode a Rhs element Vgr-like protein. The single colonies observed under microscope exhibited poor spreading as compared to wild-type and the mutant failed to digest chitin, which may indicate a defect in protein secretion (data not shown). In *Vibrio cholerae*, Vgr proteins form a complex that resembles the tail-spike complex of bacteriophage T4 and provide a conduit for T6SS mediated translocation of proteins out of and between cells (9).

Other poor spreading mutants isolated from this screen include strains with insertions in genes predicted to encode: a PSP-1 domain containing protein, a short-chain dehydrogenase/reductase family protein, a CorA-like ion transporter, and a MoxR family ATPase. Fjoh_0891, encoding the PSP-1 domain containing protein, is immediately upstream of gliding motility gene *gldH*. *gldH* mutants are nonmotile (10). It is possible that the phenotype of the mutant carrying an insertion in Fjoh_0891 is the result of a polar effect on *gldH*. This could be tested by attempting to complement this mutant with *gldH* on a plasmid. CorA is a divalent ion transporter protein and has been extensively studied because of its ability to transport magnesium and cobalt across membranes (11). In *E. coli*, MoxR family proteins are predicted to have chaperone-like activities, enabling proper maturation and activation of protein complexes (12) . It is unclear if these proteins are directly involved in gliding motility and further studies are needed to characterize the mutants and investigate their unusual poor-spreading phenotypes.

Table 1. Mutants isolated in *HimarEm1* transposon mutagenesis screen.

Strain	**Protein encoded (gene locus)**	**Phenotype**
CJ2210	Glycosyl transferase (Fjoh_0338)	Well isolated colonies are poor-spreading; clustered colonies spread more but not as well as wild-type.
CJ2214	Glycosyl transferase (Fjoh_0344)	Well isolated colonies are poor-spreading; clustered colonies spread more but not as well as wild-type.
CJ2211	Glycosyl transferase (Fjoh_0342)	Well isolated colonies are poor-spreading; clustered colonies spread more but not as well as wild-type.
CJ2304	Hypothetical protein (Fjoh_0816)	Well isolated colonies are poor spreading; clustered colonies spread more but not as well as wild-type.
CJ2030	PSP-1 domain containing protein (Fjoh_0891)	Well isolated colonies are poor spreading; clustered colonies spread more but not as well as wild-type.
CJ 2133	CorA (Fjoh_2650)	All colonies (isolated and clustered) are poor-spreading.
CJ2215	SprE (Fjoh_1051)	Well isolated colonies fail to spread; clustered colonies are poor-spreading. Fails to digest chitin, suggesting a T9SS defect.*

CJ2160	Rhs element Vgr protein (Fjoh_3155)	All colonies (isolated and clustered) are poor-spreading. Fails to digest chitin, suggesting a T9SS defect. *
CJ2308	Dehydrogenase (Fjoh_4568)	All colonies (isolated and clustered) are poor-spreading. Digests chitin. *
CJ2219	MoxR ATPase (Fjoh_0715)	All colonies (isolated and clustered) are poor-spreading. Digests chitin. *
CJ2132	DegT (Fjoh_1727)	Well isolated colonies are poor-spreading; clustered colonies spread more but not as well as wild-type; Doughnut shaped colonies after 48h. Digests chitin. *

* Chitin assay was performed only with these strains.

Table 2. Strains used in this study

Strain	Description	Source or reference
***E. coli* strains**		
DH5αmcr	Strain used for general cloning	Life Technologies (Grand Island, NY, USA)
HB101	Strain used with pRK2013 for triparental conjugation	(13, 14)
S17-1 λ *pir*	Strain used for conjugation	(15)
***F. johnsoniae* strains**		
UW101 (ATCC 17061)	Wild type	(16, 17)

References:

1. Braun TF, Khubbar MK, Saffarini DA, McBride MJ. 2005. *Flavobacterium johnsoniae* gliding motility genes identified by mariner mutagenesis. J Bacteriol 187:6943-52.
2. Shrivastava A JJ, Barren JM, McBride MJ. 2012. *Flavobacterium johnsoniae* GldK, GldL, GldM, and SprA are required for secretion of the cell suraface gliding motility adhesins SprB and RemA. J Bacteriol 195:3201-12.
3. Shoji M RD, Shi Y, Kadowaki T, Yamamoto K, Yoshimura F, Akamine A, Curtis M, Nakayama K. 2002. Construction and characterization of a nonpigmented mutant of *Porphyromonas gingivalis*: Cell surface polysaccharide as an anchorage for gingipanis. Microbiology 148:1183-1191.
4. Bollampalli S. 2011. Role of cell surface polysaccharides and proteins in *Flavobacterium johnsoniae* gliding motility Bollampalli, Sreelekha. PhD Thesis. University of Wisconsin-Milwaukee.
5. Shrivastava A, Rhodes RG, Pochiraju S, Nakane D, McBride MJ. 2012. *Flavobacterium johnsoniae* RemA is a mobile cell surface lectin involved in gliding. J Bacteriol 194:3678-88.
6. Godchaux W LI, Leadbetter ER. 1991. Defects in gliding motility in mutants of *Cytophaga johnsonae* lacking a high-molecular-weight cell surface polysaccharide. J Bacteriol 173:7607-7614.
7. Godchaux W I, GOrski L, Leadbetter ER. 1990. Outer membrane polysaccharide deficiency in two nongliding mutants of *Cytophaga johnsonae*. J Bacteriol 172:1250-1255.
8. Rhodes RG, Samarasam MN, Van Groll EJ, McBride MJ. 2011. Mutations in *Flavobacterium johnsoniae* sprE result in defects in gliding motility and protein secretion. J Bacteriol 193:5322-7.
9. Pukatzki S MA, Revel AT, Sturtevant D, Mekalanos JJ. . 2007. Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin. Proceedings of the National Academy of Sciences of the United States of America 104:15508-15513.
10. McBride M, Braun TF, Brust JL. 2003. *Flavobacterium johnsoniae* GldH is a lipoprotein that is required for gliding motility and chitin utilization. J Bacteriol 185.
11. Niegowski D, Eshaghi S. 2007. The CorA family: structure and function revisited. Cell Mol Life Sci 64:2564-74.
12. Wong KS, Snider JD, Graham C, Greenblatt JF, Emili A, Babu M, Houry WA. 2014. The MoxR ATPase RavA and its cofactor ViaA interact with the NADH:ubiquinone oxidoreductase I in *Escherichia coli*. PLoS One 9:e85529.
13. Bolivar F, Backman K. 1979. Plasmids of *Escherichia coli* as cloning vectors. Methods Enzymol 68:245-267.
14. Figurski DH, Helinski DR. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci USA 76:1648-1652.
15. de Lorenzo V, Timmis KN. 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons. Methods in enzymology 235:386-405.

16. McBride MJ BT. 2004. GldI is a lipoportein that is required for *Flavobacterium johnsoniae* gliding motility and chitin utilization. J Bacteriol 186.
17. McBride MJ, Xie G, Martens EC, Lapidus A, Henrissat B, Rhodes RG, Goltsman E, Wang W, Xu J, Hunnicutt DW, Staroscik AM, Hoover TR, Cheng YQ, Stein JL. 2009. Novel features of the polysaccharide-digesting gliding bacterium *Flavobacterium johnsoniae* as revealed by genome sequence analysis. Appl Environ Microbiol 75:6864-75.

Curriculum Vitae

Surashree S. Kulkarni
UW-Milwaukee
3209 N Maryland Ave
Milwaukee, WI-53211

Education

2011 – 2017	University of Wisconsin, Milwaukee Doctor of Philosophy, Molecular Microbiology	Milwaukee, WI
2009-2011	University of Mumbai Master of Science in Life Sciences	Mumbai, India
2006-2007	St. Xavier's College Bachelor of Science in Life Sciences	Mumbai, India

Research Experience

2011- Present — University of Wisconsin, Milwaukee: Graduate Student Researcher 2011-2015;

Graduate Research Assistant 2015-present

Advisor: Dr. Mark J. McBride

- Constructed and expressed fluorescently-tagged fusion proteins in *Flavobacterium johnsoniae* to determine how proteins are targeted to and processed by the Type IX secretion system (1 paper in press and 1 in preparation)

2010 — National Chemical Laboratory, Pune: Research Intern

Advisor: Dr. Shubhada Thengane

- Optimized *Agrobacterium rhizogenes* mediated transformation in a medicinal plant called *Nothapodytes foetida*

2007 Indian Institute of Technology, Mumbai: Research Intern

Advisor: Dr. Swati Patankar

- Identified non-coding RNAs in the genome of malarial parasite *Plasmodium falciparum* and *Plasmodium yeolii* using in-silico predictions to understand transcription regulation in *Plasmodium* spp leading to peer reviewed publication

Teaching Experience

2013-2015 University of Wisconsin, Milwaukee

Teaching Assistant Coordinator

- Coordinated 14 laboratory sections BIO150. This included composing the lab syllabus, over-seeing the procedures, equipment and chemicals needed for the same, addressing student issues, and conducting end of semester practical examinations.

2011-2013 University of Wisconsin, Milwaukee

Teaching Assistant for:

- Foundations of Biology (BIO150)

Publications

Kulkarni S, Zhu Y, Brendel C, McBride MJ. Diverse C-terminal sequences involved in *Flavobacterium johnsoniae* protein secretion [in press; https://doi.org/10.1128/JB.00884-16]

Kulkarni S and McBride MJ. Role of SprF and the conserved Carboxy-terminal domain of SprB in targeting it to the Type IX secretion system in *Flavobacterium johnsoniae* [Manuscript in preparation].

Li N, Zhu Y, Conrad R, LaFrentz B, Evenhuis J, Hunicutt D, **Kulkarni S**, Nie P and McBride MJ. The type IX secretion system is required for virulence of the fish pathogen *Flavobacterium columnare* [Manuscript in preparation]

Panneerselvam P, Bawankar P, **Kulkarni S**, Patankar S. In silico prediction of evolutionarily conserved GC-rich elements associated with antigenic proteins of *Plasmodium falciparum*. Evol Bioinformatics. 2011; 7:235–255

Poster and Podium Presentations

Kulkarni S, McBride MJ, Targeting of proteins for secretion by the Type IX Secretion System, Milwaukee Microbiology Seminar Series, School of Freshwater Sciences, University of Wisconsin, Milwaukee (2016) **[Invited Speaker].**

Kulkarni S, McBride MJ, Role of conserved Carboxy-terminal domains in targeting proteins to the Type IX secretion system of *Flavobacterium johnsoniae*, Department of Biological Sciences, University of Wisconsin, Milwaukee Symposium (2015).

Kulkarni S, McBride MJ, Targeting of proteins for secretion by the Type IX Secretion System, Flavobacterium International Conference, Auburn University, Alabama (2015).

McBride MJ, Zhu Y, **Kulkarni S**, Johnston J, Li N, Hunicutt D, The roles of *Flavobacterium* Type IX Secretion Systems in motility, virulence and polysaccharide digestion, Flavobacterium International Conference, Auburn University, Alabama (2015).

Kulkarni S, McBride MJ, Analyzing the role of *degT* in *Flavobacterium johnsoniae* gliding motility and protein secretion, Department of Biological Sciences, University of Wisconsin, Milwaukee Symposium (2013).

Awards and Funding

2015	• Graduate Student Travel Award University of Wisconsin, Milwaukee
2011-2015	• UW-Milwaukee Chancellors Graduate Award University of Wisconsin, Milwaukee
2009	• Student of the Year for excellence in undergraduate studies St. Xavier's College, Mumbai, India
2009	• Meritorious Achievement Award for procuring 1st merit rank among 300 students University of Mumbai, India

Skills and Techniques

Molecular Biology

- DNA isolation, RNA isolation, plasmid and genomic DNA isolation, gene cloning, site-directed mutagenesis, PCR, DNA sequence analysis, southern blotting, fluorescent microscopy, bacterial reporter assays**,** gene disruption and deletion in bacteria, transposon mutagenesis, phage transduction

Biochemistry

- SDS-PAGE, western blotting, recombinant protein expression and purification, bacterial lipopolysaccharide extraction and analyses, silver staining, affinity purification

Microbial techniques

- Aerobic and anaerobic bacterial cultures

Software and Miscellaneous

- MacVector, Microsoft office, Adobe Illustrator, MetaMorph, C++ programming

Professional Development/Organizations

2015 Graduate Organization of Biological Sciences

- Organized the annual research symposium event

2012 Graduate Organization of Biological Sciences

- Coordinated a mentorship program for graduate students to encourage inter-departmental collaboration in research

Professional Society Memberships

2011- Present • American Society of Microbiology

2006-2009 • Xavier's Association of Chemistry Students